

Proceedings of the XXIV Workshop
on the Developments in the Italian PhD Research
on Food Science, Technology and Biotechnology

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DEPARTMENT OF
AGRICULTURE, FOOD
ENVIRONMENT AND FORESTRY

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PhD DISSERTATION PROJECTS

Implicit and Explicit Methods to Evaluate Sensory, Affective and Attentive Responses to Plant-Based Products

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This PhD thesis research project is aimed at setting up an experimental procedure to identify individual differences in food acceptability by investigating attentive, affective and sensory responses to plant-based products in adults and pre-adolescents. Understanding why people prefer one product over another is one of the most interesting research topics in recent decades. Providing a methodological basis for measuring the interaction between multiple factors that play a role in food preferences and choice can help to bring down barriers to healthy eating.

Metodi impliciti ed espliciti di valutazione dei responsi sensoriali, affettivi e attentivi a prodotti alimentari a base vegetale

Questo progetto di dottorato mira a realizzare una procedura sperimentale volta a studiare le differenze individuale nel consumo di prodotti alimentari investigando le risposte attentive, affettive e sensoriali che vengono date a prodotti a base vegetale, negli adulti e nei bambini (pre-adolescenti). Capire perché una persona preferisca un prodotto rispetto a un altro è una delle domande di ricerca di maggior interesse degli ultimi anni, per questo motivo, sviluppare un approccio metodologico volto a misurare l'interazione tra i vari fattori in gioco nelle scelte alimentari, può aiutare ad abbattere le barriere al consumo di cibi salutari.

1. State-of-the-Art

The importance and the health benefits provided from a high consumption of plant-based foods is well known. Indeed, various studies demonstrate the specific correlation between vegetables intakes and reduced presence of many diseases. For instance, intakes of dark green leafy vegetables have been associated with reduced risk for II type diabetes, reduced risk of a number of cancers (and with reduced depression (Appleton et al., 2016)).

Although all these benefits, in the last decades a global dietary transition was registered with an increase of animal-based diet at the expense of plant-based diet. Why people prefer one product over another one? A growing body of investigations is directed to understand eating habits: this is a complex behavior in which the interaction of many variables related to the product, person and context could act. Several studies have demonstrated the influence of psychological traits in the development of food preferences, since the early age. Food neophobia, defined as the reluctance to eat, or the avoidance of novel foods (Pliner & Hobden, 1992) is a heritable personality trait, preserved during generations, that bring individuals to be extremely selective towards foods (Knaapila et al., 2007). Neophobic individuals typically reject foods characterized by bitter taste, a sensory property common to many vegetables (Appleton et al., 2016).

It has been widely demonstrated the genetic variation in taste may have a considerable influence on food acceptability. The taste sensitivity to the bitter substance 6-n-propylthiouracil (PROP) and its chemical relative phenylthiocarbamide (PTC) may be a genetic marker for individual differences. On the base of their taste responsiveness to

this substance, individuals can be divided in three samples: no-tasters, medium-tasters and super-tasters. Those considered as super-tasters are more sensitive to the bitterness of PROP as well as to other bitter compounds found in foods, astringency and other sensations (Bartoshuck, 1993; Tepper, 1998).

Attention as well may play a role in these processes of choice. Attentional bias enhances information processing of emotionally salient objects or events by selecting them from the environmental context in which they are embedded. Food cues are particularly effective at grabbing attention. The view of food pictures in contrast of non-food pictures, can enhance the attention both in normal-weight and in obese individuals and this was reported by measuring the brain activation via functional magnetic resonance imaging (fMRI) and event-related potentials. Studies with obese subjects, when compared to normal-weight subjects suggest that attentional resources are preferentially directed to high calorific foods. The presence of an attentional bias towards foods stimuli was primarily investigated on specific population, in particular in obese people, eating-disorder patients and restrained eaters. The authors found an increased visual attention bias for high-energy foods, when the attentional bias is defined as the tendency to have a selective orientation towards and/or a slowed disengagement from high-energy food cues, even in normal-weight and non-clinic samples (Burgos et al., 2017). However, the studies on attentional bias in normal-weight individual are still scarce and report controversial results.

It is still not clear if there is an interaction of the personal variables above mentioned, and in which way they can impact on food preferences and food acceptability. Moreover, all the studies realized until now had included only adults in their experimental design.

Little is known about the interaction of personality traits, taste responsiveness and attention on food preferences in children and adolescents, two populations in which vegetables intakes result to be particularly low (EFSA, 2008); this is a critical period in which eating habits start to well develop and sustain.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Assessment attentional responses** both in adults and pre-adolescent (240 subjects) through a modified version of the Stroop Task, to investigate if people with a low vegetable's intakes had develop an attentional bias towards those products and investigation of which role taste responsiveness and psychological traits may play in these attentional responses. Part of data collection will be conducted in primary and secondary schools.

A2) **Selection and development of set a product with phenolic compounds** in order to investigate sensory and affective responses to foods with critical sensory properties such as bitterness and astrincency.

A3) **Assessment of emotions elicited by foods with explicit and implicit methods**, like questionnaires and physiological recordings (skin conductance / electroencephalography)

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Assessment attentional responses																								
	1) Data collection with adults																								
	2) Data collection with children																								
A2)	Selection set of products																								
A3)	Assessment of emotions																								
A4)	Thesis and Paper Preparation																								

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High Amylose Wheat Flours for the Development of Healthy Cereal Based Foods

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This PhD thesis research project is aimed at developing innovative cereal products (bread, pizza, biscuits, rusks, fresh pasta) involving the use of new line of high amylose wheat which could potentially provide substantial health benefits through delivery of resistant starch. A complete evaluation of raw materials will be carried out and the best processes will be standardized in order to obtain innovative products with improved technological performance and able to reduce the occurrence of chronic diseases.

Utilizzo di sfarinati di frumento ad alto tenore di amilosio per lo sviluppo di alimenti salutistici a base di cereali

Questo progetto di tesi di dottorato mira allo sviluppo di prodotti a base di cereali innovativi (pane, pizza, pasta fresca, biscotti, fette biscottate) attraverso l'utilizzo di una nuova linea di frumenti ad alto tenore di amilosio i cui sfarinati possono dare effetti benefici sulla salute grazie a un elevato contenuto di amido resistente. A tal proposito, sarà effettuata un'attenta valutazione delle materie prime, standardizzando il processo di produzione per ottenere dei prodotti finiti con adeguate performance tecnologiche e in grado di contrastare l'insorgenza di malattie croniche non trasmissibili.

1. State-of-the-Art

Wheat is one of the most cultivated, consumed and traded food grains of the world with a worldwide diversity of thousands of cultivars/varieties, in particular wheat-based foods feature prominently in the cuisines, cultures and culinary histories of many countries.

Starch is the main constituent of flour, representing about 70-80% of its weight. From a biochemical point of view starch is a mixture of two polysaccharides amylose – an essentially linear polymer – and the highly branched amylopectin (Shevkani et al., 2017). Conventional starches consist of about 25% amylose, the remaining part is amylopectin. Amylose forms long-chains that are difficult for digestive enzymes to access. Conversely, amylopectin, which forms shorter chains, is more susceptible to amylolysis (Newberry et al., 2018). Modification of amylose/amylopectin ratio, by reverse genetics-based strategies, targeting genes coding key enzymes involved in starch synthesis, has succeeded in generating amylose-free wheat starch (colloquially referred to as “waxy”) and high amylose wheat starch (Botticella et al., 2018).

Production of high amylose soft wheat has raised special interest in food industry due to researches proving positive correlation between amylose content in flour, the quantity of resistant starch in derived food and the capacity of this type of starch to induce lower postprandial glycaemic responses (Hallström et al., 2011). Resistant starch is that portion of dietary starch that escapes digestion and absorption in the upper gut and consequentially reaches the large bowel where it serves as a substrate for gut microbiota. The end products of its fermentation are short chain fatty acids (SCFA), like propionate, acetate and butyrate, that are able to provide fundamental support both to the gut cell and other extra-intestinal tissue (Bird & Regina, 2018).

Therefore, the use of high amylose wheat flours could be a valid option for the development of low glycaemic index products, like bread, fresh pasta, biscuits, rusks and products of regional cuisine such as Neapolitan pizza for which the European Union has granted the official recognition to define the “ideotype” and so preserve the original characteristics (Morris & Ibba, 2018).

Thus, this PhD thesis project will be targeting to the development of innovative cereal products involving the use of new line of high amylose wheat with health benefits and improved technological performance, in terms of dough stability, tolerance to mechanical stress and starch retrogradation, which is believed to be one of the major players of the increase in crumb firmness during storage.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Bibliographic research in order to obtain specific knowledge on wheat characteristics, cereal technologies (bread, fresh pasta, biscuits, rusks, pizza making), glycaemic index, staling process and importance of high amylose and resistant starch for technological and nutritional quality of the products.

A2) Evaluation of high amylose wheat flour suitability to baking and fresh pasta making: compositional analysis of the innovative flour (moisture, protein, lipid, carbohydrates, dietary fiber, ash) and its starchy component characterization (total starch, amylose and amylopectin, damaged starch, resistant starch) (A2.1); assessment of technological aptitude of high amylose wheat flour, rheological tests: alveograph, farinograph, reofermentograph, viscoamilograph, gelatinization degree (A2.2).

A3) Development of a protocol for producing innovative healthy foods: baking performance of the high amylose flour alone and in blends with conventional flour (rheological analysis and texture analyses) (A3.1); use of pilot plant for the production of bread and pizza (“direct dough processing”, “indirect dough processing” and “sourdough” using the above-mentioned blend of flours), biscuits, rusks and fresh pasta (A3.2); comparison among the different trials and identification of the best baking and fresh pasta making processes (A3.3).

A4) Assessment of nutritional and sensory characteristics of innovative healthy high amylose products compared to “conventional” ones: complete physico-chemical and sensorial characterization of the final products (A4.1); *in vitro* and *in vivo* test for the evaluation of postprandial glucose response (A4.2); shelf-life modeling through the evaluation of the starch retrogradation and bread staling (A4.3); nutritional and healthy labeling according to EU Commission Regulations (Reg. EC 1924/2006, Reg. EU 432/2012 and Reg. EU 1169/2011) (A4.4).

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1 Bibliografic research																		
A2 Evaluation of high amylose wheat flour suitability to baking and pasta making																		
1) Compositional analysis of the innovative flour and its starch characterization																		
2) Assessment of technological aptitude of high amylose wheat flour																		
A3 Development of a protocol for the production of innovative healthy foods																		
1) Baking performance of high amylose flour alone and in blends with conventional flour																		
2) Use of pilot plant for the production of innovative products																		
3) Comparison among the different trials																		
A4 Assessment of nutritional and sensory characteristics of innovative high amylose products																		
1) Physico-chemical and sensorial characterization of the final products																		
2) Evaluation of postprandial glucose response																		
3) Shelf-life modeling																		
4) Nutritional and healthy labeling																		
A5 Reports, Thesis and Papers drafting																		

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Evaluation of Alternative Flours from Unconventional Resources in Gluten-Free Bakery Products

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The main objectives of this doctoral project are i) technological evaluation of the protein flours obtained from *Tenebrio molitor L.*, and of the starch extracted by acorns of *Quercus*; ii) the use of these flours in baking formulations, and the contemporary rheological and textural characterization of dough and bakery products. Insects will be grown on four substrates (bran, hemp, brewer's spent grains, husk), thus, their influence on processing, and related industrial scale up, and baked goods will be evaluated. Acorn fruits will be studied for the possibility to use their starch in gluten free bakery products.

Valutazione di sfarinati alternativi da risorse non convenzionali nei prodotti da forno gluten-free

Gli obiettivi di questo progetto di dottorato sono i) la valutazione tecnologica degli sfarinati proteici derivanti da *Tenebrio molitor L.*, e degli sfarinati amidacei dei frutti di *Quercus*; ii) l'uso di tali sfarinati nelle formulazioni per la panificazione, e la contestuale caratterizzazione reologica e di texture degli impasti e dei prodotti finiti. Gli insetti saranno allevati su quattro tipologie di substrato (crusca, canapa, trebbie, lolla), pertanto, sarà valutata l'influenza nei processi di trasformazione degli insetti in farine, e lo scale up industriale, e nel prodotto da forno finito. Sulle ghiande saranno valutate le proprietà dell'amido nei prodotti da forno gluten-free.

1. State-of-the-Art

The continuous demand for primary resources poses the problem of the future availability and the relative production systems of some precious food resources, such as animal proteins and starch. These needs have recently stimulated researchers to consider new types of foods that meet a low environmental impact and food safety and are nutritionally balanced. FAO indicates that insects could play an important role in both human and animal nutrition and represent one of the possible responses to this need (Van Huis et al., 2013). Entomophagy is a widespread practice in eastern countries (insects are consumed after sun drying, boiling or roasting) and in recent years the scientific interest in the applicability of this resource in western countries has increased. People in these countries often show feelings of disgust with eating insects as food (Tan et al., 2015). The use of insect flours to be included in traditional products shows, through research on emotional experiences on western people, numerous positive emotional evaluations, reducing the negative evaluations of the feelings of disgust due to the consumption of whole insects or mixtures in pieces (Gmuer et al., 2016). Roncolini et al. (2019) have evidenced positive results in the technological and nutritional features in wheat with flours of *Tenebrio molitor L.* Based on the positive data of scientific research, the European Union has redefined the marketing procedure for the new categories of foods (novel food) and feed, including those derived from insects and / or their by-products (EU regulations 2015/2283 and 2017 / 893). Compared to many animal farms, insect breeding has many advantages, including an excellent food conversion index, their potential to be bred on products that do not compete with food for humans (waste), low emissions of greenhouse gases and of ammonia, reduced surface use, low water

consumption and reduced risk of transmission of zoonoses (Veldkamp et al., 2012). In 2015 EFSA's opinion derived from the assessment of risks related to the use of insects as food or feed, is that the potential microbiological hazards are comparable to those associated with the usual animal protein sources, while the possible risk of a related transfer of chemical contaminants, this is closely linked to the different types of substrate and to the species of insects considered. The pre-image phases of the *Tenebrio molitor* L., a beetle belonging to the *Tenebrionidae* family, are easy to breed and feed and have a precious protein profile, as the crude protein content on dry basis ranges 47-60%, while fat is 31-43% and ashes are about 5% of (Makkar et al., 2014). The composition is mainly influenced by the breeding conditions and the applied technological processes. A second natural resource that presents good prospects of use for the creation of food flours is the fruits of the plants of the genus *Quercus*. Recently, several scientific publications have revealed that acorn flour or its starch could be a valid alternative to other common used starches (rice, corn) in gluten free baked products. Korus et al. (2015) have demonstrated that the addition of up to 40% of acorn flour in the formulation of gluten-free products has a positive influence on the sensory parameters and the general acceptance of the product.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Unit operations optimization for flour extraction

Different freezing, blanching, drying, grinding and defatting treatments will be tried, and the best flour storage conditions will be identified. For acorns a further starch extraction step will be considered.

A2) Chemical and physical characterization of flours

Flours will be characterized for: optimal moisture content, color (to evaluate enzymatic and non-enzymatic browning), total protein, fat, fiber and ashes.

A3) Characterization of the rheological properties of the obtained dough

Doughs will be subjected to several rheological analyses, such as reofermentographic analysis, pasting curves using Rapid Visco Analyzer (RVA).

A4) Evaluation of the impact of flours on baked products

The influence of insect flours and acorn starch on the formulations of bakery products will be studied by analyzing the texture properties of bread obtained with different percentages of unconventional flours. The nutritional properties of the final products will also be evaluated.

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Unit operations optimization for flour extraction																								
A2) Chemical and physical characterization of flours																								
A3) Characterization of the rheological properties of the obtained dough																								
A4) Evaluation of the impact of flours obtained on baked products																								
A5) Writing and Editing of the PhD thesis																								

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The Development of Flexible and Printed Biosensors to Quantify Hazards in Food Samples

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The presence of hazards in food constitutes a potential public health concern, due to their toxicological effects. Although classical detection methods allow the hazards detection (requiring expensive equipment and extensive analysis time), the danger of these compounds calls for new systems, which can rapidly identify them. In this regard, biosensors, fabricated with printing techniques, promise to overcome these limitations. This PhD thesis project aims at developing low-cost biosensors functionalized with different nanoparticles to improve sensitivity and limit of detection. To obtain high selective biosensors, antibodies will be employed as biological receptors, due to high specific chemical interaction antibody-antigen.

Sviluppo di biosensori flessibili e stampati per quantificare i rischi nei campioni alimentari

La presenza di sostanze rischiose negli alimenti costituisce un potenziale problema di salute pubblica a causa della loro tossicità. Sebbene i metodi di rilevamento convenzionali consentano il rilevamento di rischi (richiedendo apparecchiature costose e un lungo tempo di analisi), il pericolo posto da questi composti richiede nuovi sistemi in grado di identificarli rapidamente. A questo riguardo, i biosensori, realizzati con tecniche di stampa, promettono di superare questi limiti. Questo progetto di dottorato è volto a sviluppare biosensori meno costosi e funzionalizzati con di-verse nanoparticelle per migliorare la sensibilità e il limite di rilevazione. Per ottenere biosensori altamente selettivi, gli anticorpi saranno utilizzati come recettori biologici, a causa di un'alta interazione chimica specifica tra l'anticorpo e l'antigene.

1. State-of-the-Art

Every year millions of cases of food-borne diseases caused by different hazards occur worldwide (Schirone et al., 2017). Therefore, detection and quantification of these hazards in food as well as protection against food spoilage is a key task of great social, economic and public health importance. In this regard, Biogenic amines (BAs) are low molecular nitrogenous organic compounds, and they are known foodborne toxicant. These molecules are mainly formed through the decarboxylation of amino acids (fig 1.) under interaction with decarboxylase active microorganisms such as Enterobacteriaceae, Pseudomonas spp., Micrococcaceae, Enterococci and Lactic acid bacteria (Schirone et al., 2017).

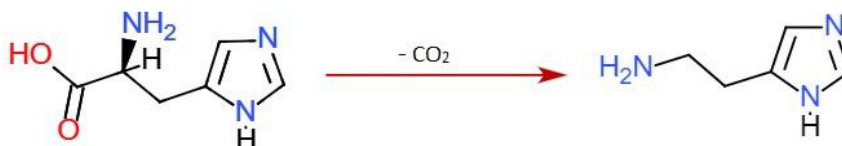


Figure 1 Decarboxylation of histidine

Fish, cheese, meats, some fruit like bananas and avocados, vegetables like mushrooms, and fermented foods such as chocolate, and soy sauce are a few examples of foods containing different concentrations of BAs (De la Torre et al., 2018).

The most known foodborne intoxication is the “scombroid poisoning”, which is caused by the large consumption of histamine. The ingestion of high concentrations of putrescine, tyramine, spermidine, and spermine can be related to the development of tumors (De la Torre et al., 2018). Quality of the meat is correlated to the Bas concentration, mainly histamine, tyramine, putrescine, and cadaverine. By summing the concentration of these four Bas, the biogenic amines index (BAI) is evaluated, $BAI < 5 \text{ mg/kg}$ meat quality is good, $BAI = 5 - 20 \text{ mg/kg}$ meat quality is acceptable, $BAI = 20 - 50 \text{ mg/kg}$ poor meat quality and $BAI > 50 \text{ mg/kg}$ spoiled meat (Triki et al. 2018).

Different analytical methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC) enzyme-linked immunosorbent assay (ELISA), have been developed for the detection and quantification of BAs (Lázaro De La Torre et al., 2013). Most of these methods required expensive equipment, extensive analysis time and specially trained personnel. Therefore, low-cost, rapid, specific, and sensitive analysis methods of BAs in a variety of samples are of widest interest. In this context, biosensors are especially interesting due to the short analysis time, the low fabrication cost, and the possibility to be used outside the laboratory (Mehrotra et al., 2016).

Biosensors are defined as analytical devices that convert a biological response into an electrical signal (Adley et al., 2014). The aim of these devices is to produce a continuous electronic signal proportional to the concentration of a specific chemical or group of chemicals. The setup of a biosensor is made of: 1) a biological receptor, for the recognition of sample analyte; 2) a transducer, which transforms the signal resulting from the interaction of the analyte with the biological element into a useable signal; 3) a detection technique, for signal analysis and processes (Kivirand et al., 2011).

Biosensors are currently very appealing in the food industry due to their capability of direct measurement of a wide range of analytes. Indeed, biosensors can provide information (Mehrotra et al., 2016). The specificity of biologically-based sensing systems against toxins makes biosensor ideal candidates in quality and safety control analysis. Nevertheless, to obtain a reliable detection of BAs, more effort is required on the improvement of their selectivity and sensitivity as well as their miniaturization. In this thesis, the aim is to obtain high selective biosensors, employing antibodies as biological receptors, due to high specific chemical interaction antibody-antigen. Furthermore, to increase the sensitivity of biosensors nanomaterials will be used. Nanomaterials are known for the amplification of conductivity, biocompatibility signals and catalytic activity of biosensor.

2. PhD Thesis Objectives and Milestones

The goal of this thesis will be the development of an integrated biosensor platform (sensor arrays), to analyze a variety of hazards or biogenic amines simultaneously. To accomplish this, the work will be divided into three main stages:

A1) Development of the biosensor for analysis of histamine in food samples.

(A1.1) Fabrication of electrochemical sensor through printed technologies, characterization of the surface morphology and electrical properties then (A1.2) electrochemical measurement of different concentrations of histamine.

A2) Development of biosensor based on field effect transistor (FET) for hazard detection in food samples. (A2.1) Fabrication and functionalization of FET, (A2.2) measuring different concentrations of hazards in food samples and (A2.3) comparison of electrochemical and FET sensors in terms of sensitivity.

A3) Development of low-cost biosensor arrays for analysis of hazards in meat samples. (A3.1) Great focus will be given to the development of the array, (A3.2) then functionalization and testing will take place.

A4) Thesis and paper preparation.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Development of an electrochemical biosensor																									
1) Surface characterization and electrical properties																									
2) Biosensor testing																									
A2) Biosensor based on FET																									
1) Fabrication and functionalization of FET																									
2) FET testing																									
3) Comparison of sensors																									
A3) Biosensor arrays																									
1) Array development																									
2) Array testing																									
A5) Thesis and Paper Preparation																									

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Potential and Pitfalls of Cellulose Nanocrystals in Advanced Packaging Materials: their Role in Food Safety and Food Protection

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The objective of this PhD research is the investigation of cellulose nanocrystals (CNCs) used in food packaging field and their role in food protection. For this purpose, potential and pitfalls of CNCs in advanced packaging materials will be studied improving safety-testing methodologies to support Risk Assessment and Enforcement of food contact nano-materials (FCM) as requested by European institutions. An in-depth physicochemical characterization of CNCs will be carried out, together with potential migration study when implemented in FCM. The study will be complemented by the development of packaging materials with “optically active” performances capable of reducing photodegradation of food and beverage.

Potenzialità e limiti di nanocristalli di cellulosa (CNC) impiegati in materiali di imballaggio avanzati: il loro ruolo nella sicurezza alimentare e nella protezione degli alimenti

L'obiettivo di questa ricerca è lo studio dei nanocristalli di cellulosa (CNC) utilizzati nel settore dell'imballaggio alimentare e il loro ruolo nella protezione degli alimenti. Saranno studiate le potenzialità e i limiti dei CNC nei materiali di confezionamento avanzati, migliorando le metodologie di test per supportare la valutazione del rischio e la loro applicazione in materiali per contatto alimentare (FCM). Verrà effettuata una caratterizzazione chimico- fisica approfondita, insieme ad uno studio di migrazione di CNC quando implementati in FCM. Lo studio sarà completato dallo sviluppo di materiali con prestazioni "otticamente attive" in grado di ridurre la fotodegradazione di alimenti.

1. State-of-the-Art

Nanotechnology is the ability to work on a scale of about 1–100 nm in order to understand, create, characterize and use material structures, devices and systems with new properties derived from their nanostructures (De Azeredo, 2009). New properties and functions of nanoscaled engineered materials present new opportunities to enhance traditional product performance; thus, they have been considered as alternative novel materials across a wide range of fields. The food and beverage industry has been a recent focus for nanomaterial applications. In Europe, packaging applications represent about 40% of the total plastics demand, representing the largest application sector. There is an increasing trend for the use of bio-based materials due to plastic accumulation problem in the environment. However, the use of biopolymers as food packaging materials has been limited by poorer mechanical, thermal, and barrier properties, as compared to conventional materials. A way to overcome these disadvantages is proposed by using engineered nanoparticles which are supposed to have better mechanical and barrier properties (Vilarinho et al., 2018). Being cellulose the most common and widespread polymer resource available in nature, more attention has been paid on its use within the engineered nanomaterials. Especially application of CNCs into plastic and paper materials has led to the improvement of flexibility, barrier properties, mechanical strength, thermal resistance and anti-fog properties of materials (Khan et al., 2012; Li et al., 2013; Nair et al., 2014; Mascheroni et al., 2016; Rampazzo et al., 2017)

but while these properties have already been improved, a few research focused on the ability of CNCs to modify optical properties of a plastic material to protect the food from detrimental effect of light exposure. Furthermore, all these functionalities obtained by using CNCs in packaging materials are not well correlated with whether these improvements can extend the shelf-life or not. With a specific reference to food packaging applications, the crystalline and nanoscale forms of cellulose seem to be a resource not studied and exploited in an appropriate manner, especially from a safety point of view. When CNCs are intended to use as food contact material (FCM), they must fulfill some regulatory requirements. Indeed, nanocellulose has properties that may be associated with health risks due to its needle-like shape and biopersistence in the body similarly to inorganic fibers. However, in contrast to rigid mineral inorganic fibres, CNC has a flexible structure, which may eliminate the safety concerns. Nevertheless, no study is present in the literature about CNCs safety in food packaging application because a comprehensive risk assessment based on their potential of migration into food is necessary to support the application as FCM. This lack of information is also highlighted by European Food Safety Authority (EFSA).

On the basis of these remarks, this PhD study will be divided into two main parts, namely, i) food safety assessment of CNCs; ii) food protection conveyed by special properties of CNCs applied to plastic materials. Mainly, the first task aims at developing a structured pathway useful to carry out safety assessment of this kind of organic CNCs, providing also suggestions for useful methods for physicochemical characterization, exposure assessment and hazard identification. Moreover, on the front of new functionality of CNCs, the use of these crystals layered onto plastic films will be investigated exploiting unique optical properties of CNCs in aqueous solutions able to create selective absorption of visible wavelengths, thus protecting foods from photodegradation.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Safety assessment of CNCs as pristine material for food contact applications: Selection of appropriate techniques for the CNCs characterization, physico-chemical characterization of pristine CNCs coming from different sources, characterization of CNCs in FCM and stability assessment.

A2) Safety assessment of CNCs in food contact materials: CNCs migration potential into simulants in different conditions, potential indirect exposure estimation and hazard identification.

A3) Functionalization of CNCs to protect food products: Investigation of liquid crystalline properties of CNCs (i.e. CNCs morphology, chemistry and spatial distribution) functionalization and development of «optically active» films; role of «optically active films» in protecting light-sensitive food products.

A4) Dissemination: Writing and editing of the PhD thesis, scientific papers and oral and/or poster communications, knowledge sharing with the official bodies (i.e. EFSA) and final users of FCM.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Safety Assessment of CNCs: 1 st part																								
1) Physico-chemical characterization of pristine CNCs																								
2) Stability assessment																								
A2) Safety Assessment of CNCs: 2 nd part																								
1) Migration and exposure assessment																								
2) Risk characterization																								
A3) Food Protection																								
1) Investigation of liquid crystalline properties																								
2) Optically active film development																								
3) Shelf-life assessment																								
A4) Thesis and Paper Preparation																								

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Sustainable Development of Craft Beer in Sardinia: the Reuse of Brewer's Spent Grains

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The general objective of the PhD thesis is to increase the national and international competitiveness of regional brewing productions by creating an innovative and virtuous model of circular economy. Particularly, the specific objective is focused on the possible re-use of brewing wastes. Among these, brewers' spent grains, the main by-product of brewing, require a proper valorization and a practical alternative to their disposal. Through the transformation of wastes into resources and the implementation of research activities into production systems, it will be possible to propose productive models based on environmental and economic sustainability

Sviluppo Sostenibile della birra artigianale in Sardegna: il recupero delle trebbie di Birra

Questo progetto di ricerca di tesi di dottorato si occuperà dei diversi aspetti dell'intera filiera della birra artigianale. L'obiettivo generale del progetto è quello di rendere le produzioni birrarie regionali più competitive sul mercato nazionale e internazionale creando un modello innovativo e virtuoso di economia circolare. In questo contesto, l'obiettivo specifico di questa tesi è di concentrarsi sul possibile reimpiego dei prodotti di scarto (le trebbie e le acque reflue). L'attività di ricerca si concentrerà sulla trasformazione delle trebbie da un prodotto di scarto a una nuova materia prima. I residui della produzione di birra verranno trasformati in risorse.

1. State-of-the-Art

The production of beer is constantly increasing all over the world. In Italy in 2017 it reached a historical maximum of 15.6 million hectoliters of beer, with a per capita consumption of 31.8 liters. Consequently, a massive increase of wastes and by-products deriving from the brewing process has been observed. In Sardinia the craft beer sector is in continuous exponential growth in economic terms. Today there are more than 40 breweries (artisans, agricultural and beerfirm) surveyed by Confartigianato Imprese Sardegna (there were 19 in 2009). On average, the production of each brewery does not exceed 1,000 hectoliters per year. A world, that of small breweries, which enriches the Sardinian territory, where quality and craftsmanship are the fundamental coordinates in which the processing method, the quality of the raw materials used as well as the professionalism, passion and competence of the master brewer are key elements. Despite the ever-increasing numbers, innovations in the production chain and the definition of craft beer 100% "made in Sardinia" are considered strategic. For craft breweries, it is mandatory to utilize high quality raw materials from local producers, to strengthen the link between beer and the production area. In addition, the increase in the sustainability of production could be attained through the proper valorization of by-products. Brewers' spent grains (BSG) are the most abundant by-product of brewing (217,000 tons in Italy in 2017), representing 85% of the total by-products; with an average yield of about 20 kg per hectolitre of beer produced. BSG originate from the hot extraction of malt and are produced throughout the year, both by large and small breweries. The chemical and microbiological composition of BSG depends on the raw materials and on

the fermentation process: barley variety, year of harvest, malt and maceration conditions. BSG are nutrient rich substrates that can be used in different sectors, thus they could become a source of income for the brewery. In addition, BSG are interesting raw materials for food ingredients, they have high fibre content, proteins and potential phenolic antioxidants. The proteins in BSG are rich in glutamine which has been reported to promote the recovery and preservation of intestinal mucosa and to prevent bacterial translocation from the gastrointestinal tract. BSG are potential sources of soluble xylo-oligosaccharides, provided that the hemicelluloses are first hydrolysed into oligosaccharides. Xylo-oligosaccharides are indigestible by human enzymes in the small intestine, but are extensively fermented by desirable bacterial species, such as Bifidobacteria, in the large bowel and therefore have prebiotic activity. However, all the described uses of BSG are limited by their high-water content, their microbiological instability and the difficulties in their storage for long time. Thus appropriate biotechnologies should be implemented for their possible valorization.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) Brewers' spent grains will be used as raw substrates to produce vermicompost, a high-value agricultural fertilizer A.1.1) Chemical (enzymatic assay, mycotoxin content, total nitrogen and carbon, humic and fulvic acids) and microbiological analyses of the BSG before the process; A.1.2) Start-up of breeding vermibeds and monitoring of vermicomposting; A.1.3) Chemical (enzymatic assay, mycotoxin content, total nitrogen and carbon, humic and fulvic acids) and microbiological analyses of the BSG after the process

A2) A composted organic fertilizer enriched with rhizosphere microorganisms will be obtained from Brewers' spent grains; A.2.1) Chemical analysis of BSG and zootechnical waste to be used for composting; A.2.2) Monitoring of the composting process of different BSG / waste mixtures; A.2.3) Chemical analysis (Organic Substance, pH, Nitrogen, Phosphorus, C / N, electrical conductivity, heavy metals, humification index) of the final composts and identification of the two mixes with the best chemical characteristics; A.2.4) Pelletizing of the final composts and subsequent enrichment of the pelleted material by adding selected microbial consortia

A3) Brewers' spent grains will be used as substrates for microbial growth to produce Single Cell Proteins. A.3.1) isolation and identification of microorganisms able to produce proteins from the carbon sources in BSG; A.3.2) use of the selected microorganisms in solid state fermentation and submerged culture (preparation of the substrates, optimization of fermentation conditions); A.3.3) scale up from laboratory to industrial scale; A.3.4) Chemical analyzes of the final product (determination of total proteins, fatty acids)

A4) Brewers' spent grain will be used as ingredients to obtain functional food for human and animal nutrition. The following activities will be performed: A.4.1) preparation of BSG; A.4.2) Immobilization of microorganisms such as Bifidobacteria on BSG; the main factors influencing the growth and metabolic activity of each strain used in association with the BSG substrate will be identified; A.4.3) Manufacturing and formulation of the functional food: incorporation of the compound microorganisms / BSG; A.4.4) Evaluation of the nutraceutical effects of the microorganism / BSG association with in vitro trials; (bioavailability, toxicity, stability, solubility, formulation); A.4.5) Animal testing: the functional food containing BSG and the immobilized microorganisms will be used to feed mice. The effect of the functional food on mouse microbiota will be studied in the faeces of mice by next-generation sequencing, DGGE, quantitative qPCR with specie specific primers.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A1) vermicompost																									
1) Chemical and microbiological analysis before vermicomposting																									
2) vermibeds and vermicomposting																									
3) Chemical and microbiological analysis after vermicomposting																									
A2) Soil improver																									
1) Chemical analysis																									
2) Monitoring composting process																									
3) Chemical analysis aftercomposting																									
4) Enrichment of the pelleted																									
A3) Low-cost substrate																									
1) isolation and identification of microorganisms																									
2) preparation of the substrates																									
3) scale up																									
4) Chemical analyzes of the final product																									
A4) Food ingredient																									
1) preparation of BSG																									
2) Immobilization of microorganisms																									
3) Manufacturing and formulation																									
4) Evaluation of the nutraceutical effects																									
5) Animal testing																									
A5) Thesis and Paper Preparation																									

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Novel Approaches in Healthy Cream Development

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The aim of the present PhD project is to develop and characterize new health creams using novel approaches such as the introduction of oleogels, the use of alternative natural sweeteners and bioactive ingredients in order to reduce the caloric value and improve the nutritional profile.

Approcci innovativi nello sviluppo di creme salutistiche

Lo scopo del presente progetto di dottorato è lo sviluppo e la caratterizzazione di nuove creme salutistiche attraverso approcci innovativi, come l'introduzione di oleogels, l'utilizzo di dolcificanti naturali alternativi e ingredienti ricchi in composti bioattivi per ridurre il potere calorico e migliorare il profilo nutrizionale.

1. State-of-the-Art

Creams are generally characterized by a medium-high calorific power and their sensory performance is strictly related to the presence of a large amount of saturated fat and sugar providing taste, texture, mouthfeel and flavour to the product. It is well known that the consumption of excessive amounts of saturated fats increase the incidence of cardiovascular and other related diseases. Solid fats produced from oils by hydrogenization, interesterification, and fractionation processes are widely used in different foodstuffs.

An emerging strategy is to replace solid fat with unsaturated oils solidified through molecules (oleogelators) forming self-assembly networks. These systems called oleogels are thermo-reversible, anhydrous and viscoelastic materials. Recently, the use of oleogels has been studied in ice cream, chocolate containing products, bakery products, frankfurters and margarine (Marangoni & Garti, 2018). There is a large number of oleogelators that can be used to gel oils. Saturated monoglycerides and natural waxes are a promising strategy in food applications because they are able to gel oil at low concentrations (<10%) and are inexpensive (Pehlivanoglu et al., 2018). Several type of vegetable oil, such as sunflower oil, corn oil, olive oil, canola oil, and hazelnut oil, can be used to prepare oleogels; Potential application of pomegranate seed oil oleogels based on different type of waxes as partial substitutes of palm oil in functional chocolate spread has been investigated (Fayaz et al., 2017). Oleogel of ethylcellulose and beeswax, prepared with a lipid mixture of olive, linseed and fish oils, have been used as substitutes for animal fat for pork liver pâté (Gómez-Estaca et al., 2019). In the work of Moghtadaei et al., (2018), oleogels were prepared from sesame oil and beeswax and employed in the formulation of beef burgers. Therefore, an innovative strategy could be to create oleogels from polyunsaturated oils naturally rich in vitamins and bioactive compounds, e.g. pomegranate oil, walnut oil, hemp oil, sesame oil and chia seed oil, to improve the overall nutrition profile of the final product.

Several studies have shown that a diet high in sucrose is associated with several diseases; replacing sugars seem to reduce the energy intake decreasing body weight. Current focus to reducing sucrose intake concerns the replacement by artificial sweeteners which, even if they are calorie free, can impart bitter flavour to the product.

The combined use of alternative sweeteners and bulking agents is still one of the most used approach to reduce sugar content in food, e.g. intense sweeteners together with fibers. An innovative strategy is the use of multisensory integration principle (Di Monaco et al., 2018). Another approach could consist in the application of a natural ingredient with a low glycemic index, such as maple syrup, agave nectar, brown rice syrup, honey; lucuma (*Pouteria obovata*) and yacon (*Smallanthus sonchifolius*), naturally rich in potentially beneficial bioactive compounds (Ak-Cvitanović et al., 2015). Through this strategy the caloric content will be reduced and the nutritional profile improved.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Literature analysis

A2) **Market online survey** to gather information on consumer expectations and to define the concept of healthy cream to develop.

A3) **Fat and sugar phases improvement.** Different oils/oleogelators mixtures will be selected and characterized, by means of rheological, thermal and microstructure determinations (A3.1), in order to use the minimum concentration of molecules forming networks and to obtain the optimal oleogel formulation. Several sweetening systems will be selected and characterized, by means of physical and sensory evaluations (A3.2).

A4) **Formula and process optimization of creams.** Process conditions of new creams will be developed and new ingredients will be balanced to obtain the optimal replacer levels of solid fat and sugar (A4.1). Physico-chemical and sensory properties of new creams will be studied (A4.2) and the actual bioavailability of the bioactive compounds will be verified (A4.3).

A5) **Pilot production of optimal creams.** A pilot production of the optimal formulations will be performed and the oxidative stability of spreads will be evaluated, by means of accelerated shelf life tests (A5.1). Innovative methodologies of consumer science will be used to investigate. The effect of using new ingredients on consumers behaviour and on the acceptability of new products (A5.2).

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Literature analysis																								
A2)	Market online survey																								
A3)	Fat and sugar phase improvement																								
	1) Oleogels development and rheological, thermal and microstructure determinations																								
	2) Physical and sensory characterization of different sweetening system																								
A4)	Cream optimization																								
	1) Process condition and ingredient level																								
	2) Physico-chemical and sensory properties																								
	3) In vitro bioavailability																								
A5)	Pilot production of optimal creams																								
	1) Shelf life prediction																								
	2) Consumer test																								
A6)	Thesis and Paper Preparation																								

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Exploring the Virulence of *Arcobacter butzleri* during Simulated Infection of Human Gut Models

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This PhD thesis research project aims on *Arcobacter butzleri* that is an emerging foodborne pathogen often isolated from pork, chicken and beef meat, which causes different gastrointestinal diseases, such as diarrhoea and abdominal pain, in human and animals due to its invasive behaviour. However, the pathogenicity of *A. butzleri* is still underestimated due to a substantial lack of information on its virulence mechanisms, metabolic and genomic features. In this study we obtain a first description of the mucus role in the infection and different design of analysis protocols for the future test.

Esplorando la virulenza di *Arcobacter butzleri* durante l'infezione simulata di modelli intestinali

Questo progetto di tesi di dottorato si occupa di *Arcobacter butzleri*, un patogeno emergente presente nel cibo frequentemente isolato da carne di suina e bovina e di pollo e che causa differenti sintomi gastrointestinali come diarrea e dolori addominali in uomo e animali a causa del suo comportamento invasivo. Nonostante ciò la patogenicità di *A. butzleri* è sottostimata a causa della penuria di informazioni riguardanti il suo meccanismo di virulenza e aspetti metabolici e genomici. In questo studio abbiamo ottenuto informazioni riguardanti il ruolo del muco nell'infezione e messo appunto differenti protocolli d'analisi per le prove future.

1. State-of-the-Art

Arcobacter butzleri (Vandamme P *et al.*, 1991) is an emerging foodborne pathogen included in Campylobacteraceae family often isolated from pork, chicken, beef meat and water, which causes different gastrointestinal symptoms such as abdominal pain, vomit, diarrhea, in humans and animals due to its invasive behavior. However, the pathogenicity of this gram negative bacteria is still underestimated due to a substantial lack of information on its virulence mechanisms, in bibliography are presents different ecological work about these bacteria but few work on the virulence mechanism, in particular about genomics and transcriptomics data (Ferreira, Queiroz, Oleastro, & Domingues, 2014). The ecological works show the presence of *A. butzleri* in different environmental compartment and host such as waste and marine water, plants, animals (pigs, cattle, poultry) and in clinical cases. In the case of *A. butzleri* infection in addition to the previously cited symptoms has occurred cases of septicemia. For the wide presence on food and for the pathogenicity of this bacteria specie, new study about virulence mechanism will be useful to describe its role in clinical cases (Ramees *et al.*, 2017).

2. PhD Thesis Objectives and Milestones

This study aims to explore, in simulated host-pathogen interactions, colonization and invasion capability of different strains of *A. butzleri*, thanks the use of different intestinal models (mucus producers and not mucus produces cell models). In addition, an initial study virulence gene expression was performed on the type strain, to obtain first data useful for following test, such as genomics and transcriptomics studies on other strains.

The aims mentioned for my PhD project can be subdivided into the following activities according to the Gantt diagram (Figure 1).

2.1 Colonization and invasion assay

To determinate the colonization and invasion ability of the bacteria specie in object, after 14 days from the models cellular confluence, a load of $6.5 \pm 0.5 \text{ Log CFU cm}^{-2}$ was inoculated on three no mucus (Caco-2) and mucus producers (Caco-2/HT29 MTX, 9/1) cell models. After 90 minutes from the inoculum colonization and invasion have been determinated with an omogeneization (Triton-X 0,25%) and with gentamicin application followed from an omogeneization respectively.

2.2 Study of bacteria translocation

To determinate the traslocational ability of the bacteria through the cell layer 3D models have been produced and inoculated at the attainment of a TEER (trans epithelial endothelial resistance) of $1000 \Omega \text{ cm}^{-2}$. To determinate the bacteria passage, after 1, 2, 4, 6, 24, 30 and 48 hours, microbiological load sampling has been performed in the basolateral compartment. In parallel a TEER mesurament was executed to determinate the cell layer integrity.

2.3 RT-qPCR gene expression study

An *ex novo* primer design (Thornton & Basu, 2011) was performed for nine putative virulence gene (*cadF*, *ciaB*, *cj1349*, *irgA*, *hecA*, *hecB*, *mviN*, *pldA*, *tlyA*) (Doudah et al., 2012) on *A. butzleri* genome (type strain LMG 10828^T), their relative expression was quantified by Reverse Transcriptase (RT)- quantitative (q)PCR under simulated host-pathogen interaction conditions. To collect cDNA to analyzable, *in vitro* gut models of mucus-producing and not producing human cells were inoculated with the pathogen, and total RNA was obtained at different timeframes. The gene expression determination has been calculated with the relative gene expression methods “double delta cq”.

2.4 Genomics

The genomes of the strains in object will be sequenced with Illumina Myseq (NGS) to obtain data about different genomes from strains of this specie to link at the colonization and invasion tests.

2.5 Gene annotation

To obtain information about the role of genes sequenced from the previous step, the genomes obtain will be processed with the bioinformatic software Prokka, this to have a gene annotation fundamental to predict the function of *A. butzleri* genes (Seemann, 2014).

2.6 Transcriptomics

RNA collected at different times form the initial inoculum will be study with RNAseq, this to obtain data about the real expression of possible virulence gene. The collecting of RNA will be performed on no mucus and mucus producers cell models, this to botain data about the role of intestinal mucus on *A. butzleri* virulence mechanism.

2.7 Transcriptomics confirmation

The confirmation of trascriptomic data will be performed through different qPCR on the more relevant genes. This adpting the methodology at the point 2.3.

2.8 Proteomics

In conclusion a proteomics study will be executed to obtain data about the most interesting protein in dependence of the gene expression data. This will be important to obtain a further data confirmation and to obtain sequence and structure of the most relevant proteins linked to the *A. butzleri* virulence mechanism.

2.9 Writing and presentations

My PhD work will be presented at different workshop and congress such as IAFP symposium, as well as at the final thesis discussion. The results from my project will be published on specialized journals.

Table 2 Gantt diagram for this PhD thesis project.

year	First year											Second year											Third year													
month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
2.1 Colonization and invasion assay																																				
2.2 Study of bacteria translocation																																				
2.3 RT-qPCR gene expression study																																				
2.4 Genomics																																				
2.5 Gene annotation																																				
2.6 Transcriptomics																																				
2.7 Transcriptomics confirmation																																				
2.8 Proteomics																																				
2.9 Writing and presentations																																				

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Combined Antimicrobial Effect of Essential Oils and other Natural Products (bacteriocins, Propolis, Polyphenols, chitosan, etc.) against *Listeria monocytogenes* in Ready-to-eat Seafood

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Doctorate in Agri-Food Sciences, Technologies and Biotechnologies

This PhD thesis research project aimed to isolate and characterize Lactic Acid Bacteria (LAB) strains from selected seafood in order to demonstrate the ability of some of them to produce bacteriocins with antibacterial activity. Subsequently, the antibacterial activity of EOs was investigated both alone and in combination with other natural products bacteriocins, nisin, chitosan, propolis and polyphenol, to evaluate a possible synergistic effect between various natural products against the psychrotropic pathogen *Listeria monocytogenes*.

Effetto antimicrobico combinato tra oli essenziali e prodotti naturali (batteriocine, propoli, polifenoli, chitosano, ecc.) contro *Listeria monocytogenes* in frutti di mare pronti al consumo

Questo progetto di tesi di dottorato mira a isolare e caratterizzare ceppi di LAB da prodotti di mare (sardine, ecc.), precedentemente, selezionati per dimostrare l'abilità dei LAB nel produrre batteriocine con attività antibatteriche. Successivamente viene valutata l'attività antibatterica di oli essenziali, da soli o in combinazione con altri prodotti naturali come batteriocine, nisin, chitosano, propoli e polifenoli contro batteri psicotropici, come *Listeria monocytogenes*.

1. State-of-the-Art

The increased resistance of fish pathogens to conventional treatments has lead researchers to investigate the antibacterial properties of natural resources, such as essential oil (EOs) of plants (da Cunha et al., 2018) and bacteriocins, produced by LAB (Lactic Acid Bacteria), alone or combined among them and with nisin, Polyphenols, chitosan and propolis, in an effort to find products that are less harmful to the environment and human health. The trend towards the consumption of minimally processed ready-to-eat (RTE) or foods consumed raw has affected the incidence of diseases caused by psychrotropic bacteria, such as *Listeria monocytogenes* (Anacarso et al., 2014). Various strategies based on the addition of chemical preservatives have been applied, but these types of treatments have many drawbacks and limitation, including the already known toxicity of many chemical preservatives. For this reason and to harmonize the consumer demands for necessary safety standards, the research has been oriented toward a natural preservation approach. Both bacteriocins and most EOs are recognized as safe (GRAS) by the FDA and have shown an important role in the control of pathogens (including antibiotic-resistant bacteria), and spoilage bacteria, with feasible application in various foods (Lacroix, 2007). The first purpose in this study the LAB strains were to isolate from selected seafood and characterize, in order to demonstrate the ability of some of them to produce bacteriocins. Safety aspects of bacteriocinogenic strains and the properties of the best antibacterial substances were also investigated for their potential use in food preservation. The second purpose was to use EOs alone and in

combination with bacteriocins, nisin, chitosan, propolis and polyphenols in order to demonstrate the synergistic antibacterial activity against pathogenic strains and their potential combined use in food storage.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activity:

A1) Isolation of LAB strains and bacteriocins purification: One-hundred and forty-three (143) *Enterococcus* spp. were isolated from different commercial seafood products.

A2) Determination of the antibacterial activity: Bacteriocin production was screened by the deferred antagonism method using as indicators Gram-positive and Gram-negative bacteria belonging to different genera or species. The antibacterial ability of the best producer strains was confirmed by agar well diffusion assay.

A3) Agar disk diffusion assay: this test was used to screen the antibacterial activity of eight Essential Oils (EOs): *Mentha piperita*, *Melaleuca alternifolia*, *Salvia officinalis*, *Thymus vulgaris*, *Citrus limon*, *Citrus aurantium*, *Zingiber officinalis* and *Rosmarinus officinalis* against *Listeria monocytogenes* spp. Studies are underway on the antibacterial activity of other natural substances such as: chitosan, propolis, nisin and polyphenols.

A4) Minimum Inhibitory Concentration (MIC): the MIC of *Thymus vulgaris*, *Salvia officinalis* and *Melaleuca alternifolia* against *Listeria monocytogenes* spp. were determined using the microwell dilution method.

A5) Fractional inhibitory concentration index (FICI): The combined antibacterial activity between *Thymus vulgaris*-*Salvia officinalis* and EOs- bacteriocins was determined with 96 microplates method and future studies with other essential oils will be performed.

A6) Biofilm: the activity of *Salvia officinalis* and *Thymus vulgaris* against a complex aggregation (biofilm) of *Listeria monocytogenes* spp. was evaluated. In the future other essential oils and natural compounds will also be analyzed

A7) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	LAB strains and bacteriocins analysis																								
	1) Isolation of LAB																								
	2) Purification of bacteriocins																								
A2)	Antibacterial activity																								
	1) Deferred antagonism																								
	2) Agar well diffusion assay																								
A3)	Agar disk diffusion assay																								
	1) Antibacterial activity of 8 EO's against <i>Listeria monocytogenes</i>																								
	2) Antibacterial activity of chitosan, propolis, nisin and polyphenolis against <i>Listeria monocytogenes</i>																								
A4)	MIC of <i>Thymus vulgaris</i>, <i>Salvia officinalis</i> and <i>Melaleuca alternifolia</i> against <i>Listeria monocytogenes</i>																								
A5)	Fractional inhibitory concentration index (FICI)																								
A6)	Biofilm																								
A7)	Thesis and Paper Preparation																								

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- Anacarso, I., Messi, P., Condò, C., Iseppi, R., Bondi, M., Sabia, C., & de Niederhäusern, S. (2014). A bacteriocin-like substance produced from *Lactobacillus pentosus* 39 is a natural antagonist for the control of *Aeromonas hydrophila* and *Listeria monocytogenes* in fresh salmon fillets. *LWT Food Science and Technology*, 55, 604-611.
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Innovative Technological Methodologies as a Tool to Improve Food Nutritional Properties and Palatability: Legumes for All!

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It's well known that legumes represent one of the cornerstones of the Mediterranean Diet due to their nutritional properties. Nowadays, legumes consumption is not common in Italian population above all due to the low palatability, digeribility and time-consuming preparations. In this regard, food habits will be further investigated, and innovative functional foods will be developed to promote legumes consumption. In addition, effects of different technological processes, cooking and storage methods on the nutritional properties of legumes will be evaluated. In parallel, nutritional education will represent a key point to improve knowledge and awareness of consumers.

Metodologie Innovative Come Strumento per Migliorare le Proprietà Nutrizionali e il Gusto dei Legumi

Com'è ormai ben noto, i legumi rappresentano un elemento cardine della Dieta Mediterranea. Nonostante i legumi presentino numerose proprietà, la loro assunzione non è comune nella popolazione italiana, a causa di diversi fattori: scarsa palatabilità, digeribilità e del tempo necessario per la loro preparazione in cucina. In parallelo è necessario valutare le abitudini alimentari e il consumo di legumi in modo da elaborare nuovi prodotti funzionali per favorirne l'assunzione. Inoltre, verranno analizzati anche gli effetti che diversi metodi di conservazione e cottura dei legumi hanno sulle loro caratteristiche nutrizionali. Tale studio sarà poi volto a promuovere l'educazione alimentare, utile per descrivere al consumatore le proprietà benefiche dei legumi, incentivarne l'uso e al tempo stesso sviluppare consapevolezza delle proprie scelte alimentari.

1. State-of-the-Art

Many studies demonstrate that conscious food choices and lifestyle correlate with health (Jezewska-Zychowicz et al., 2018;). In this context, the principles of the Mediterranean dietary pyramid are the universally accepted guideline for human nutrition and legumes are a cornerstone of the Mediterranean diet. Legumes are a source of vegetable proteins, fiber, minerals, vitamins and antioxidants. Scientific evidence has demonstrated that Mediterranean diet reduces the risk of cardiovascular diseases (Ahmad et al, 2018) and chronic conditions, such as diabetes and metabolic disorders and legumes have an important preventive role (Papandreou et al., 2018).

Nutritional surveys reported that Italians general trend is based on an inadequate or absent legumes consumption because of several factors such as long cooking preparation time, meteorism and scarce palatability.

Moreover, it is useful to take into account there are different types of legumes available in the market: dry, fresh, steamed, canned and flaked. The transformation process used to produce them can differently impact on their nutritional properties (Güzel et al., 2012).

To promote legumes intake, it could be convenient to offer to consumers new nutritionally balanced and palatable dietary products with legumes, that do not require time for cooking and, if possible, that are not disturbing for intestine. These combinations could participate in covering daily nutritional needs and could aid to optimize legumes nutritional power.

2. PhD Thesis Objectives and Milestones

This PhD project will be addressed to improve legumes consumption through alternative legumes-based food preparation.

One of the primary goals will be studying balanced and tasty nutritional combinations to introduce legumes in the diet. New combinations will be proposed for different types of consumers and practical factors (daily time available for cooking, palatability...). In parallel, the impact of different cooking methods on nutritional properties and anti-nutritional factors of legumes will be evaluated using specific analytical techniques.

The PhD thesis project can be divided into the following activities according to the Gantt diagram provided in Table 1:

A1) Analyse food habits and behaviour in the population by using specific food questionnaires. The definition of habits and food choices (A1.2) is fundamental to analyse legumes intake using validated food questionnaires and to elaborate strategies to improve legumes use in the diet (A1.2). Data will be collected also implementing statistical analysis (A1.3).

A2) Evaluation of innovative food combinations with legumes to improve their nutritional properties and optimize their consumption. It is of fundamental importance to find associations that can satisfy nutritional needs of population (A2.1) and to study the best timing and occasions of intake (A2.2). Effects of the intervention will be evaluated by using ad hoc questionnaires and all data will be processed and statistically analysed (A2.3).

A3) Impact of different technological methods on legumes nutritional properties. The positive effects of legumes are due to their nutrients (A3.1) but these are not maintained using some types of transformation processes. Processed legumes (for example steamed, soaked, flaked and puffed) will be characterized by chemical and biochemical analyses (A3.2) to evaluate changes in nutritional and anti-nutritional properties. All data will be processed and statistically analysed (A3.3).

A4) Nutritional education approach as a tool to sensitize population. Nowadays, fake news about nutrition is common and the effectiveness of nutritional tips is still low (A1.1). Therefore, it is fundamental to find new strategies to easily communicate with all the population (A4.2) to promote nutritional knowledge. Effects of the intervention will be evaluated by using ad hoc questionnaires (A4.3).

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications (A5).

Table 1 Gantt diagram for this PhD thesis project.

Activity Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Analyse population food habits using specific food questionnaires.																								
	1) Literature review																								
	2) Elaboration of food questionnaires																								
	3) Data analysis																								
A2)	Evaluation of innovative food combinations with legumes																								
	1) Formulations development																								
	2) Optimization																								
	3) Data processing																								
A3)	Impact of different technological methods on legumes nutritional properties.																								
	1) Literature review																								
	2) Analytical Characterization of processed legumes																								
	3) Data processing																								
A4)	Nutritional education approach as a tool to sensitize population.																								
	1) Literature review																								
	2) Elaboration of activities to promote nutritional information																								
	3) Data analysis																								
A5)	Writina and Editing																								

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Biocatalytic and Fermentative Approaches for the Production of Added Value Compounds Using Wild Type and Genetically Modified Acetic Acid Bacteria (AAB)

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The goal of the current PhD research project is the production of added value molecules using wild type and genetically modified acetic acid bacteria. Different biocatalytic and fermentative approaches will be studied using agro-food wastes as feedstocks.

Utilizzo di batteri acetici per il riutilizzo di scarti agro-alimentari

Questo progetto di dottorato si propone di utilizzare batteri acetici wild type e geneticamente modificati per la valorizzazione di scarti di origine agro-alimentare verso la produzione di molecole e biopolimeri ad alto valore aggiunto.

1. State-of-the-Art

Acetic acid bacteria (AAB) are gram-negative obligate aerobe bacteria, in the family of *Acetobacteraceae*, which can oxidize in a regio and stereo-selective manner different substrates, such as carbohydrates and alcohols, releasing the corresponding acids into the medium. For this reason, AAB have been always used in the food and biotechnology industry for their ability to perform incomplete oxidative fermentations: typical applications of AAB are the 2-keto L-gluconic acid and vitamin C production.

These reactions are catalyzed by membrane-bound dehydrogenase (ADH) that are stick to the periplasmic side of the bacterial membrane. Those enzymes are highly specific for alcohols (ADH) and aldehydes (ALDH) and lead to the complete oxidation of substrates by sequential reactions. For instance, ethanol is first oxidized to acetaldehyde and sequentially to acetate respectively by the quinoprotein-cytochrome complex (ADH-PQQ) and molybdoprotein-cytochrome complex (ALDH). (La, Luciana, and Maria 2018)

Another important matter is the ability of different AAB to produce cellulose. The polysaccharide is secreted outside the cells by an enzymatic complex called Bacterial Cellulose Synthase (BCS) and lead to the formation of a thick biofilm. The CSs enzymatic complex is composed of different specific subunits involved in the synthesis and exportation of the cellulose chain outside the cytoplasmic membrane. (Gullo 2018)

The main peculiarities of this material are the extreme purity and crystallinity, the high-water holding abilities and the mechanical stress resistance. Due to the mentioned peculiarities, this material represents an optimal alternative to plant-derived cellulose for specific applications. This material finds application in the food industry as an additive, in the medical field is used as drug delivery and for wound healing. (Rovera et al. 2018) *Komagataeibacter xylinus* is the most used strain for bacterial cellulose production because of its ability to secrete a good amount of this exopolysaccharide starting from different substrates.

Despite the wide use of AAB in different application, hitherto only few examples of metabolically engineered Acetic Acid Bacteria are used in large scale processes. Genetic

engineering of these microorganisms could expand the amount of feedstocks convertible into value-added compounds by whole-cell biocatalysis. The aim of this research is to improve the native biotechnological features of different acetic acid bacteria. One topic of this project will be focused on the chromosomal integration and overexpression of BCS operon in Acetic Acid Bacteria for enhanced expression of the biofilm.

Another topic of this PhD work will be the cloning of gene encoding for a limonene monooxygenase (cytochrome P450) in different AAB strains in order to obtain a biological system able to perform regioselective hydroxylation and oxidation by whole-cells biotransformation (Scheme 1). Cheap and highly available compounds such as Limonene, a compound of the terpene family, present in the orange peels and in the citrus pulp will be used for the production of high added value molecules like Perillyl acid and Perillaldehyde, thanks to a one-pot hydroxylation (scheme 1, reaction 1) and oxidation (fig. 1, reaction 2).

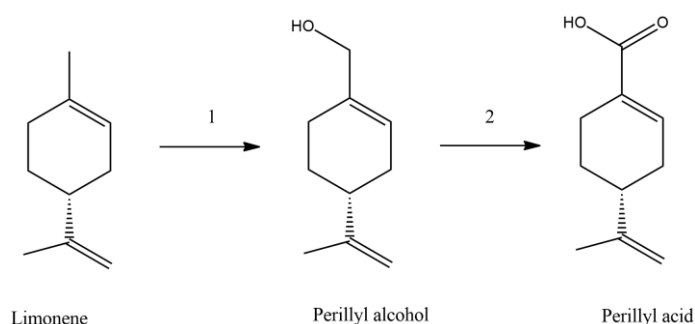


Figure 1 One-pot synthesis of Perillyl acid starting from Limonene.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Molecular biology study.** Construction of new plasmid vectors optimized for acetic acid bacteria and Chromosomal integration studies

A2) **Genes acquisition and protein expression studies.** The stability of the new vector in AAB will be studied, transformation protocols will be tailored for the studied strains, protein expression will be investigated in different conditions.

A3) **Substrates and growing conditions optimization.** The optimal conditions for new obtained strains grown and bioconversion will be investigated.

A4) **Analytical studies:** Biotransformation and cellulose production yields will be studied through HPLC and GC analysis.

A5) **Thesis and paper preparation**

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Genetical study																								
	1) plasmid constructions																								
	2) chromosomal integration																								
A2)	Genes acquisition and protein expression studies																								
	1) plasmids stability																								
	2) chromosomal integration studies																								
A3)	Substrates and growing condition optimization																								
	1) Substrate usability																								
	2) growing condition investigation																								
A4)	Analytical studies																								
A5)	Thesis and Paper Preparation																								

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Evaluation of the Chemical and Nutritional Quality of Foods Through Meta-Omics Approaches

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This PhD thesis research project is aimed at assessing the chemical and nutritional quality of foods through meta-omic approaches, studying the relationship between some foods components and human health. In particular, the aim of the PhD thesis is to study the effects of dark chocolate and the contribution of its components on the possible beneficial effects on health.

Valutazione della qualità chimica e nutrizionale degli alimenti mediante approcci meta-omici

Questo progetto di tesi di dottorato mira a valutare la qualità chimica e nutrizionale degli alimenti mediante approcci meta-omici, studiando la relazione tra alcuni componenti alimentari e la salute umana. In particolare, il progetto di tesi di dottorato mira a studiare gli effetti del cioccolato fondente e il contributo dei suoi componenti sui possibili effetti benefici sulla salute umana.

1. State-of-the-Art

The chemical quality of foods is given by their content in proteins, lipids and carbohydrates while the nutritional quality depends both on the quantity of chemical energy that it brings and the qualitative aspect that is given by the combination of the nutritive elements contained in them. The nutritional quality, therefore, relates to the molecules and nutrients contained in foods.

Diet plays a very important role in modulating various metabolic functions. In fact, in addition to provide the energy necessary for the normal metabolic processes of the body, foods are a unique source of "active ingredients" such as antioxidants, vitamins, minerals, polyunsaturated fatty acids with beneficial effects on health. This means that the diet and its components can contribute to a state of well-being, to a reduction of the risks related to certain pathologies and to an improvement in the quality of life (El Sohaimy, 2012).

There is growing attention to the value and nutritional risk of foods, as well as to their possible beneficial effects on health. Foods consist of macronutrients (proteins, fats, sugars) and micronutrients (vitamins, mineral salts, polyphenols, dietary fiber). The presence of micronutrients, in many cases, increases the nutritional value of the foods, as these compounds are often related to an increase in the well-being of the organism. Among the micronutrients, polyphenols are the great interest for researchers due to the well-known health-beneficial effects, such as anti-carcinogenic, anti-atherogenic, anti-inflammatory, anti-microbial. Polyphenols in green and black tea, grape seeds, grapes and red wine have raised much attention but chocolate has not been investigated intensively until now. Some studies highlighted that the consumption of foods containing cocoa shows positive health benefits. For example, cocoa can lower blood pressure, resulting in a lower risk of cardiovascular disease, endothelial dysfunction reversal and the sensitivity of insulin beta improvement (Keen et al., 2005; Latif, 2013).

The "omics" sciences are a set of biomolecular disciplines that deal with the study of pools of biological molecules (eg, ions, nucleic acids, proteins, enzymes) in certain biological samples (for example, serum, urine, faeces, saliva, tissues). These disciplines use analytical technologies that allow the production of information, in very large numbers and at the same time, useful for the description and interpretation of the biological system studied. The "omics" sciences encompass disciplines such as genomics, transcriptomics, proteomics and metabolomics (Aebersold and Mann, 2003). The aim of this holistic approach is to understand higher level operating principles, which overall define the biology of the systems working with integrative approaches. This in order to be able to answer hierarchically more complicated biological questions (for example, pathogenesis, natural history or therapeutic success and prognosis of a disease).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Determination of the chemical-physical and sensory properties of dark chocolate by analyses of proteins, peptides, amino acids, fatty acids, antioxidants, polyphenols, fibers, and evaluation of the organoleptic characteristics.

A2) Study of gastrointestinal motility by enrollment of at least 16 healthy subjects (A2.1) and evaluation of effects on motility and gastrointestinal symptoms in a cohort of healthy subjects tested with dark chocolate and standard meal (A2.2).

A3) Pilot study on patients with mild hypercholesterolemia following to the daily intake of dark chocolate for 30 days. Enrollment of at least 50 patients with mild hypercholesterolemia (A3.1), soministration of diets, writing of alimentary diaries (A3.2) and evaluation, on biological samples, of glycemia, insulinemia, lipids (LDL, HDL, total cholesterol), anti-inflammatory effect and pro-inflammatory effect, anti-oxidative effect, presence of bioactive peptides, metabolomic analysis (A3.3).

A4) Pilot study on patients with mild hypercholesterolemia following to the daily intake of fiber-enriched dark chocolate combined with statin intake for 3 months. Enrollment of at least 50 patients with mild hypercholesterolemia (A4.1), soministration of diets, writing of alimentary diaries (A4.2), and evaluation, on biological samples, of lipids (LDL, HDL, total cholesterol), anti-inflammatory effect and pro-inflammatory effect, anti-oxidative effect, presence of bioactive peptides, metabolomic analysis (A4.3).

A5) Statistical analysis of data

A6) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	/	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Determination of chemical-physical and sensory properties of dark chocolate																									
A2)	Study of gastrointestinal motility																									
	1) Enrollment of healthy subjects																									
	2) Evaluation of effects on gastrointestinal motility																									
A3)	Pilot study on patients with mild hypercholesterolemia																									
	1) Enrollment of patients																									
	2) Soministration of diets and writing of alimentary diaries																									
	3) Evaluation of anti and pro-inflammatory effect, anti-oxidative effect, presence of bioactive peptides, metabolomic analysis																									
A4)	Pilot study on patients with mild hypercholesterolemia																									
	1) Enrollment of patients																									
	2) Soministration of diets and writing of alimentary diaries																									
	3) Evaluation of anti and pro-inflammatory effect, anti-oxidative effect, presence of bioactive peptides, metabolomic analysis																									
A5)	Statistical analysis of data																									
A6)	Thesis and Paper Preparation																									

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Biotechnological Conversion of Residual Biomasses in Value Added Products

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This PhD project aims to exploit the residual biomasses from the agricultural and agri-food sectors of the Basilicata's region. These biomasses can be used to produce, through biotechnological processes, value added molecules such as triglycerides, polyols, organic acids, carotenoids etc, to be used in food production. The project involves the optimization of fermentation processes of biomass platform streams from laboratory to pilot scale. Statistical tools for the experimental design, Design Of Experiment (DOE) and molecular biology techniques will be used to optimize the conversion processes and relevant efficiencies.

Conversione biotecnologica di biomasse residuali in prodotti ad alto valore aggiunto

Il presente progetto di dottorato ha lo scopo di valorizzare le biomasse residuali del comparto agricolo e agro-alimentare della regione Basilicata. Tali biomasse possono essere utilizzate per produrre, attraverso processi biotecnologici, molecole ad alto valore aggiunto come trigliceridi, polioli, acidi organici, carotenoidi etc, da impiegare nel settore alimentare. Il progetto prevede l'ottimizzazione del processo fermentativo su scala da banco e su scala pilota. Verranno utilizzati strumenti statistici per il design sperimentale, Design Of Experiment (DOE) e tecniche di biologia molecolare per ottimizzare il processo e l'efficienza di conversione.

1. State-of-the-Art

The International Energy Agency (IEA) defines biorefinery as a production system aimed at the sustainable conversion of biomass into a variety of marketable products and energy (IEA, 2008). According to the European Directive 2009/28 / CE on the promotion use of energy from renewable sources, the term biomass includes the biodegradable fraction of products, waste and residues of biological origin from agriculture, forestry and related industries, as well as the biodegradable fraction of urban and industrial waste.

Among the waste from the Lucania agricultural and food supply chain, the most interesting by-products include wheat straw, olive mill wastewaters and whey. Today, these matrices represent wastes and, in some cases, need expensive pretreatments before disposal. In recent years, many efforts have been made for the valorization of these matrices, but the economic feasibility remains the bottleneck of the process.

Several studies have shown the ability of some oleaginous yeasts to uptake and metabolize several organic substrates, such as biomass derived sugars, and to convert them into a wide variety of products, such as organic acids, triglycerides, polyols and carotenoids (Sitepu et al., 2014; Makkar et al., 2002). These molecules represent

interesting raw materials for the food, nutraceutical and chemical industries Maina et al., 2017).

The present PhD project aims to study and optimize the metabolic performance of selected microorganisms on organic substrates in order to increase the microorganism robustness, improve the selectivity toward target products and overall efficiency of the process.

2. PhD Thesis Objectives and Milestones

The PhD thesis project can be subdivided into the following activities schematized in the Gantt diagram given in Table 1:

A1) Study, pretreatment and characterization of biomass

The biomass feedstocks will be selected, studied and characterized. Lignocellulosic biomass will be pretreated through the technological units available at the ENEA centre in Basilicata, that include the steam explosion technology and several plants for thermal processes. The main scope of this step is to produce intermediates from biomass which can be used to feed fermentation process.

A2) Yeasts screening and metabolic studies

A2.1 Screening of oleaginous yeasts and selection of the strains showing the best performances in term of resistance to inhibitors, productivity and ability to transform substrates into products. **A2.2** Detailed study of the selected yeasts and optimization of the various process parameters using DOE statistical software.

A3) Engineering of microorganisms

Engineering of the microorganism that shows the best production performances. Use of genome editing technique aimed to increase the microbial production and external secretion of selected compounds.

A4) Bioreactor scale-up processes

A4.1 Scale-up the process from laboratory to pilot scale 50 L with microorganisms previously selected.

A5) Writing and Editing of the PhD thesis, scientific papers and oral poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Study, pretreatment and characterization of biomass																								
	1.1 lignocellulotic biomasses																								
	1.2 whey and olive mill wastewater																								
A2)	Yeasts screening and metabolic studies																								
	A2.1 screening																								
	A2.2 optimization process parameters																								
A3)	Engineering of microorganisms																								
	3.1 metabolic flow																								
	3.2 secretion products																								
A4)	Bioreactor scale-up processes																								
	10 L trial																								
	50 L trial																								
A5)	Thesis and Paper Preparation																								

3. Selected References

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Effect of Sprouting on the Technological Properties of Grains

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This PhD project aims at understanding the effects of sprouting - carried out under controlled conditions of time and temperature - on the technological properties of grains (i.e. cereals, pseudocereals, and pulses). The project focuses on the relation between structural/functional changes in starch, protein, and fiber and the characteristics of cereal-based products (i.e. bread and/or pasta). Understanding the relationship between molecular changes and grain functionality will help in re-designing new cereal-based products with improved nutritional, sensory, and textural characteristics.

Effetto della germinazione sulle proprietà tecnologiche dei semi

Questo progetto di dottorato ha come obiettivo la comprensione degli effetti del processo di germinazione - effettuato in condizioni controllate di tempo e temperatura - sulle proprietà tecnologiche di cereali, pseudocereali e legumi. Il progetto ha come focus la relazione tra i cambiamenti strutturali/funzionali delle principali macromolecole dei cereali, ovvero amido, proteine e fibra e le caratteristiche dei prodotti finiti quali pane e pasta. La comprensione della relazione tra modificazioni strutturali e caratteristiche tecnologiche degli sfarinati risulta peculiare nella formulazione di nuovi prodotti a base di cereali con migliori specifiche nutrizionali, sensoriali e di *texture*.

1. State-of-the-Art

Sprouting (or germination) is a natural process that transforms seeds into plants triggering different physiological changes in the seed. Enzymes such as amylases and proteases are synthesized during sprouting to break down the storage macromolecules (i.e. starch and proteins) and transform them into soluble nutrients (i.e. simple sugars, small peptides and amino acids) in the sprout (Khan & Shewry, 2016).

Wheat sprouting is often considered negatively because of the high accumulation of hydrolytic enzymes, which are responsible for the worsening of the technological performance of the related flours. This phenomenon - also known as pre-harvest sprouting - happens when wheat is exposed to prolonged wet or foggy conditions in the field. The resulting dough is characterized by high stickiness and low stability, making sprouted wheat unsuitable for producing bread and other cereal-based foods (Paulsen & Auld, 2004).

On the other hand, for centuries, especially in many oriental countries, sprouting has been carried out at a domestic level in order to soften the structure of grains (i.e. pulses), making their cooking easier and shorter (Uwaegbute et al., 2012). At the same time, sprouting may improve sensory traits, likely due to sugar production by starch hydrolysis. In addition, sprouting promotes various important modifications of nutritional relevance, including the increase in phenolic compounds with antioxidant activity, mineral and vitamin bioavailability, and soluble compounds (i.e. amino acids and sugars), while decreasing antinutritional factors, such as enzyme inhibitors and phytates (Nelson et al., 2013).

Evidence of the relationship between sprouting and nutritional enhancements, together with the search for “natural” and traditional processes/foods, accounts for the increasing interest in sprouted grains on the part of Western consumers.

The biochemical changes detectable in sprouted grains are closely and deeply associated with a rich enzymatic framework, whose activity must be understood and controlled, especially when sprouted grains are used as ingredients in cereal-based formulations. Thus, the set-up of processing conditions in terms of time, temperature and relative humidity – which are specific for each type of grain - is the key for balancing the sensory, nutritional and technological performance of sprouted cereals, pseudo-cereals and pulses.

Despite current knowledge about sprouting, more information on its impact on cereals, pseudo-cereals and pulses is required. In particular, this PhD project aims at understanding the effects of controlled sprouting on starch, protein and fiber functionality and their relationship with the characteristics of the related dough, bread and pasta. Cereals (i.e. common and durum wheat), pseudo-cereals (i.e. quinoa) and pulses (i.e. chickpea) will be sprouted in order to provide an overview of the effects of sprouting on different types of grains.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Literature review. The purpose of this phase is to complete the literature review that was started during the preparation of the project.

A2) Setting up of the sprouting process conditions. Optimized conditions of time, temperature, and relative humidity adopted during each step of the process (i.e. soaking, sprouting, and drying) will be assessed on both lab and pilot scales. Samples will be collected at different sprouting times (from 12 h to 72 h), to study the changes in chemical composition (A2.1) and enzymatic activities (A2.2) over time.

A3) Study of the impact of sprouting on the main components of grains. This activity will focus on understanding the effects of sprouting on starch (A3.1), protein (A3.2), and fiber (A3.3).

Pasting and gelatinization and retrogradation properties will be assessed by Micro-Visco-AmyloGraph and by Differential Scanning Calorimetry, respectively. Potential changes in amorphous and crystalline order will be measured by Nuclear Magnetic Resonance solid state (A3.1).

The impact of sprouting on gluten proteins will be assessed by measuring the amount in quality-related gluten fractions (gliadin and glutenin) using Reverse Phase-HPLC. Moreover, Attenuated Total Reflectance Fourier Transform Infrared spectroscopy will be used to evaluate changes in protein secondary structure upon sprouting (A3.2).

As regards bran from sprouted wheat, changes in soluble/insoluble dietary fiber content, fiber-water and fiber-protein interactions will be assessed. As regards fiber-water interactions, bran water absorption and binding capacity will be evaluated. Finally, the resulting changes in fiber-protein interaction will be evaluated by measuring the changes in gluten aggregation kinetics (A3.3).

A4) Study of the impact of sprouting on dough properties and product quality. This activity will focus on relating the structural/functional changes measured in A3 to the rheological properties of doughs made from the related flours (both wholemeal and refined flours). Dough tenacity (Alveograph test), stability (Farinograph test),

extensibility (Extensograph test), elasticity (Glutograph test), and stickiness (Texture Profile Analysis test - TPA) will be considered (A4.1). Finally, sprouted grains will be used alone or in mixtures to produce bread and/or pasta. Bread will be assessed through specific volume (sesame method), crumb porosity (image analysis) and crumb firmness (TPA) after baking and during storage (A4.2). As regards pasta, the samples will be characterized in terms of cooking losses, water absorption and firmness (AACCI 66-50.01) (A4.3).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 *Gantt diagram for this PhD thesis project.*

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Literature review																									
A2) Setting up of sprouting conditions																									
1) Chemical composition																									
2) Enzymatic activities																									
A3) Study of the impact of sprouting on:																									
1) Starch: structure and functionality																									
2) Gluten proteins: structure and functionality																									
3) Fiber-protein interaction																									
A4) Study of the impact of sprouting on:																									
1) Dough properties																									
2) Bread-making and characterization																									
3) Pasta-making and characterization																									
A5) Thesis and Paper Preparation																									

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Selection of Probiotic Microorganisms for their Potential Psychobiotic Activity

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The study aims at selecting psychobiotic microorganisms, capable of producing neuroactive molecules such as GABA, serotonin, acetylcholine and catecholamine, able to modulate the gut-brain axis, so the connection between the functions of the colon and brain. The ultimate goal of the study is to develop food supplements in order to delay the onset of different diseases, to improve the quality of life and reduce the social costs related to health care.

Selezione di microrganismi probiotici con potenziali attività psicobiotiche

Lo studio si propone di selezionare microrganismi psicobiotici, che attraverso la loro capacità di produrre mediatori neuroattivi (GABA, serotonina, acetilcolina e catecolamine) siano in grado di modulare l'asse intestino-cervello, ovvero la connessione tra la funzionalità del microbiota del colon e il cervello. Il fine ultimo dello studio è porre le basi per lo sviluppo di integratori alimentari o alimenti funzionali che insieme ad una corretta alimentazione possano prevenire o ritardare l'insorgenza di diverse patologie, in particolare neurologiche, migliorando la qualità della vita e riducendo i costi sociali legati all'assistenza sanitaria.

1. State-of-the-Art

Recent work has found that the trillion of bacteria located in the gastrointestinal tract, also known as gut microbiota, seems to influence the development of diseases of the enteric and central nervous system, including neurodegenerative diseases and several neuropsychiatric and behavioral disorders, like anxiety and depression. This bidirectional relationship between gut and brain function is established by several different pathways of communications identified along the "gut-brain axis". Mediators of microbiota-gut-brain communication affected by microbial metabolism include short-chain fatty acids (for example, butyrate), a wide range of mammalian neurotransmitters (for example, serotonin and γ -aminobutyric acid (GABA), hormones (for example, cortisol) and immune system modulators (for example, quinolinic acid) (Mireia Valles-Colomer et al. 2019). Support for regulatory roles of intestinal microbes in human disease is provided by studies of probiotics (Strandwitz et al. 2018, Brain research). Due to the increasing commercial demand for GABA, various chemical and biological synthesis methods for GABA have been studied, showing that *Lactobacillus* and *Bifidobacterium* species are reported as GABA-producing bacteria. GABA act as the major inhibitory neurotransmitter in the central nervous system and has several physiological function such as neurotransmission, induction of hypotensive effects, diuretic effects, treatment of epilepsy and tranquilizing effects as well as strong secretagogue of insulin from pancreas, preventing diabetes (Huang 2007; Boricha et al. 2019; Pokusaeva 2016). GABA secretion has been reported to serve as an acid-resistance mechanism, that consist in the decarboxylation of glutamate induced at a low pH, which is then exported from the cell in a protonated form, alkanizing the cytoplasm (Strandwitz, et al. 2018). On an industrial scale, using the cheaper sodium L-glutamate (L-MSG) is a valuable process to producing

γ -aminobutyric acid (GABA) whose conversion rate of L-MSG to GABA can be calculated as follow (Zhang et al. 2012)(1):

$$\text{Conversion rate} = \frac{\text{Final GABA concentration (mM)}}{\text{Initial MSG concentration (mM)}} \times 100 \quad (1)$$

The function of serotonin and dopamine in relationship to lactic acid bacteria strain have been also studying even if without many relevant results in literature. One work has shown the capacity of *Streptococcus thermophilus* (NCFB2392) to produce serotonin and dopamine using arginine decarboxylase broth (ADB) as well as other biogenic amines (Ozogul 2012). Quantification of GABA and other neurotransmitter can be done by Mass Spectrometry, HPLC or again colorimetric methods in the case of GABA.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Study of the ability of bacterial cultures to produce neuroactive mediators (Screening phase). It will take place at the University of Naples in collaboration with Proge Farm, with the specific objective of selecting the microbial cultures of lactic bacteria to be used in the study to verify the production of GABA, serotonin, acetylcholine and other neurotransmitters such as norepinephrine and Dopamine. The tests will be conducted starting from a minimum of 100 strains of lactic bacteria, several of which come from the Proge Farm collection.

A2) Production optimization of selected strains to proceed with the study of formulations that contain the strains or mixtures, which have shown the best results in vitro. It will be held at Proge Farm in collaboration with UNINA and will have the specific objective of carrying out growth tests, on an industrial level, of different cultures of *Lactobacillus* and *Streptococcus*, previously identified as potentially probiotic and capable of producing neuroactive substances. The goal is to obtain at least 3 strains and one or more combinations of probiotic strains capable of producing one or more neuroactive substances.

A3) Finalization of the formulation of the finished product. In this phase we will proceed with the study of formulations (maximum 3) that contain the strains or mixtures, which have shown the best results in vitro, and any other ingredients that can contribute to this activity.

A4) Performance study in SHIME. It will take place at the University of Wageningen with the aim of evaluating (i) the effect on the composition of the microbiota in short and medium term experiments (up to 4 weeks) and (ii) the production kinetics of inflammation mediators intestinal, or the response to the time of consumption and the accumulation of mediators over time in different parts of the colon.

A5) Human study for the validation of selected psychobiotics so It will take place at the University of Wageningen with the aim of testing the effect of two combinations of psychobiotics on cognitive performance. The methods of administration (criteria for inclusion of duration of the intervention and quantity of psychobiotics) as well as the entire study design will be established based on the results of the previous

activities. Only cognitive performance parameters that will be measured through computer tests and questionnaires will be evaluated.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A1) <i>Isolation and study of the ability of lactic bacteria to produce neuroactive mediators (Screening activity)</i>																									
A2) <i>Production optimization of selected strains</i>																									
1) Industrial growth test																									
2) Studies of growth synergies																									
A3) <i>Development of the finished product</i>																									
A4) <i>Performance study in SHIME</i>																									
1) Effect on the composition of the microbiota																									
2) Production kinetics of intestinal inflammation mediators																									
A5) <i>Human study for the validation of selected psychobiotics (Cognitive test)</i>																									
A6) <i>Literature analysis, thesis and paper preparation</i>																									

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Bioactive Compounds from Lactic Acid Bacteria: Beneficial Effects of Fermented Foods

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This PhD research project is aimed to study fermented food, produced by lactic acid bacteria (LAB) from a new perspective. LAB are involved in food fermentation since the beginning of human history. Fermentation is a well-known system for food preservation and is exploited also for improving sensory characteristics of food. However, literature is paying increasingly attention to nutritional properties of fermented foods. In this optic this project will investigate the ability of LAB isolated from food and belonging to collection of food microbiology group of University of Parma to produce bioactive compounds with potentially prebiotic, antimicrobial and health related effects.

Composti Bioattivi Prodotti da Batteri Lattici: gli Effetti Benefici degli Alimenti Fermentati

Questo progetto di dottorato mira allo studio degli alimenti fermentati, prodotti da batteri lattici una nuova prospettiva. I batteri lattici sono stati impiegati nella fermentazione degli alimenti fin dagli albori della storia umana. La fermentazione è un sistema molto diffuso per la conservazione degli alimenti ed è impiegata nel miglioramento delle proprietà sensoriali. La letteratura scientifica sta recentemente tributando una sempre maggiore attenzione alle proprietà nutrizionali degli alimenti fermentati. In quest'ottica questo progetto verterà sull'investigare gli effetti positivi degli alimenti fermentati da batteri lattici e la produzione di composti bioattivi, con potenzialità prebiotiche, antimicrobiche ed effetti sulla salute.

1. State-of-the-Art

Lactic acid fermentation is exploited by men since ancient times to prolong shelf-life of food products and to improve taste. However, the ability of LAB to produce bioactive compounds in fermented foods has recently gained the literature attention, shedding the light on the relation between fermented foods and health. Bioactive compounds are subject of great interest because of their potential activity as: prebiotic, antimicrobic and for their potential positive effects on consumers health. LAB can ferment a multitude of substrates, such as: milk, fruits and vegetables (Yoon et al 2005). During fermentation of the substrates, LAB are able to release a variety of bioactive compounds, according to the environmental characteristics (different fermented foods, different ecological niche). LAB can release both in fermented milk and vegetables bioactive peptides, with a variety of function. Bioactive peptides are defined as: inactive polypeptide included in the sequence of a protein that when released by mean of enzymatic hydrolysis gains various physiological roles. Bioactivity of the peptides depends on composition and conformation and according to them, bioactive peptides can exert different activity like immunomodulatory, antimicrobial, antioxidative, antithrombotic, hypocholesterolemic and hypertensive activities (Sarmadi & Ismail, 2010). LAB can also produce during fermentation functional lipids such as conjugated linoleic acid with a well-known anti-inflammatory and anti-carcinogenic effect. LAB can produce also γ -aminobutyric acid, a non-proteic aminoacid that have many documented effects both physiological and psychological such as: tranquilizing effect, help in treatment of alcoholism, depression and anxiety state (Santiago-López et al., 2018). Another class of molecules produced by LAB during fermentation are Exopolysaccharides (EPS). EPS are heterogenic

polyssacharides produced by LAB during fermentation. EPS can be very different depending on molecular composition, size and structural characteristics. EPS can be exploited in improving textural and sensory attributes of fermented foods and beverages. EPS show also healthy features such as: modulation of immune functionality, positive influence on level of beneficial microorganisms in the intestine, cholesterol lowering effect and antioxidant activity (Lynch, *et al* 2018). LAB metabolisms have been exploited by men since ancient time to preserve food, but nowadays the opportunity to deepen the knowledge about the possible healthy features of LAB fermented foods, can offer a new perspective on this ancestral bond between man and LAB.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Evaluation of the production of bioactive compounds** by LAB belonging to the University of Parma food microbiology group's microbial collection, in different environmental condition and ecosystems (i.e: different fermented foods).

A2) **Study of isolation methods** and the operating conditions to maximize the production level of the bioactive compounds

A3) **Study of positive effect of bioactive compounds** on other microbial population and human health

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Evaluation of the production of bioactive compounds																								
1) Selection of the strains																								
2) Evaluation of the production																								
A2) Study of isolation methods																								
1) Selection of the method of isolation																								
2) Screening of methods to improve the production																								
A3) Study of positive effect of bioactive compounds																								
1) Study of LAB bioactive compounds effect on food microorganisms																								
2) Study of LAB bioactive compounds effect on human health																								
A5) Thesis and Paper Preparation																								

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New Synthetic Approaches for the Preparation of Antimicrobial Compounds

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This PhD thesis research project aims at synthesizing new bio-inspired molecules endowed with potential antimicrobial activity. In particular, the approach in the field of agrochemicals is based on the preparation of hybrid compounds combining two different pharmacophores whose target has already been validated. In an extended multidisciplinary point of view this project involves not only synthetic chemistry, but also molecular modelling for the structure-activity relationship (SAR) studies and biochemical and microbiological investigations to test the new synthesized compounds.

Nuovi approcci sintetici per la preparazione di composti antimicrobici

Questo progetto di tesi di dottorato è rivolto alla sintesi di nuove molecole ispirate a prodotti di origine naturale che possiedono una potenziale attività antimicrobica. In particolare, l'approccio nel campo dell'agrochimica consiste nel creare composti ibridi combinando due diversi farmacofori di cui è già conosciuto il target. Considerato da un punto di vista più esteso e multidisciplinare, questo progetto non solo coinvolge la chimica sintetica, ma anche la modellistica molecolare per studi di relazione struttura-attività (SAR) e investigazioni biochimiche e microbiologiche per testare i nuovi composti sintetizzati.

1. State-of-the-Art

Nowadays an increasing problem for human health that involves all the food supply chain is the presence of pathogens that developed in the years antimicrobial resistance (AMR). Since 1945 Sir Alexander Fleming, about the potential for AMR to become a public threat, said "there is the danger that the ignorant man may easily under dose himself and by exposing his microbes to not-lethal quantities of the drug make them resistant". In fact, antimicrobial resistance can be transmitted from bacterium to bacterium in different ways (phages, trasposoms and plasmins) resulting in horizontal gene transfer (HGT); this process occurs in the environment, food and the gastrointestinal tract of humans (Hudson, J. A. et al., 2017). In simple words the antimicrobial resistance is the capability of a specific microbe to resist to an antimicrobial at which it was sensitive before.

It's important to highlight that antimicrobials are included in all the food chain: as antifungals and pesticides to maximise the crops (the data suggest that the crop-destroying fungi account for perennial yield losses of ~20% worldwide, with a further 10% loss postharvest) (Fisher, M.C. et al., 2018); as antibiotics in animal production (therapeutic treatment of animals, growth promotion, metaphylaxis and prophylaxis) and also in aquaculture; at the end as preservatives for the shelves food life. In particular biopreservation is focused on the utilization of natural preservatives from sources like bacteria, fungi, plants, animals, having the ability to ensure food safety due to their antimicrobial activity applied against a large spectrum of foodborne pathogens (Pisoschi, A. M. et al., 2018).

These evidences show the link between the food security and the requirement to find new antimicrobial compounds overcoming the antimicrobial resistance. The WHO

and others prompt not only to reduce the use of antimicrobials, but at the same time invite the researchers to work hard to obtain new classes of antimicrobials. In the chemistry field new approach are investigated to develop new molecules that overcome cross-resistance. Considerable efforts are directed to 1) explore new chemical classes of antimicrobials, for example isolating new natural products 2) attack different and still unknown target and 3) study the unknown mechanisms of action of already used compounds (World Health Organization, 2017).

Our innovative approach in the field of agrochemicals is focused to create new hybrid molecules with dual antimicrobial activity. The hybrids contain two pharmacophores, antimicrobial A and B whose targets are already validated. The two pharmacophores act on two different targets once they cross the cell membrane.

Hybrid antimicrobials for dual targeting



Figure 1 Hybridization approach for the development of novel antimicrobials (Klahn P. and Brönstrup M., 2017).

These conjugates can be divided in two major categories: antimicrobial hybrids, in which both moieties of the hybrid exert antimicrobial activity and antimicrobial conjugates in which one functional part controls the accumulation of the other part of the conjugate, for example mediating an active transport into the bacterial cell or stopping the efflux pumps.

The advantages of this molecular design are several; first of all, the dual targeting molecules could have an improved antimicrobial potency due to synergistic effects of the single components, second, the hybrid antimicrobials could go beyond the efficacy of their component drugs, either alone or in combination, by maintaining their antimicrobial activity and also decreasing the frequency of resistant mutations. Furthermore, the physico-chemical properties of covalently linked drug moieties have been reported to be beneficial for their uptake and retention in the bacterial cell. (Klahn P. and Brönstrup M., 2017)

The covalent linkage of the two antimicrobial compounds plays a key role. A crucial step in the design will be the identification of an appropriate combination and outdistancing between the pharmacophoric moieties to drive to compounds characterized by a suitable size and geometrical shape to fit both targets.

The challenge of this work is to identify two classes of natural products with known antimicrobial activity and develop new synthetic approaches to link their pharmacophoric moieties. Optimization of the structures and the architecture of new molecules, based on SAR and molecular modelling studies, will be fundamental to reach the goal of obtaining hybrid compounds endowed not only with antimicrobial activity, but also able to overcome resistance.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Design of new molecules by molecular modelling**

A2) **Development of synthetic and biomimetic procedures**

A3) **Biological evaluation:** tests of obtained compounds as antimicrobials and antifungals and studies of the interaction with microbiota

A4) **Modification of active compounds for SAR studies on a particular biological target**

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Design of new molecules																									
A2) Synthesis and purification																									
A3) Biological evaluation																									
A4) Chemical modification of selected compounds																									
1) design of analogues																									
2) synthesis for SAR studies																									
A5) Thesis and Papers Preparation																									

3. Selected References

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Use of Legume Flours for New Food Applications

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The research project of this PhD thesis aims at increasing the consumption of legumes through the lab-scale development and subsequent semi-industrial application of production protocols of legume flour-based foods, such as bakery products, snacks, pasta and meal solutions (e.g. burgers). The project involves the rheological characterization of legume flours, from different species, and nutritional and sensory analysis of the end-products.

Impiego di farine di legumi per nuove applicazioni alimentari

Questo progetto di dottorato mira all'incremento del consumo di legumi attraverso la messa a punto su scala di laboratorio e la successiva applicazione semi-industriale di protocolli di produzione di alimenti a base di farine di legumi, quali prodotti da forno, snacks, pasta e soluzioni pasto (es. burger). Il progetto prevede anche la caratterizzazione reologica degli sfarinati di legumi, ottenuti da specie diverse, e la successiva analisi nutrizionale e sensoriale dei prodotti finiti.

1. State of the Art

In a scenario which involves the doubling of the world population by 2050, the role of legumes is very important due to their environmental sustainability and good nutritional performances. Different studies have shown the beneficial role of legumes in cropping system, by increasing biological nitrogen fixation, reducing energy costs, improving soil physical conditions and biodiversity, and in the production of healthy food products. Legumes are a rich source of proteins (20–37%, w/w), dietary fibers (3–31% w/w) and resistant starch (11–20% w/w) (Asif *et al.*, 2013). However, a drawback of legumes is the need of prolonged cooking, hardly meeting the modern expectation for foods requiring short preparation times. Moreover, despite several studies have highlighted the possibility of obtaining food products by using legumes as raw material (Summo *et al.*, 2019; Summo *et al.*, 2016; Foschia *et al.*, 2017), there are few industrial applications, which mainly include local productions characterized by low economic impact. This gap is due to different reasons, such as i) the high variability of compositional, nutritional and technological characteristics of different species of legumes and their genotypes; ii) the need of designing new technological production protocols suited to the characteristics of the raw materials; iii) the need of deepening the knowledge of sensory characteristics of the end-products. In a strategy of a circular economy, the characteristics of legume by-products should be studied also for their reuse in the production chain.

Therefore, the aim of this project is to increase the consumption of legumes by producing food products such as bakery products, pasta, snacks and meal solutions (e.g. burgers), also gluten-free, based on legumes or fortified with their flours, trying to reduce the gap between basic research and industrial application. The work will be conducted in

collaboration with the Andriani SpA company, specialized in legume milling and in the production of legume-based gluten-free pasta, and the Latvia University of Life Sciences and Technologies, with skills in the production of extruded food products. The project will involve the rheological characterization of legume flours, from different species, and the nutritional and sensory analysis of the end-products.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1. All data, as output of each step, will be subjected to statistical analysis.

A1) Research and Bibliographic study

A2) Selection and nutritional characterization of legume samples. Thirty legume accessions will be identified and selected into a worldwide collection of over 400 legume accessions available at the Dept. of Soil, Plant and Food Sciences (DiSSPA) of the University of Bari, Italy comprising peas, lentils, faba beans and chickpeas. The selection will be carried out on the basis of genetic diversity and agricultural performance in collaboration with the Plant breeding research group of the same Dept., and the selected legume accessions will be characterized from the nutritional point of view in terms of: i) proximate composition; ii) fatty acids and amino acids profile; iii) content of bioactive compounds; iv) antioxidant activity; v) content of antinutritional compounds.

A3) Set-up of milling processes and rheological characterization of the flour. The set-up of milling processes will be developed together with the managers of the Andriani SpA Company evaluating the different types of native and modified legume flours (taking advantage of the presence, in the Company, of an extrusion-cooking plant suitable for producing modified flours). The waste will be characterized for possible reuse, in a logic of circular economy. The rheological properties and functional features of legume flours (WSI, WAI, water absorption and solubility index, degree of damage to starches) will be evaluated.

A4) Set-up of technological processes for the production of legume flour-based products, also gluten-free. The activity will be conducted partly at the Department of Food Technology of Latvia University of Life Sciences and Technologies (from the 19th to the 24th month) and partly at the DiSSPA Department (from the 25th to the 28th month). During the period abroad, based on the results obtained of the A1 and A2, extruded legume-based products (sweet snacks and savory, legume flakes) will be developed by cooking-extrusion pilot plants, which will be made available by the host University. In the second period, new applications of legume flours in bakery products (bread and bread substitutes, pizza crust and others) and meal solutions will be developed. At the end of the A3, at least 9 overall references will be produced.

A5) Nutritional and sensory evaluation. This activity will allow to evaluate the improvement of the nutritional value of the legume-based food products, aiming at labeling them with at least two health claims related to the reduction of the glycemic index, to the increase in the unsaturated fatty acid content, and to the high contents of fiber. Furthermore, sensory evaluation will be carried out, as well as shelf-life studies by means of accelerated shelf-life tests.

A6) Semi-industrial scale-up. In this finale phase the protocols developed in the A3 will be transferred on a semi-industrial scale at the Andriani SpA company, for the production of at least 3 prototypes with nutritional characteristics as defined in the A4.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2 ÷ 4	4 ÷ 9	10 ÷ 2	13 ÷ 15	16 ÷ 17	16 ÷ 18	19 ÷ 21	22 ÷ 23	24	25 ÷ 27	27 ÷ 30	31 ÷ 33	34 ÷ 36
A1)	Research and Bibliographic study														
A2)	Selection and nutritional characterization of legume samples														
	1) Selection														
	2) Characterization														
	3) Study of nutritional profile														
A3)	Set-up of milling processes and rheological characterization of the flour														
	1) Set-up of milling processes														
	2) Technological characterization														
	3) Nutritional characterization of milling waste														
A4)	Set-up of technological processes for production of legume flour-based products														
	1) Set-up of process conditions for bakery-extruded products														
	2) Laboratory-scale production of extruded products														
	3) Rheological and sensory characterization of extruded products														
A5)	Nutritional and sensory evaluation														
	1) Evaluation Nutritional and sensory evaluation														
	2) Accelerated shelf-life tests														
A6)	Semi-industrial scale-up														
	1) Set-up of production processes														
	2) Stabilization and packaging processes of the prototypes														

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Study, Analysis, Biological Evaluation and Formulation of Nutraceuticals from Unconventional Foods and by-Products of Food Matrices into Mediterranean Area

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This research project is focused on the chemical characterization and evaluation of the biological activity of unconventional vegetable matrices and by-products of the Mediterranean area. The unconventional matrix is *Phyllostachys pubescens* (Bamboo Moso), rich in many nutrients including proteins, minerals, carbohydrates, phytosterols, fibers and low fat. The second matrix is wastewater of Extra Virgin Olive Oil, a by-product of Mediterranean area. Some researchers have tested the possibility to extract from wastewater oil some particular organic compounds: sugars, polyphenols, organic acids, pectins, fats, nitrogenous substances, polyalcohols, polyacids and mineral elements such as phosphorus, potassium, magnesium and calcium.

Studio, Analisi, Valutazione Biologica e Formulazione di Nutraceutici a partire da Cibi non Convenzionali e da sottoprodotti di matrici dell'Area Mediterranea

Questo progetto di ricerca è incentrato sulla caratterizzazione chimica e sulla valutazione dell'attività biologica di matrici vegetali non convenzionali e sottoprodotti dell'Area Mediterranea. La matrice non convenzionale in questione è *Phyllostachys pubescens* (Bamboo Moso), ricco di molti nutrienti tra cui proteine, minerali, carboidrati, fitosteroli, fibre e basso contenuto di grassi. La seconda matrice presa in esame è costituita dalle acque madri di vegetazione dell'Olio Extra Vergine d'Oliva, un sottoprodotto dell'area Mediterranea. Alcuni ricercatori hanno testato la possibilità di estrarre dalle acque madri alcuni particolari composti organici quali: zuccheri, polifenoli, acidi organici, pectine, grassi, sostanze azotate, polialcoli, poliacidi ed elementi minerali come fosforo, potassio, magnesio e calcio.

1. State-of-the-Art

Bamboo is a giant plant, belonging to the Poaceae family, a subfamily of the Bambusoideae. It is a plantation cultivated all over the world, in fact there are more than 1250 species of bamboo belonging to 23 different genera. Bamboo is mainly cultivated in India, in fact 12.8% of the country is covered by Bamboo forests (1). The evergreen bamboo plant consists of aerial stems known as culms, which arise from a network of rhizomes that produce branches and leaves. The young shoots are tightly bound by means of overlapping sheaths which must be eliminated in order to reach the edible part (2). In particular, *Phyllostachys Pubescens* is a bamboo giant originating in China and Japan, it grows mainly in tropical and subtropical areas (3). Currently, it represents for over 2 billion people a widespread resource able to satisfy primary needs as it is: renewable, productive, versatile, low cost or without costs and easily accessible. All this because bamboo is able to grow in 3 years, a much shorter period than most wood species, which is why bamboo represents a rapidly renewable resource. In rural areas of the developing world it represents a potential for improving life. With the time and the development of Bamboo plantations as an alternative crop, we are trying to understand the possible and multiple uses and benefits that can be drawn from such plantations (4). In fact, in countries like Japan, strong development is becoming a problem for which we

try to use it in many different fields (5). Currently more than 1500 different uses of bamboo have been documented, which constitute a source of: food, shelter, medicines, raw materials for construction, wood substitutes and paper and pulp for industry. For example, for many centuries the Japanese have used leaves as fodder for livestock. Many bamboo, due to their beauty, are commonly used in homes and gardens as ornamental plants. Its intertwined system of rhizomes and roots allows intervention in certain environmental conditions such as soil erosion control, water conservation, soil rehabilitation and carbon sequestration. In China, the decoction of shoots was used in traditional medicine to treat infections, clean wounds, wounds infected with larvae and ulcers. In Java, sap from shoots is used to treat jaundice. Bamboo has been used for more than 2500 years in China as a food, in particular its juvenile shoots. Of all the existing species, many of which are edible, only 100 species are cultivated and used in the food sector. Bamboo shoots are not only delicious but are also rich in nutrients including proteins, minerals, carbohydrates, phytosterols, fiber and low fat. The portion of edible bamboo represents 27% and of this part the nutritional value is determined. Bamboo shoot contains about 88.8% water, over 3.9% protein, 17 amino acids, and is rich in fiber and low in fat. In the bamboo shoot the amino acid content is very high compared to other vegetables, in fact the 8 essential amino acids that are introduced by man through the diet are all available in the shoots. The bamboo shoot contains 17 different types of enzymes and over 10 mineral elements such as Cr, Zn, Mn, Fe, Mg, Ni, Co, Cu (6). The sprouts are free of residual toxicity because during growth they do not need dangerous fertilizers or pesticides.

2. PhD Thesis Objectives and Milestones

The present project will consist of four work packages and many tasks that will be carried out at the Department of Pharmacy - Pharmaceutical Sciences of the University of Bari and at other research laboratories in Italy and abroad. The activities are reported in the Gantt chart shown in table 1:

A1) Chemical characterization of the matrices examined A1.1: development of analytical methods in particular: fat-soluble vitamins, water-soluble vitamins, fibers, nitrogen, sugars, lipids, vitamin C, vitamin D.

A2) Preparation of polyphenolic extracts A2.1: extraction, characterization and development of the polyphenolic fraction of the plant matrix with the use of conventional methods. A2.2: extraction, characterization and development of the polyphenolic fraction of the plant matrix with unconventional methods.

A3) Use of in vitro and in vivo protocols to evaluate the nutraceutical activity of the extracts A3.1: Choice and development of experimental protocols for antioxidant activity with the methods: MEDINA, ORAC, Oxidative stress, DPPH and comparison between them. Evaluation of the biological activity of the matrices on cell lines. A3.2: Ex vivo studies, choice of experimental protocols and preparation of scientific work based on the results obtained from biological activities conducted on cell lines.

A4) Study of innovative formulations A4.1: This objective envisages, following all the studies conducted, the choice of the most suitable formulation for the matrices.

A 5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Chemical characterization																									
1) Analytical methods																									
A2) Preparation of polyphenolic extracts																									
1) Conventional methods																									
2) Unconventional methods																									
A3) Nutraceutical activity of the extracts																									
1) Protocols for antioxidant activity																									
2) Ex vivo studies																									
A4) Innovative formulations																									
1) formulation for the matrices																									
A5) Thesis and Paper Preparation																									

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Evaluation of the Influence of Technological Treatments on Bioactive Compounds in Vegetable Foods

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This PhD thesis research project is aimed at assessing the effects of technological processes on the qualitative/quantitative content of bioactive compounds in vegetable foods. Bioactive compounds and their degradation products will be used as potential markers of product/process. The study of these products could help in predicting losses during processing and in identifying the best technological conditions for process optimization and food preservation of their content and their bioavailability.

Valutazione dell'influenza dei trattamenti tecnologici sui composti bioattivi negli alimenti vegetali

Questo progetto di tesi di dottorato è volto a valutare gli effetti dei processi tecnologici sul contenuto qualitativo/quantitativo dei composti bioattivi presenti negli alimenti vegetali. I composti bioattivi e i loro prodotti di degradazione potranno essere utilizzati come potenziali marcatori di prodotto/processo. Lo studio di questi prodotti potrebbe aiutare a prevedere le perdite durante la lavorazione e ad identificare le migliori condizioni tecnologiche per l'ottimizzazione dei processi e la conservazione degli alimenti del loro contenuto e della loro biodisponibilità.

1. State-of-the-Art

Growing evidence suggests that vegetables and vegetable-based products contain notable levels of bioactive compounds (BSc) of health benefits, among which carotenoids, phenolic compounds, dietary, fibre and vitamins (such as tocopherols, Vitamin C, etc.) have been given particular attention in recent years. Most of these compounds have shown antioxidant activities, associated with health benefits due to the reduction in cardiovascular disease risk factors, the decrease of the incidence of many different types of cancers besides their protective role against a wide range of other chronic diseases (Fiedor et al., 2014; Shahidi et al., 2016). The concentration of these compounds in vegetables widely varies within botanical families and it is also influenced by growing conditions and, then, storage and food-processing.

Tocopherols (tocopherols or tocopherol derivatives) according to their saturation or unsaturation of the carbonaceous chain (Phytol) forming part of their chemical structure, they are divided into two groups: α , β , γ and δ -tocopherol (T) and α , β , γ and δ -tocotrienol (T3). Vegetable oils are the main source of tocopherols, but substantial amounts of these compounds are also reported in most cereal grains including barley, oats, wheat, rye, rice (Adom et al., 2005; Panfili et al., 2003).

Carotenoids are pigments of fruits and vegetables and minor constituents in cereals, responsible for the colour of a wide variety of foods (Panfili, et al., 2004). Due to the strong evidence of their biological activities and their importance in human nutrition, carotenoids have had their food content in several databases. Different factors make the analysis of these compounds assay difficult, because, the carotenoids are highly sensitive to light, heat, air, acids and their isolation and analysis may be accompanied by

degradation, formation of stereoisomers, structural rearrangements, and other physicochemical reactions.

Phenolic compounds, with a diverse group structure, include flavonoids (flavonols, flavanols, flavanones, isoflavones, and anthocyanins) phenolic acids, tannins, stilbenes, among others. This fraction also includes isoflavones with a phytoestrogenic action, present in soy, plums, figs, strawberries, melon or pears.

Dietary fibre it represents the undigested or unabsorbed fraction from the body, including: cellulose, hemicellulose, lignin, gums and pectins, etc. Are presents in pigments that are found in fruits, vegetables, whole grains and legumes (chickpeas, lentils, carrots, fennel, apples, etc). The level of BSc could depend on the food processing carried out.

In vegetable products many factors influence the degradation of the bioactive compounds during food processing: temperature, air, light, moisture content, water activity, pH and enzymes related to the food spoilage. In addition, all the above factors can strongly affect the BSc estimation intake in terms of bioaccessibility/bioavailability. Consequently, this PhD thesis research project aims to identifyofthe influence of technological treatments and innovative technological processes on the qualitative/quantitative content of BSc their degradation products in vegetable foods and their bioaccessibility/bioavailability. Moreover, to evaluate of the possibility to use some of them BSc as process/product indicators. the database of bioactive compounds, in particular tocols and carotenoids, in vegetable foods will be also investigating and increased.

In addition, all the above factors can strongly affect the BSc estimation intake in terms of bioaccessibility/bioavailability.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Optimization of analytical methods

(A1.1) Optimization/setting up of analytical methods for the evaluation of BSc and their degradation products in different vegetable products (A1.2) and realization of appropriate databases of BSc may be considered of particular importance for vegetable matrices where the existing knowledge is absent or weak.

A2) Study of the influence of technological treatments on BSc

(A2.1) Selection of the most suitable samples for the technological treatments under investigation (A2.2) and study of the influence of traditional and innovative technological treatments and pre-treatments on the qualitative/quantitative content of BSc (A2.3) Identification of the technologies that may limit structural damage, preserving the properties of products. (A2.4) Evaluation of the possibility to use some of BSc as as process/product indicators.

A3) Bioaccessibility/bioavailability of on BSc

(A3.1) Choosing and setting up of methods for the evaluation of the bioaccessibility/bioavailability of BSc under investigation. (A3.2) The investigation of the effect of technological treatments on the bioaccessibility/bioavailability of BSc from vegetable products will be made using an “in vitro” approach.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Optimization of analytical methods																									
b) Optimization of methods																									
c) Realization of database																									
A2) Study of the influence of technological treatments on BSc																									
a) Setting up of technological treatments, optimization of the different process parameters.																									
b) Study and analysis of the effects of technological treatments and pre-treatments on the qualitative/quantitative content of BSc																									
c) Identification of the technologies that may limit structural damage, preserving the properties of products																									
d) Evaluation of the possibility to use some of BSc as process/product indicators																									
A3) Bioaccessibility/bioavailability of BSc																									
a) Choosing and setting up of methods for the evaluation of the bioaccessibility/bioavailability of BSc																									
b) Study of bioaccessibility/bioavailability in vitro																									
A4) Thesis and Paper Preparation																									

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New Pulses-Based Ingredients Obtained by Dry Fractionation to Improve the Nutritional and Technological Quality of Food

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Dry fractionation (DF) is a method to produce vegetable protein. It is more sustainable than the most common and more energy consuming wet extraction. Currently, DF is unusual and limited to few species, with a consistent lack of food applications. The aims of this PhD project are: I) the production of new protein rich ingredient obtained DF of pulses flours; II) the assessment of the influence of the raw material characteristics on the chemical and technological properties of the separated fractions; III) the application of these fractions as ingredient in food to improve the nutritional and technological quality.

Utilizzo di Nuovi Ingredienti Ottenuti Tramite Dry Fractionation per il Miglioramento del Valore Nutrizionale e della Qualità Tecnologica degli Alimenti

Il processo di dry fractionation (DF) è un metodo fisico per produrre proteine vegetali più sostenibile del metodo “chimico” comunemente utilizzato (wet extraction). Gli studi sul DF riguardano la sua applicazione su poche specie con scarsi impieghi alimentari. Gli scopi del progetto di dottorato sono: I) produzione di ingredienti innovativi ottenuti mediante DF di farine di legumi; II) valutazione dell'influenza delle caratteristiche della materia prima sulle proprietà fisico-chimiche e tecnologiche delle frazioni separate; III) uso delle frazioni come ingrediente per il miglioramento del valore nutrizionale e tecnologico degli alimenti.

1. State-of-the-Art

Pulses are commonly processed and consumed as whole or milled as flour. Pulses consumption is highly recommended by nutritionists (Oliveira et al., 2019) although they need a long cooking that rarely meet the current lifestyles (Summo et al., 2019). New strategies to increase the pulses consumption in developed countries concern the use of new pulses-derived ingredients such as starch, protein, or fiber sources (Sozer et al., 2017). Proteins indeed are widely used by the food industry as emulsifiers, due to their ability to reduce the interfacial tension and stabilize O/W emulsions. Whereas, starches are used for their thickening and gelling properties. Most of the proteins used as ingredient by the food industry are animal-based such as whey protein, casein, egg protein. However, the substitution of these products by vegetable alternatives is widely encouraged by researches, considering both the emerging healthy aspects and the sustainability of the productions. Therefore, the food industry requires new strategies to find consumer friendly and sustainable plant protein ingredients to replace those derived from animal sources.

This scenario explains the growing interest for pulses protein that can be used as functional ingredient to enhance the nutritional and technological value of food. Moreover, the demand of texturized protein used as meat analogues constantly increases.

The majority of pulses-derived proteins available on the market are conventionally obtained by wet extraction (Schutyser et al., 2015), in which the pulses flour is dispersed in water in order to separate the starch. Proteins are solubilized by using a strong alkali (pH 9) and then precipitated to the iso-electric point (pH 4.5-4.8). The pH is adjusted to 7 and the protein isolate is dried, providing a protein concentration of 75-90 %. The

drawbacks of this process consist in the use of a considerable quantity of water and chemicals. Moreover, it involves high energy consumption (Schutyser et al., 2015).

To overcome these problems, a dry fractionation method was studied in the last years. A fine milling of pulses causes the detachment of the starch granules (20-30 μm) from the protein body (1-3 μm), therefore these fractions can be separated on the basis of both density and size by the air classification (Schutyser et al., 2015). The flour is fed in a cyclone and separated in two fractions by using a calibrated stream of air. Finer fraction is rich in protein (43-56 %) whereas the coarse fraction is composed by the 70 % of starch.

Since the dry fractionation has received a renewed interest from the scientific community, the studies currently present in literature are limited and focused on few pulses species such as pea (Rempel et al. 2019), lupin (Pelgrom et al., 2014) and faba bean (Felix et al., 2018), furthermore the effectiveness of these fractions to produce meat analogues was just theorized (Schutyser et al., 2015).

Therefore, the aim of this PhD project is to investigate the use of air-classified flours as ingredient in order to improve the nutritional and technological quality, with a particular emphasis on meat products and meat analogues. To reach this aim, the influence of the raw material on the technological properties of the air-classified fractions will be also studied.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1. The data obtained as output of each activity will be submitted to statistical analysis.

A1) Chemical and physical characterization of the raw materials and of their air classified fractions in order to assess the influence of the physical characteristics (A1.1) and chemical composition (A1.2) of raw material on the chemical and technological properties of fractions. The activities will be performed on a wide set of samples (already available and produced in collaboration with a local food company) of different species and obtained by the application of different processing parameters;

A2) Selection and production of dry fractionated fractions: on the basis of the A1 results, DF fractions from the most suitable pulses species and the optimal processing parameters for dry fractionation will be produced. This activity will be performed in collaboration with a local food company and/or using a pilot plant for DF available in our department;

A3) Application of air classified fractions as ingredient: fractions produced in A2 will be used for (A3.1.) the production and optimization of a meat analogue and/or meat extender. The optimal formulation and the process will be selected on the basis of sensorial and structural properties. (A3.2) Use of A3.1 output as ingredient in a target food. The set-up of technological protocol and optimization of formulation will be carried out. (A3.3) Use of air classified fraction directly as ingredient (emulsifier/thickener) in target food (salad dressing or vegan cheese) in order to improve the nutritional value;

A4) Nutritional, sensorial and textural evaluation of the products and shelf life assessment;

A5) Planning the industrial scale-up: at least on one product derived from A3 will be performed the develop of a plan for the semi-industrial implementation of the process. The activity will be carried out in cooperation with a local engineering company;

A6) Data Analysis, Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Physico-chemical characterization of raw material and DF fractions																								
	1.1 Influence of physical characteristics																								
	2.1 Influence of the chemical composition																								
A2)	Selection and production of ingredients by DF																								
A3)	Use of DF fractions as ingredient																								
	3.1 Production of a meat-analogue/extender																								
	3.2 Use of meat-analogue in food																								
	3.3 Use of fractions directly as ingredient in target food																								
A4)	Nutritional, sensorial and textural evaluation																								
	4.1 Chemical and textural properties																								
	4.2 Shelf life assessment																								
A5)	Planning the industrial scale-up of at least one product																								
A6)	Data Analysis, Thesis Writing and Editing																								

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Characterization of Ancient Abruzzi Wheat Grains and Flours Intended for the Production of High Quality Foods

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This PhD thesis research project is aimed to characterize ancient grains typically grown in Abruzzo by comparing them with modern varieties. The whole "supply chain" will be followed, from sowing, harvesting and milling to the production of pasta, bread and beer. The objective is therefore to valorize the biodiversity of the territory and, at the same time, to take advantage of those genotypes capable of growing in the most marginal lands, providing new products that meet the needs of modern society.

Caratterizzazione di granelle e farine di frumenti antichi abruzzesi per la produzione di alimenti di qualità

Questo progetto di tesi di dottorato mira a caratterizzare grani antichi tipicamente coltivati in Abruzzo confrontandoli con varietà moderne. Si seguirà tutta la "filiera", dalla semina, raccolta e macinazione fino alla produzione di pasta, pane e birra. L'obiettivo è quindi quello di valorizzare la biodiversità del territorio e allo stesso tempo di sfruttare quei genotipi capaci di crescere nelle terre più marginali, fornendo nuovi prodotti che soddisfino le esigenze della società moderna.

1. State-of-the-Art

The durum and soft wheats represent in our country and in the world the most important sustainable resource for food production. In Italy the cultivation of wheat has always had an enormous importance. The new cultivars developed by agronomists and geneticists in the last 100 years allowed to feed many more people. As a result, ancient cultivars were slowly abandoned. However, the modern cultivars were implemented under modern agronomic practices using high agricultural inputs (Boukid et al., 2018) with the aim of improving yield, proteins and technological food features to the detriment of phytochemical and nutritional ones (Di Silvestro et al., 2012). In addition, more than 95% of the organic products derives from cultivars bred for the conventional farming and there is a lack in cultivars suitable for low input agronomic techniques as the organic system (Lammerts van Bueren et al., 2011). Ancient wheats could help agriculture in marginal areas thanks to their high adaptability in disadvantageous growing conditions (such as wet, cold soils, high altitudes, and poor soils) (Konvalina et al., 2010). This work aims to characterize two ancient wheats typically cultivated in Abruzzo: the soft wheat (*Triticum aestivum* L. subsp. *aestivum*) Solina and the durum wheat (*Triticum turgidum* L. subsp. *durum*) Senatore Cappelli and to use their milling products to produce high quality foods.

Solina is a soft local wheat cultivated in Abruzzo at least since the sixteenth century. From the agronomic point of view Solina is very rustic, well suited to poor and consisting of stone soils, very resistant to cold and to the spring-summer drought, of limited productivity (on average 2 t/ha) but from constant production. It does not tolerate the nitrogenous fertilizations and the very fertile soils as sensitive to lodging, this because of the thin straw and of the high size, characteristics which give a good competitive ability against weeds (Solina Consortium's Production Regulations, 2008). Solina flour is

attributable to the category of biscuits flour with a W of 66.0 ± 12.4 and a P/L of 0.17 ± 0.02 (Porfiri et al., 2004).

Senatore Cappelli durum wheat is a variety obtained by the agronomist Nazareno Strampelli in 1915 and published in 1923 from a late-maturing pure line selected from the North African landrace Jean Retifah (Lorenzetti R., 2012). Senatore Cappelli had a wide adaptability, rusticity, greater number of seeds per spike (40-60) and semolina quality, on the other hand it was tall, late, and susceptible to lodging and rust (Scarascia Mugnozza, 2003; Bocci et al., 2018). According to Fois et al. (2011) Senatore Cappelli, given its high protein content, would allow to produce an excellent high temperature dried pasta, despite its low gluten index (GI) compared to modern varieties.

Given the importance that such crops constitute, it is appropriate to analyse all aspects related to their characterization, collection and use. The results will constitute the scientific support for the acquisition of quality brands, for the definition of disciplinary aimed at optimizing production procedures and for the commercial enhancement of the products themselves.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Characterization and study of the functional properties of raw materials (grain, flour, bran) to identify the difference between the modern and old wheat cultivars grown in different years and at different altitudes, monitoring climate data, cultivation techniques and harvesting. The planned analyses range from the classic commercial ones (thousand seeds weight, hectolitre weight, impurities, dimensions) to the chemical-physical ones (hardness, protein, ash, gluten, moisture, fat and carbohydrate). For the flours, the classical empirical-imitative analyses will also be carried out (Brabender farinograph, Chopin alveograph). An important goal will also be to obtain a clear protein and genetic characterization of Solina wheat (SDS-PAGE, genetic analysis using molecular markers such as Infinium SNP array, DaRT seq technology e GBS).

A2) Characterization and quality improvement of bread based on traditional cereals. To comply with this aim, wheat flours, either stone or roller-milled, in purity or blends will be kneaded and leaven with yeast or sourdough in order to study their suitability for bread making. The composition, gluten quality, rheological properties and functional properties of the flours will be analysed as well as the physical and functional properties, volatiles profile and sensory properties of breads.

A3) Characterization and quality improvement of pasta products based on traditional cereals: To comply with this aim, durum wheat semolina, either stone or roller-milled, in purity or blends will be extruded and dried either at low and high temperature in order to study their suitability for pasta making. The composition, gluten quality and functional properties of the semolinas will be analysed as well as the physical and functional properties, volatiles profile and sensory properties of pasta.

A4) Characterization and improvement / enhancement of craft beers based on traditional cereals To comply with this aim, wheats and durum wheats will be processed to obtain either Weizen beers or ale beers. The composition, physical and functional properties, volatiles profile and sensory properties of beers will be analysed.

A5) Bibliographic research, writing and editing of the PhD thesis, scientific papers and oral and/or poster communications

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2 ÷ 8	9 ÷ 10	11	12	13	14	15	16	17 ÷ 20	21	22	23	24	25	26 ÷ 28	29 ÷ 32	33	34	35	36
A1) Characterization of raw materials																					
A2) Characterization of bakery products																					
A3) Characterization of pasta																					
A4) Characterization of craft beers																					
A5) Bibliographic research, writing and editing																					

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The Development of a Biodegradable Smart Package

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This proposed PhD project is aimed to develop a fully smart package to control the freshness and extend the shelf life of food products, constituted by a real-time multiplexed flexible printed gas sensor that employs aptamers as bioreceptors, and then develop an active package employing smart polymers which release anti-oxidants upon application of a voltage. This new generation of smart packages will have a great advantage as compared to the old class of packaging materials in terms of environmental-friendliness, performance, accuracy and shelf life extension.

Lo Sviluppo di Imballaggi Intelligenti e Biodegradabili

Questo progetto di dottorato è volto a sviluppare un imballaggio completamente intelligente per estendere la durata di conservazione dei prodotti alimentari usando un biosensore di gas stampato, flessibile e multiplexato e anche usiamo aptameri come biorecettore, e poi sviluppando un imballaggio attivo utilizzando polimeri intelligenti che rilasciano antiossidanti mentre sono sottoposti a un campo elettrico. Questa nuova generazione di imballaggi intelligenti avrà un grande vantaggio rispetto alla vecchia classe di materiali di imballaggio in termini di compatibilità ambientale, prestazioni, precisione e estensione della durata di conservazione.

1. State-of-the-Art

Food waste is a major global concern since one-third of all food produced on the planet is wasted. There are, however, large differences across different regions and along different stages of the supply chain. In particular, the yearly amount of food waste in the European Union is around 88 million tonnes of food. This waste and loss of food creates huge environmental, economic and social problems (Kummu et al., 2012). The freshness of meat decreases with time mainly due to the microbial spoilage and biochemical reactions which occur during storage. Thus, meat with different freshness status will generate different kinds of gases. Conventionally the freshness of meat is evaluated by the concentration of the total volatile basic nitrogen (total VBN, TVB-N), usually in the units of mg/100 g. VBN includes mostly three kinds of gas molecules, ammonia (NH_3), dimethylamine ($(\text{CH}_3)_2\text{NH}$, DMA), and trimethylamine ($\text{N}(\text{CH}_3)_3$, TMA). After the animal death, the spoilage bacteria and the enzymes in the meat will convert the trimethylamine oxide (TMAO) into DMA and TMA.

Hence to decrease the food waste and improve the safety of the food products new solutions have to be adopted for monitoring and controlling food products where we'll be able to monitor and control the freshness and the quality of the food products. Intelligent packaging systems can carry out intelligent functions (like detecting, sensing, recording) to facilitate decision making, to extend shelf-life, enhance safety, improve quality, provide information. At the same time, intelligent packages contain sensors or indicators able to monitor the food condition during its life cycle and able to communicate information related to the quality of the product. There are multiple types of "smart devices" that can be used in intelligent packaging and their functions have been explored

by multiple studies (Zhang et al., 2016). Furthermore, Active packaging is a further extension of the concept of intelligent packaging, as it enables an interaction of the product and its environment with the packaging material to extend the product shelf life and to ensure its microbial safety. The active packaging maintains the quality of the packed food by absorbing food derived chemicals from the food or the environment within the packaging surrounding the food; or releasing substances into the food or the environment surrounding the food such as preservatives, antioxidants, and flavorings (Kerry et al., 2006).

One class of biosensors that could be included in food packaging is Field effect transistor (FET), are electronic devices where two electrodes called source and drain are separated by a semi conductive channel whose conductivity can be increased or decreased as a function of the potential applied to a third electrode called gate. By functionalizing the gate with bioreceptors (aptamers in our case) and in the presence of the analyte, the current flowing from the source to the drain (I_{ds}) will change, allowing a direct correlation between the change in the current with a concentration of the analyte. Compared to other types of biosensors a transistor amplifies the signal, so we can detect much lower concentrations. This on the other side related to a much more complicated fabrication process (Shi et al., 2018). One of the bioreceptors that could be implanted in sensors are aptamers, Aptamers are in vitro-selected single-stranded DNA or RNA oligonucleotides exhibiting high affinity to specific targets including proteins, cell surfaces, and even small molecules. Due to their three-dimensional folding, aptamers are able to bind their targets with high selectivity. Especially for small molecule targets, they are promising bioreceptors, since they can be selected for a variety of small analytes including those with high toxicity or low immunogenicity (Stoltenburg et al., 2005).

Stimuli-responsive polymers are materials which mimic biological systems in the capacity to change under external stimulation. These smart polymer biomaterials should present their response within biological conditions. Typical stimuli are temperature, pH, light, electric field, and electrolytes (Li et al., 2018). The responses triggering the drug release can be: dissolution/precipitation, degradation, change in hydration state, swelling/collapsing, hydrophilic/hydrophobic surface, change in shape, conformational change and micellization. Recently Marturano (2019) developed a light-responsive active packaging which releases antioxidant after emission with ultra-violet light.

Thus, this PhD thesis project will be directed to isolate aptamers for spoilage gases (Ammonia, Trimethylamine, Dimethylamine.) and then develop a transistor using the aptamers isolated before, and finally developing an active packaging which releases anti-oxidants and antimicrobial upon application of a voltage.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) Isolation of aptamers for (Ammonia, Trimethylamine, Dimethylamine) by using The SLEX process.

A2) Development of a printed flexible transistor for Real-time detection of Ammonia by functionalizing the transistor gate with the specific aptamers developed before, (A2.1) Development of a multiplexed detection system with different transistors functionalized with different aptamer types. (A2.2) Coupling the sensor with a printed RFID to avoid using the battery.

A3) **Development of an active packaging** by using a smart polymer or silver nanoparticles (responsive materials), which will release anti-oxidants while subjected to an electric field.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) SELEX Process																								
1) Aptamers for Ammonia, Trimethylamine, Dimethylamine.																								
A2) Flexible Transistor Gas Sensor																								
1) Multiplexed Gas Sensor.																								
2) Coupling the Gas Sensor with RFID																								
A3) Active Packaging																								
1) Smart nano-materials																								
A4) Thesis and Paper Preparation																								

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Using Advanced Techniques for Studying the Rheological and Tribological Behavior of Complex Food Systems

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This PhD thesis research project is aimed at using the advance techniques for understanding the rheological and tribological properties of different food complexes and enumerating the effect of addition of different solutes on the friction profile of different food matrixes. Furthermore, linking the oral processing technological data with the traditional sensory evaluation would also be a goal to achieve in this PhD project

Strategia sperimentale per la progettazione ottimale di unità di ultrafiltrazione per il recupero di biopolimeri di interesse alimentare

Questo progetto di ricerca ha come scopo principale quello di impiegare tecnologie avanzate ed innovative per la comprensione delle proprietà reologiche e tribologiche di matrici alimentari complesse, evidenziando/identificando inoltre l'effetto dei soluti di diversa natura sul 'friction profile' degli alimenti. Inoltre questo studio ha l'obiettivo di mantenere un legame tra l'elaborazione dei dati ottenuti da un processo tecnologico ed innovativo e la tradizionale analisi sensoriale, garantendone una correlazione ottimale.

1. State-of-the-Art

Food processing heavily relies on complex flow processes hence rheological characterization of the individual ingredients as well as the composed food product found on supermarket shelves is an integral part of food science. Rheological research in food science is linked to the development of food products and could address the industrial process such as (stirring, pumping, dosing, dispersing, spraying), cooking as well as consumption of food (oral perception, digestion, well-being). The close link of rheology and structure also introduces the relationship to flow processing, which determines the dynamic conditions under which the food material flows. Rheology by determining the viscous and elastic components of foods plays an important role because (i) flow properties define food structure during manufacturing (factory) or preparation (kitchen) and (ii) physiologically in mouth, stomach, and intestine where food structure is perceived and digested. Eventually rheology impacts directly on perception and digestion by influencing the flow characteristics during mastication and digestion but also triggers other quality characteristics such as flavor or nutrient release at specific sites (Fischer & Windhab, 2011).

Tribology is a term of Greek origin word 'tribo' (sliding or rubbing) which represents a new branch of rheology dealing with the lubrication, wear and friction of the interacting surfaces when they are in the state of relative motion. These surface interactions are of vital importance for the proper functioning of the manmade machines.

In the food sector tribology is gaining importance as it can contribute to better understand some sensory properties of foods when present and chewed in the mouth (e.g. mouthfeel, stickiness) and to optimize and develop food formulation for target rheological properties. Moreover, oral tribology and the lubrication occurring in mouth in

the presence of saliva is still unexplored parts in the studies of tribology applied to foods. In the last stages of oral processing delicate tribological changes take place in the leftover food particles and salivary film along which defines the aftertaste and mouth feel of taken food. (Chen & Engelen, 2012). In some foods the sensory perception is highly dependent on elasticity or firmness of foods which could be described with the help of sensory evaluation; however, this method has its own limitation as it can't be used to define the mouthfeel attributes which are primarily dependent on the stickiness of food. It is believed that tribological measurements by mimicking the food-tongue/saliva-plate system could be used to correlate instrumental data and the sensory ones that at the moment are very scarce in the literature.

With the increasing quantity and variety of powders being produced in industry, there is a need for information about their handling and processing characteristics, especially for food powders, because of their complexity. Powders or granular materials can be produced with a variety of different physical properties. The measurement of physical properties is important because these properties help define the powder and because they intrinsically effect its behavior during storage, handling and processing. The flow ability of powders and their flow behavior under pressure, temperature and humidity are important in handling and processing operations, such as storage in hoppers and silos, transportation, formulation and mixing, compression and packaging. For such purposes a newly developed powder cell rheometer is a very effective and valuable tool. Powder rheometer is an effective tool for calculating the stress in loosely compacted powders as it provides a consider edge while enumerating the low normal stress in powders which is of vital importance in many industrial applications e.g. mixing, fluidization or pneumatic transportation. This gives an in depth understanding for powders which are to be processed in different packing states (Opaliński, Chutkowski, & Hassanpour, 2016)

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Optimization of new methods for tribological measurements (A1.1) and rheology (A1.2) of powders to be applied on model and real food systems to evaluate the effects of variables like composition, physical and colloidal state and presence of saliva

A2) Characterization of tribological behavior of real food and beverage samples (e.g. coffee, wine); results will be related to composition and physical properties including the rheological behavior, as well as sensory properties. Tribological analysis would be performed also in presence of saliva

A3) Tribological study on colloidal systems (emulsions): 6 months' internship will be performed in a high ranking research center to study tribology behavior of model and real food emulsions

A4) Food powders rheology: flow measurements will be carried out to characterize powders physical and technological properties; followability, fluidization, compression and adhesion strength of powders equilibrated at different RH % will be also performed to determine stickiness and collapse phenomena

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Methods optimization																									
1) Tribological methods																									
2) Powders rehology																									
A2) Tribology: application to real systems																									
1) Rehological aspects																									
A3) Powders characterization																									
1) Characterization																									
2) Rehological measurements																									
A4) Mobility																									
A5) Thesis and Paper Preparation																									

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Biomarkers of Food Intake: a Metabolomics Approach

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This PhD research project is aimed at identifying biomarkers of intake of specific compounds, namely flavan-3-ols, and of dietary patterns using targeted metabolomics. Flavan-3-ols are secondary plant metabolites and are therefore characteristic compounds of plant-based foods. Many *in vivo* studies demonstrated potential beneficial effects of these compounds on human health. Identifying specific biomarkers of intake of flavan-3-ols would enable a more accurate assessment of their dietary intake and, potentially, a deeper understanding of the existing association between intake and health effects. Similarly, identifying metabolic profiles associated with dietary patterns will improve the evaluation of dietary exposure and nutritional status.

Studio dei Biomarkers di Consumo Alimentare: un Approccio Metabolomico

Questo progetto di dottorato mira ad individuare biomarkers di consumo sia di specifici composti, i flavan-3-oli, che di modelli alimentari, adottando un approccio metabolomico di tipo targeted. I flavan-3-oli sono metaboliti secondari delle piante e di conseguenza composti caratteristici degli alimenti di origine vegetale. Numerosi studi *in vivo* hanno dimostrato come questi composti esercitino effetti potenzialmente positivi sulla salute umana. Individuare specifici biomarkers di introduzione di flavan-3-oli permetterà una stima più accurata del loro consumo, e, potenzialmente una comprensione più approfondita delle associazioni esistenti tra il loro consumo e gli effetti sulla salute. Allo stesso modo, l'identificazione di biomarkers di consumo associati a diversi modelli alimentari permetterà di migliorare la valutazione della dieta e dello stato nutrizionale.

1. State-of-the-Art

Flavan-3-ols are the characteristic polyphenols of tea, cocoa, wine, pome fruits (as apple and pear), berries, and nuts, but they are also found in stone fruits and legumes (Zamora-Ros et al., 2013). This subclass of compounds is the main source of flavonoids in Western diets (Zamora-Ros et al., 2016).

Phenyl- γ -valerolactones (PVLs) and phenylvaleric acids (PVAs) are the main catabolites of colonic origin of flavan-3-ols and are specific of this subclass of compounds (Mena et al., 2019). In particular, glucuronide- and sulfate-conjugates of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, are the most important flavan-3-ol metabolites, regardless of the food source, and have therefore been proposed as biomarker of flavan-3-ol intake (Ottaviani et al., 2018). However, information on their production after cranberry consumption is limited.

Concerning dietary patterns, little is known about their identification through the application of metabolomics (Esko et al., 2017), while the amount of information related to the intake of specific foodstuffs has been notably increasing during the last decade (Edmands et al., 2015).

Usually, dietary assessment has been performed using self-reported methodologies such as food frequency questionnaires (FFQs), 24-h dietary recalls or food records (Garcia-Aloy et al., 2017). However, the accuracy of dietary intake and nutritional status is frequently challenged by participant misreporting, non-compliance and bias.

These limitations can be overcome by the application of metabolomics to characterize dietary exposure.

Combining the information collected thanks to food intake biomarkers with traditional tools used in dietary assessment, this approach will represent a step forward towards improving the evaluation of dietary exposure and nutritional status.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD project can be subdivided into the following activities, according to the Gantt diagram given in Table 2.

Table 2 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Biomarkers of cranberry flavan-3-ols intake																									
1) Sample preparation and analysis through UHPLC-ESI-MS/MS																									
2) Data analysis																									
A2) Selection of food intake biomarkers																									
1) Systematic search																									
2) Assessment of suitability of food intake biomarkers																									
3) Final list of food intake biomarkers																									
A3) Validation of selected food intake biomarkers																									
1) Method preparation																									
2) Sample preparation and analysis through UHPLC-ESI-MS/MS																									
3) Data analysis																									
4) Validation																									
A4) Assessment of dietary patterns																									
1) Sample preparation and analysis through UHPLC-ESI-MS/MS																									
2) Data analysis																									
3) Assessment of dietary patterns																									
A5) Thesis and Paper Preparation																									

A1) Study of phenyl- γ -valerolactones and their related phenylvaleric acids as biomarkers of flavan-3-ols intake: the case of cranberry flavan-3-ols, employing secondary analysis of plasma and urinary samples collected in three cranberry feeding studies.

A2) Selection of the most suitable biomarkers associated with consumption of food groups or specific foodstuffs according to the literature using the FoodBall reviews and reports, doing a systematic search on Scopus and also additional researches.

A3) Validation of selected food intake biomarkers employing secondary analysis of urinary samples obtained from prior studies and by comparison with information collected in traditional dietary assessment tools.

A4) Assessment of dietary patterns through the use of validated food intake biomarkers employing secondary analysis of urinary samples obtained from prior studies.

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

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Main Roles of Agro-Industries in the Agroecological Transition: Public Policies and Private initiatives

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This PhD thesis research project is aimed to define and evaluate how the agro-industry can be recognized as a co-leader actor in the agro-ecological transition dynamics across agricultural productive systems and rural communities. To achieve this goal will be analyse public policies and private initiatives which currently promote agro-ecological principles evaluating if are working in duality or synergic mode. Using a quantitative approach special attention will be dedicated to evaluating how private sustainability initiatives could affected contract power balance and economic value distribution between supply chain actors and society.

Il principale ruolo dell'agro industria nella transizione verso l'agroecologia: politiche pubbliche e iniziative private

Questo progetto di tesi di dottorato mira a comprendere e valutare quanto l'agroindustria possa essere riconosciuta come uno dei soggetti trainanti nella transizione agro-ecologica del settore agricolo. Tale obiettivo verrà perseguito valutando la congruità e la complementarità di iniziative private e politiche che attualmente promuovono principi agro-ecologici. Attraverso metodologie analitiche basate su schemi di analisi quantitativa, particolare attenzione sarà dedicata alla valutazione di come l'adozione di iniziative private promosse dall'agroindustria possano influenzare l'equilibrio del potere contrattuale e la distribuzione del valore aggiunto tra gli attori della filiera e la società.

1. State-of-the-Art

Since the 70s, agricultural systems have focused on maximizing productivity and profitability. However, this intensive model in a few years has accelerated the exploitation of natural resources. During the opening intervention of the 2nd International Agro-ecology Symposium (FAO, Rome 3-5 April 2018), the FAO's director general, José Graziano da Silva, urged the transition to healthier and more sustainable food systems he declared that only the agro-ecology can contribute to this transition. According to Gliessman (1998) the agro-ecology is defined like the science of applying ecological concepts and principles for the design and management of sustainable agro-food systems. Among the most suitable strategies for this step, there is the diversification of agricultural systems, through the introduction of new agronomic practices and organizational models at farm and supply chain level (Francis et al., 2003).

This reading is also confirmed by the new Common Agricultural Policy (CAP) 1st june 2018 Commission 216 reform proposal that continues to move on Fischler reform path. In fact, this has strengthened tools such as cross-compliance, greening, new proposal on ecological payment schemes (1st pillar) and has plan again agro-environmental schemes for farmers and others actor of the supply chain (2nd pillar).

The research of sustainability attribute in the food products seems to be very interesting looking at market shares increasing and opportunities for future golden shopper demand. The results of Grunert et al. (2014) shows that consumers associate the sustainability concept to aspects of environmental protection. There is a growth in attention on individuals as a means of tackling environmental problems in Western democracies (Barr et al., 2011). So, an increasing number of consumers demand and prefer food with sustainability claims.

The agri-food companies answer at this demand with sustainable products by adopting new approaches to the supply of raw materials. A first approach consists in using raw materials certificated by sustainability certification schemes. The use of this tools during the last years (from 2011 to 2016) is testified by the increase of hectares of agricultural areas affected by different certification schemes that call for sustainability in the world: from a minimum of 26.8% (sugarcane) to a maximum of 187% (cocoa) (Lernoud et al., 2018). Recently agro-industries have promoted others approaches based on private sustainable supply chain initiatives, and they have started communicating sustainability-claim related information about food to consumers, introducing labels and logos in-store and on-pack (Blasi, Ruini, & Monotti, 2017; Matopoulos et al.; 2001).

However, it is very important to understand which are the certifications and private initiatives that facilitate the introduction of agroecological principles and practices at farm level, and if, at the same time, that are recognized by consumers as “sustainable attributes”. So, the transition of the agricultural sector towards agro-ecology and sustainability seems to be an ongoing process. The agricultural sector is “pushed” by a regulatory framework more focused on environment issues and “pulled” by subsidies linked to the adoption of environmental practices supported by policies and private supply chain initiatives promoted by the market. The overall objective of this PhD thesis project will be directed to define how agro-industry could be a player of agroecological transition of the agricultural sector between public policy and private initiatives (Figure 1).

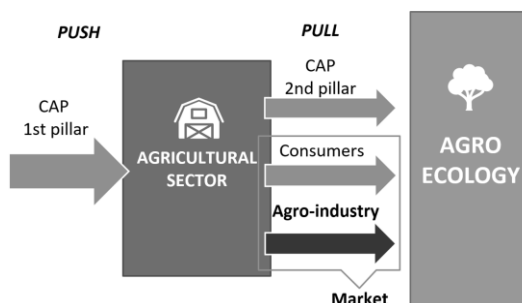


Figure 1 *Transition of the agricultural sector to the agro-ecology.*

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Certification schemes and private initiatives review. Define which are the certification schemes (A1.1) and the private initiatives adopted, or to be adopted (A1.2), by the agro-industry, that influence the agro-ecological transition of the agricultural sector. The deliverable is to obtain a qualitative ranking of certifications and private initiatives that respect agroecological principles at farm level (A1.3).

A2) Public policies review. Define which are the public policies at farm level that influence the agro-ecological transition of the agricultural sector (A2.1). The deliverable is to obtain a qualitative ranking of measures that respect agroecological principles (A2.2).

A3) Complementarity between private and political initiatives. Evaluate the congruity and complementarity between private and political initiatives that currently promote agro-ecological principles. The deliverable is a comparative analysis of convergences and divergences in order to identify supply chain tools that can be used synergistically.

A4) **Economic sustainability of private initiatives.** Define if the adoptions of private initiatives can improve the economic sustainability for the actors of the supply chain. This goal will be done by identifying two case studies representatives of the Italian agro-industrial sector: the supply chains of pasta and bakery products (A4.1). After a data collection of both cases study (A4.2), it will be identified and applied a business model to verify the improvement of supply chain actor's performances (A4.3). Finally, it will be done an analysis of results (A4.4).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Quater	1	2	3	4	6	7	8	9	10
A1)	Certifications and private initiatives review									
	1) Certification schemes									
	2) Private Initiatives									
	3) Qualitative ranking									
A2)	Public policies review.									
	1) Public policies									
	2) Qualitative ranking									
A3)	Complementarity private-political initiatives									
A4)	Economic sustainability of private initiatives									
	1) Identification of cases study									
	2) Data collection									
	3) Business model									
	4) Analysis of results									
A5)	Thesis and Paper Preparation									

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Application of Pulsed Electric Fields (PEF) for the Modulation of Chemo-Physical and Rheological Properties of Different Foods

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This PhD thesis research project is aimed at the application and optimization of pulsed electric field (PEF) technology, particularly as pre-treatment, for the improvement of mass transfers phenomena in plant and animal tissues.

Utilizzo dei campi elettrici pulsati (PEF) per la modulazione delle caratteristiche chimico-fisiche e reologiche di differenti prodotti alimentari

Il presente progetto di ricerca si propone di valutare l'applicazione di campi elettrici pulsati (PEF) a differenti tipologie di alimenti, in particolare come pre-trattamento/intervento di trasformazione per l'aumento della velocità dei fenomeni di trasferimento di massa in fase di processo.

1. State-of-the-Art

Among all emerging non-thermal technologies, pulsed electric fields (PEF) is one of the most appealing due to its reduced heating effects and short treatment times with respect to other technologies.

Pulsed electric fields (PEF) technology consists on an electrical treatment of short time (from several nanoseconds to several milliseconds) with electric field strengths from 100 to 300 V/cm to 20–80 kV/cm (Vorobiev & Lebovka, 2008). High electric fields (> 20 kV/cm) are generally used to inactivate alterative and pathogenic microorganisms and quality related enzymes (Sánchez-Vega *et al.*, 2014); while lower electric field strenghts (0.5 – 1 kV/cm) are used alone or in combination with other food process unit operations and they could lead to the lost, temporarily or permanently, of cell membrane semi-permeability. As cell membranes could be considered a physical barrier to the diffusion processes, their permeabilization degree could influence mass transfer phenomena. Cell membrane electroporation promote the release of intracellular components (Lebovka & Vorobiev, 2010).

It has been widely demonstrated that pulsed electric field can be used in food processes as a pre-treatment method in order to enhance the subsequent process kinetics or to modify the quality of the final product. It can be used in combination with the most energy-consuming processes, such as freezing (Jalte *et al.*, 2009), drying (Ade-Omowaye *et al.*, 2001), extraction (Loginova *et al.*, 2011), which are based on heat and/or mass transfer. Due to electrically induced disintegration of the cell membrane (and some other organelles), the progress of drying, osmotic dehydration, or freezing can be enhanced, and thus the specific energy consumption of aforementioned processes can be reduced.

The energy consumption is an important factor for the estimation of industrial attractiveness of the electrical pulsed energy application in the food industry. PEF treatment require a moderate energy consumption, generally between 1 and 15 kJ/kg of product (Vorobiev & Lebovka, 2010), vantageous aspects in terms of sustainability.

Electroporation mechanisms have been widely investigated in model systems and static operation conditions; however, how dynamic processes and the complex geometry of matrices could influence the outcome is not clear and need further explanations.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Literature review																								
A2) Toxicants reduction in frying processes																								
1) PEF protocol optimization for the production of potato chips																								
2) Quantification of the formation of probably carcinogenic compounds																								
A3) Innovative vegetable snacks																								
1) Combination of PEF and <i>mild</i> conventional treatments																								
2) Quality evaluation of <i>snack</i> products																								
A4) Minimally processed vegetable and animal products																								
1) Evaluation of structure and metabolic modifications due to electroporation																								
2) PEF protocol optimization to enhance mass transfers in animal tissues																								
A5) Shelf life study of minimally processed products																								
A6) Industrial scale-up																								
A7) Conferences participation, writing of scientific papers and thesis																								

A1) **Literature review** on the application of pulsed electric field technology in food processes.

A2) **Toxicants reduction in frying processes**, optimization of PEF parameters in order to enhance the release of Maillard reaction substrates from the potato tissue and mitigate the concentration of probably carcinogenic compounds (e.g. acrylamide) in fried potato crisps.

A3) **Production of innovative vegetable snacks** evaluating the possible combination of pulsed electric field pre-treatment with *mild* conventional processes for the production of high functional foods.

A4) **Minimally processed vegetable and animal products**. Comprehension of electroporation / electropermeabilization phenomena due to the application of an external electric field to animal and plant cells, and evaluation of structural and metabolic modifications.

A5) Shelf life study of minimally processed products.

A6) Industrial scale-up of the most promising pre-treatments found thanks to the collaboration with high voltage systems producers.

A7) Conferences participation with poster and oral presentation, writing of scientific papers and thesis.

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Fermentation as a Strategy to Produce High Value Compounds from Byproducts and Waste

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The aim of this Phd research project is to apply steered fermentation processes on waste materials in order to obtain high value biochemical molecules. The first part of the research will be focused on the exploitation of strain biodiversity, exploration of different waste/by-products and the optimization of fermentation parameters. Chemical profiling and identification of metabolites of interest will be performed in a second step. The last part of the project will be oriented to find the right application of these compounds in different fields, from the food/feed industries to the chemical and pharmaceutical one.

Fermentazione di Scarti come Strategia per la Produzione di Composti ad Alto Valore

Lo scopo del progetto di ricerca di questo dottorato, è quello di studiare i processi mirati di fermentazione partendo da materiali di scarto al fine di ottenere molecole bioattive. La prima parte della ricerca sarà rivolta a esplorare la diversità tra i ceppi, sfruttando scarti e sottoprodotti di differente origine e all'ottimizzazione dei parametri di fermentazione. Seguiranno le analisi relative alla caratterizzazione molecolare dei fermentati e all'identificazione dei composti bioattivi di interesse. L'ultima fase del progetto sarà diretta all'impiego delle molecole ottenute in diversi settori industriali, tra cui quello alimentare, mangimistico, chimico e farmaceutico.

1. State-of-the-Art

Solid state fermentation (SSF) is defined as a technique of cultivating microorganisms on solid material which acts as physical support and as source of nutrient, in the absence of a free aqueous phase.

Different metabolites are produced during fermentation like bacteriocin with an antimicrobial activity against different pathogens, aroma compounds, secondary metabolites of industrial interest. Fermentation is also note to improve food digestibility, removal toxins and antinutritional compounds, enhance antioxidant activity (Swain & Ray, 2016).

Solid state fermentation offers several advantages such as low cost, simple equipment and most important, it permits the use of agricultural and agro industrial byproducts as substrates (Martinez-Avila et al., 2014). The use of these materials as the culture medium of microorganisms represents a potential significant reduction in the cost of bioprocesses and the conversion of the waste into products with commercial value-added.

In the last decade several studies have investigated the use of SSF on industrial waste, using different strains of *Lactobacillus* for the production of lactic acid (Djukić-Vuković et al., 2016) or to improve nutritional composition (Gupta et al., 2018), and using yeast to modulate the aromatic profile (Vong & Liu, 2017).

We will focus on both plant by-products such as brewer's spent grain and okara, a soybean by-product, derived from the manufacturing of soybean foods such as soymilk and tofu, as well as animals such as waste from insect farms of which there are no studies in the literature.

Table 1 Recent studies (since 2014) on fermented byproducts for different purpose

Byproduct/ waste	Microorganism used	Properties studied			Reference
		Antimicrobial activity	Volatile compounds	Other properties	
Insect derived waste	Untested	Untested	Untested	Untested	-
Okara	<i>Lactobacillus plantarum</i>	Untested	Untested	Improve nutritional composition	(Gupta et al., 2018)
	Yeast		Decrease off- odours	Untested	(Vong & Liu, 2017)
Brewer's spent grain	<i>L. rhamnosus</i>	Untested	Untested	Production of lactic acid	(Djukić-Vuković et al., 2016)

In this context, this PhD project will be direct to explore the characteristics of SSF system and variables that affect the product formation by this process, as well as the variety of substrates and microorganism that can be used for the production of different bioactive compounds. We want to consider the analysis of various aspects: production of antimicrobials, characterization of volatile compounds, nutritional aspects such as the reduction of anti-nutritional factors present in the starting raw materials, production and extraction of secondary metabolites of industrial interest.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Grow ability																								
A2)	<i>In vitro</i> antimicrobial activity																								
A3)	Identification of metabolites																								
A4)	Characterize the volatile compounds																								
A5)	Thesis and Paper Preparation																								

A1) Establish grow ability

A2) Test the antimicrobial activity *in vitro*

A3) Identify the metabolites produced during fermentation and evaluate the reduction of antinutritional compound using different techniques (HPLC, mass spectrometry)

A4) Characterize the volatile compounds by GC

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

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Measurement of Organic Table Grape Nutraceuticals Using Non-Destructive Methods

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This PhD thesis research project is aimed to compare between destructive and non-destructive analytical techniques that measure the organic table grapes quality attributes and bioactive compounds. All these latter provide nutraceutical effects that will be evaluated by means of in vivo trials. The accuracy of NIR spectroscopy and Multiplex® will be tested to do the non-destructive measurements directly in the field. Additionally, this work aims to maintain the quality of these grapes for long term cold storage. Therefore, Blow® breathing device, combined with modified atmosphere packaging, will be implemented to the grape packages over 75 days under cold storage.

Misurazioni dei componenti nutraceutici in uva da tavola biologica usando metodi non distruttivi

Scopo della tesi di dottorato è di paragonare tecniche di analisi distruttive e non-distruttive che misurano la qualità dell'uva da tavola biologica e i suoi composti bioattivi. Tutti questi composti presentano effetti nutraceutici che saranno valutati attraverso prove in vitro. L'accuratezza della metodica spettroscopica NIR e dell'uso del Multiplex® saranno testati direttamente in campo al fine di produrre misurazioni con tecniche non-distruttive. Inoltre, questo lavoro mira a mantenere la qualità di queste uve più a lungo durante la conservazione in celle frigorifere. Allo scopo, un sistema di etichettatura, denominato Blow®, combinato con l'imballaggio in atmosfera modificata, sarà implementato sulle confezioni di uva conservate per 75 giorni in cella frigorifera.

1. State-of-the-Art

Table grapes are rich in many bioactive compounds, such as polyphenols and flavonoids, characterized by many biological properties that provide health benefits including antioxidant, antimicrobial, anti-inflammatory, anticancer, antiviral, cardio-protective, and hepato-protective activities (Tenore et al., 2014). Therefore, table grapes have a great potential to provide various nutraceutical effects.

The majority of researchers consider total soluble solids (TSS), titratable acidity (TA), TSS/TA ratio, colour and volatile compounds the main factors to be measured as maturity indices in order to determine the optimal harvest time. The assessment of these parameters is routinely performed in laboratory on grape samples and done by classical analytical methods known as wet chemistry. However, despite being expensive and time consuming, these methods work only for a limited number of samples (Piazzolla et al., 2017). Therefore, postharvest researchers become oriented toward non-destructive analytical techniques which are fast, accurate and enable real time analyses for a large number of samples.

As alternatives to the traditional analytical methods, the non-destructive techniques, including Near-Infrared Spectroscopy (NIRS), can be used to determine the maturity indexes, permitting the possibility of on-field application. Single and global

models were developed for the non-invasive prediction of phenolic compounds of grape skin, pH, TA and TSS. Additionally, the excellent potential of using a Vis-NIR hyperspectral imaging device was reported to predict the maturity and discriminate the harvest times of table grapes (Piazzolla et al., 2017). Moreover, most recently, techniques based on the fruit auto-fluorescence have been successfully exercised on grapes. Based on the comparison of chlorophyll fluorescence excited at two different wavelengths, fluorescence indices proved to reflect the epidermal phenolic content in leaves and also the skin anthocyanin content in olives and grape berries (Agati et al., 2007). Multiplex® is an industrial version and commercially available tool that includes the chlorophyll fluorescence screening technique and the fluorescence emission ratios. In 2010, a study showed that Multiplex® can be used to assess the anthocyanin content of grape intact clusters directly on the vine in a non-destructive way, and now it can be applied on other fruits and vegetables based on the same model (Ghozlen et al., 2010).

From the other hand, table grapes are highly perishable fruits, thus they are exposed to serious quality problems during the postharvest period. Due to the increased demand for fresh organic products, many studies were done in order to develop safe strategies to control the postharvest decay. Among these strategies, The Modified Atmosphere Packaging (MAP) is commonly used as non-toxic method to preserve the quality of fresh fruit and vegetables. Moreover, recent findings have shown the effect of a smart breathing device, called Blow® micro-technology, on the extension of the storage period for perishable commodities. It controls the internal package atmosphere through the regulation of the gaseous exchange between inside and outside the packages (Altieri et al., 2018).

While the majority of the studies refer to wine grapes, the objectives of this work were to evaluate the feasibility of using non-destructive techniques in quality parameter assessment of organic table grapes, with main focus on the nutraceutical components and their effects, and to maintain their quality and extend their storage life.

2. PhD Thesis Objectives and Milestones

Wince the first year of PhD still on going, the research activities that will be carried out in the following two years are presented in the Gantt diagram given in Table 1. All the analyses will be done on Scarlotta Seedless®, organic late-season table grape variety produced in Apulia region, south of Italy.

A1) Assessment of grape quality parameters near the harvest period using the classical destructive analytical techniques (A1.1), therefore grape sampling should occur periodically in order to monitor the grape maturity indices (pH, total soluble solids and titratable acidity). The phenol compounds will be measured by UHPLC-MS, the antioxidants by ABTS and DDPH, and the volatile compounds by SPME-GC-MS. In parallel, all the above mentioned parameters will be tested using NIR spectroscopy and Multiplex®, which are non-destructive analytical tools (A1.2)

A2) Harvesting of table grapes in the optimal harvesting time that will be defined based on the measured maturity indices.

A3) Packaging and cold storage after MAP and Blow® treatments (A3.1) will be implemented before the initiation of the storage period. Gases concentrations, quality and sensorial analyses (A3.2) should be repeated at a regular time intervals in order to test the quality along the extended storage period (75 days).

A4) Test the grape nutraceutical effects by means of in-vivo trials.

A5) Training abroad where all the above mentioned analyses should be done on table grapes produced in the southern hemisphere.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Assessment of grape quality parameters																									
1) Destructive methods																									
2) Non-destructive methods																									
A2) Harvesting																									
A3) Packaging and cold storage																									
1) MAP and Blow® treatments																									
2) Quality monitoring																									
A4) Test the nutraceutical effects																									
A5) Training abroad																									
A6) Thesis and Paper Preparation																									

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Exploitation of Functional and Healthy Properties of Protein Hydrolysates in Food System

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This PhD research project aims at study the potentialities of protein hydrolysates, containing the derived peptides, to improve food functionalities. To this aim, proteins from different sources will be enzymatically hydrolysed and the derived products will be characterized in terms of chemical composition, healthy and technological properties. Finally, selected protein hydrolysates will be delivered into functional foods. At the moment, whey proteins have been considered as protein source in the research activities.

Sfruttamento delle Proprietà Funzionali e Salutistiche degli Idrolizzati Proteici nel Sistema Alimentare

Questo progetto di dottorato mira a studiare il possibile impiego di idrolizzati proteici e peptidi da loro derivati quali ingredienti funzionali in alimenti. A tale scopo, proteine derivanti da diverse fonti proteiche verranno idrolizzate per via enzimatica e i prodotti derivati verranno caratterizzati in termini di proprietà chimiche, salutistiche e tecnologiche. Infine, si procederà all'utilizzo di alcuni idrolizzati in alimenti funzionali. Al momento, sono state considerate come matrice proteica di partenza le proteine del siero.

1. State-of-the-Art

The past decades has seen a burgeoning of literature on food-derived peptides and protein hydrolysates, this is mainly due to the evidences that protein hydrolyzed and in particular certain peptides demonstrated different biological activities (Mine et al, 2010). Specific protein fragments that have a positive influence on physicochemical and metabolic function/condition of the body or may have ultimate beneficial effects on human health are called bioactive peptides (Kitts & Weiler, 2003).

Bioactive peptides possess very important biological activities and functionalities, including antimicrobial, antihypertensive, antioxidative, opioid and mineral-carrying activities (Sánchez-Rivera et al, 2014).

It is a matter of fact that many of the functionalities of proteins are encrypted within their native protein sequences and can thus be liberated only by protein fragmentation (Pihlanto-Leppälä et al, 2000; Schanbacher et al, 1998). For this reason, there is a need to develop hydrolysis processes to generate the desired protein fragments. Enzymatic hydrolysis is one of the most widely applied method to this aim (Kitts & Weiler, 2003). Interestingly, peptide bioactivities could be tailored by properly designing the hydrolysis process.

Among different protein sources, whey proteins resulted one of the most promising source of bioactive peptides. Moreover, these proteins have the additional advantage to exhibit some technologically fascinating characteristics, such as high solubility, heat-induced and cold-set gelation, foaming, emulsifying and water-binding

capacity. For these reasons, a commercial whey protein isolate has been considered as the first choice in this research.

2. PhD Thesis Objectives and Milestones

The proposed project collocates within this context aiming to developing protein hydrolysates and derived peptides, as functional ingredients to be used to improve food functionalities. To this aim, the protein hydrolysis process will be firstly developed by exploiting different enzymes and selecting the best conditions able to generate the desired hydrolysis degree. Then, protein hydrolyzated will be characterized in terms of chemical composition by HPLC, protein/peptides molecular weight by SDS-page, particle size and z-potential by DLS analysis. Moreover, biological activities, such as ACE inhibitory, antioxidant and antimicrobial capacity will be tested to select the hydrolysis processing conditions able to maximize the biological functionalities of generated peptides.

Finally, selected protein hydrolysates will be delivered into functional foods. During the first year activities, whey proteins have been considered as protein source in the experiments. At the moment, the enzymatic hydrolysis conditions to be further used to generated whey protein hydrolysates have been designed.

Within the aim of this PhD thesis project, the following activities can be identified (table 1):

- A1) Bibliographic research on state of art**
- A2) Enzymatic hydrolysis process design**
- A3) Study of the functionalities of protein hydrolysates generated in A2 phase**
- A4) Development of food prototype** containing protein hydrolyzates
- A5) Paper writing and thesis development**

Table 1 *Gantt diagram for this PhD thesis project*

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Bibliographic research on state of art																									
A2) Enzymatic Hydrolysis process design																									
Analytical measurements																									
Functional and emulsion properties																									
Evaluation of Antioxidant activity and Hypertensive peptides																									
A3) Study of the functionalities of protein hydrolysates generated in A2 phase																									
A4) Development of food prototype																									
1) Food functionality evaluation																									
2) Evaluation of shelf-life of the product																									
3) Industrial compatibility and cost evaluation																									
A5) Paper writing and thesis development																									

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Shelf Life of Bakery Products Formulated with the Addition of Antioxidant Compounds Obtained from Food Industry By-Products

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The aim of this PhD project is to study the shelf life of bakery products enriched with different antioxidant extracts obtained from different food industry by-products. After a preliminary characterization of the antioxidant extracts, the physico-chemical characterization and the antioxidant activity of the final bakery products will be evaluated: indeed, the addition of such extracts could be a valid alternative to the use of synthetic preservatives. In addition, these natural antioxidants compounds will also be incorporated into the food coating formulation, verifying the effectiveness of their antioxidant activity in the packaged food products during storage.

Shelf life di prodotti da forno formulati con l'aggiunta di composti antiossidanti ottenuti da sottoprodotti dell'industria alimentare

Lo scopo del presente progetto di dottorato consiste nello studio della shelf life di prodotti da forno arricchiti con differenti estratti antiossidanti ottenuti da sottoprodotti dell'industria alimentare. Nello specifico, dopo una iniziale caratterizzazione degli estratti antiossidanti, sarà determinata la composizione fisico-chimica e l'attività antiossidante dei prodotti da forno finali: l'aggiunta di tali estratti, infatti, potrebbe essere una valida alternativa all'uso di conservanti di natura sintetica. Inoltre, gli stessi estratti antiossidanti precedentemente ottenuti saranno addizionati nella formulazione di coating alimentare, verificando l'efficacia della loro attività antiossidante nei riguardi degli alimenti confezionati nel corso della conservazione.

1. State-of-the-Art

By-products of the agri-food industry are a critical issue at world level, due to the large quantity produced every year and the possible toxicity of some of them. On the other hand, food by-products are important sources of natural antioxidants, because of the high content of active compounds found in them, such as polyphenols, flavonoids or anthocyanins. The exploitation of agri-food industry by-products through the extraction of the antioxidant components contained therein and their subsequent use as food ingredients, monitoring behaviour in enriched food matrices, is an innovative approach, which allows, on one side, the industrial use of biological resistant food by-products and, on the other side, the abatement of the polluting power through a sustainable and environmentally vision.

For the enrichment of food matrices with antioxidant compounds, obtained mainly from fruit and vegetable food by-products, a food grade solvent extraction is one of the most commonly used procedure, due to its ease of use, efficiency and wide applicability. The yield of extraction depends on the nature of solvents used (e.g. solvent polarity), the extraction time and temperature, sample-to-solvent ratio and on the chemical composition and physical characteristics of the samples (Sharma et al., 2018). Furthermore, several efficient extraction methods have been recently developed, such as microwave-assisted extraction and ultrasound-assisted extraction (Papoutsis et al., 2018; Chianoti & Tzia,

2018), as well as techniques based on the use of compressed fluids, e.g. subcritical water extraction (Lachos-Perez et al., 2018).

The beneficial properties of antioxidant compounds from food by-products have already been demonstrated in different food applications, reporting significant results as potentially promising ingredients for value-added food applications (Baiano et al., 2015).

Moreover, another application of the antioxidant extracts obtained by food by-products consists in the addition of them in edible coatings formulations, which can be used to reduce the use of synthetic preservatives and replace conventional polymers. Edible coatings are formed directly onto food surface by dipping or spraying and can be used to extend food shelf life by reducing exchange of moisture, gasses and volatiles with the surrounding environment and by preventing surface contamination, helping to improve the efficiency of food packaging (Otoni et al., 2017).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

Table 1 Gantt diagram for this PhD thesis project

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) By-products valorization																									
1) Antioxidants compounds extraction																									
2) Antioxidant extracts characterization																									
A2) Bakery products formulation and enrichment																									
1) Production process elaboration																									
2) Food matrix enrichment																									
3) Characterization and shelf life study																									
A3) Food coating realization and application																									
1) Edible coating formulation and enrichment																									
2) Edible coating application and shelf life study																									
A4) Thesis and Paper Preparation																									

A1) **Valorization of food industry by-products** through a food grade extraction and

A1) **Valorization of food industry by-products** through a food grade extraction and purification of antioxidant compounds in order to add them into food matrices. The extracts characterisation will include the evaluation of antioxidant compounds and antioxidant activity.

A2) **Formulation and enrichment of the bakery products** with the antioxidant extracts previously obtained, which are added at different concentrations in order to

evaluate their additional effects on the final products over time (shelf life). The elaboration of the most suitable formulation of the final dough will be evaluated referring to the best way in which extracts could be added during the preparation phase and the stability of the bioactive compounds.

A3) The realization and application of the food coating involve the use of food grade compounds and its enrichment with natural antioxidant extracts previously obtained (A1). This will be followed using the coating on food products and the study of their shelf life.

A4) Writing and Editing of the PhD thesis, scientific papers and oral and poster communications.

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Quality and Authenticity of Mountain Milk Products

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The aim of this PhD thesis research is to apply high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) to study markers for quality and authenticity analysis of milk and milk products. The final goal is to achieve a better understanding of chemical reactions in milk and to provide fast and reliable methods for milk analysis. This thesis falls within the FESR-EFRE-project Heumilch FESR1129 (CUP: H36H19000000007; Linea di finanziamento "Investimenti a favore della crescita e dell'occupazione FESR 2014-2020"): Analytical indicators in milk associable with silage in cow's forage.

Qualità e autenticità di prodotti lattiero-caseari di montagna

Questo progetto di tesi di dottorato mira ad applicare la cromatografia liquida ad alte prestazioni accoppiata con spettrometria di massa ad alta risoluzione (HPLC-HRMS) per studiare i marcatori per l'analisi di qualità e autenticità del latte e dei prodotti lattiero-caseari. L'obiettivo finale è ottenere una migliore comprensione delle reazioni chimiche nel latte e fornire metodi rapidi e affidabili per l'analisi del latte. La tesi rientra nel progetto FESR-EFRE Heumilch FESR1129 (CUP: H36H19000000007; Linea di finanziamento "Investimenti a favore della crescita e dell'occupazione FESR 2014-2020"): Indicatori analitici nel latte associabili all'impiego di insilati nella razione delle lattifere.

1. State-of-the-Art

In recent years, there has been an increasing interest in the study of milk, its nutritional value and how to preserve its quality and authenticity. Regionality and traceability of milk products are important aspects for the production of milk. Valorization of milk from mountain areas and the protection of milk from the Alpine region are of interest of the Alpine European states and the EU commission. Being a mountainous region, mountain dairy farming is a substantial part of the South Tyrolean farming landscape. A harmonious combination of innovative technology with tradition and authenticity is what characterizes the South Tyrolean dairy industry (*Südtiroler Sennereiverband*).

Quality, safety and authenticity of milk and dairy products are key priorities for the agro-industrial sectors of South Tyrol, Italy and the EU. Producers and consumers value high quality standards and research should provide reliable methods for monitoring quality and authenticity of milk products.

Thermal treatment of milk is an important procedure to deliver a safe product, and for processing milk products, such as cheeses, desserts, creams and milk formulations. However, heating can affect the quality and nutritional value of milk products. The influence of the thermal treatment on the nutritional value of milk is generally assessed by high performance liquid chromatography (HPLC) coupled with UV detection, following the standard procedure of Resmini et al. (1990), which follows the production of the Maillard reaction product furosine. Furosine is commonly used as an indicator for thermal treatment in milk (Ritota et al., 2017). However, when milk is intensively heat-treated to produce i.e. dairy desserts and milk creams, the standard procedure may suffer from the

extensive production of Maillard reaction products and the accumulation of a myriad of furosine analogues. The complex nature of the resulting heated dairy product may hamper the quantitative evaluation. For this reason, in this project we have proposed an untargeted analysis of the heated dairy products based on high-resolution mass spectrometry equipped with Q-Exactive orbitrap technology. The high mass accuracy of the Q-Exactive orbitrap, coupled with a multivariate statistical analysis, can be a valuable approach to study the quality changes occurring to milk during extensive heat treatment.

Likewise, the authenticity of milk products can be analysed via HPLC-HRMS. This is in line with the incorporated FESR-EFRE Heumilch project which deals with the analysis of the authenticity of hay milk. The project aims to develop new tools able to trace the traceability and authenticity of typical South Tyrolean food products - in this specific case hay milk - with a view to enhancing local products. These elements of quality, authenticity, safety and traceability can be achieved by implementing the methods established through the PhD thesis research project via HPLC-HRMS.

The combination of HPLC with high-resolution MS results in an extremely powerful analytical tool. Mass spectrometers of the last generation deliver high-resolution, accurate-mass determination of compounds, allowing for a precise assignment of markers in analysed samples. This enables the accurate determination of target but also non-target molecules, allowing with the aid of multivariate statistical analysis, for the study of not only established, but also new markers for milk quality and authenticity (Matissek and Baltes, 2016; Finot et al., 1968; Resmini et al., 2003; van Boekel, 1998).

2. PhD Thesis Objectives and Milestones

The main objectives mentioned above will be subdivided into the following research activities and are summarized in the Gantt diagram (Table 1):

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Method Development																								
1) Methods from literature research and their adaptation																								
2) Methods developed unibz Food Quality Lab																								
3) Investigating and establishing markers and biomarkers																								
A2) Analysis of Milk Products																								
1) Optimizing milk sample preparation																								
2) Applying markers and biomarkers																								
3) Applying HPLC-HRMS																								
A3) Period Abroad																								
A4) Further Literature Review, Thesis and Paper Preparation																								

A1) Method development. Establishing methods using high-performance-liquid-chromatography coupled to high-resolution mass-spectrometry (HPLC-HRMS). (A1.1) Methods from literature research and their adaptation and modification to meet the requirements of the present project. (A1.2) Methods that will be developed at unibz Food Quality Lab: Developing new methods and assays for the assessment of milk quality and authenticity. Comparison of methods described in the literature, modification and adaptation to the needs and equipment of the unibz Food Quality Lab. (A1.3) Investigating and establishing markers and biomarkers for milk quality and authenticity.

A2) Analysis of milk products. (A2.1) Optimizing milk sample preparation using solid phase extraction (SPE). (A2.2) Applying markers and biomarkers for the assessment of milk quality and authenticity. (A2.3) Applying HPLC-HRMS with the established methods.

A3) Period abroad (6-12 month between 2nd and 3rd year) at a working group relevant to the thesis' research field.

A4) Further literature review, writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

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Development of Carbon Nanotube-Based Field Effect Transistor Flexible Biosensors for Additive Detection in Industrial Beverages

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This PhD thesis project aims at fabricating of carbon nanotube-based field effect transistor (CNTFET) biosensors on flexible substrate to detect nitrate, sulfite, benzoic acid and aspartame in industrial beverages and wine. In order to develop biosensors sensitive to the abovementioned analytes, selective and specific enzymes will be immobilized and used to react with the analytes. As a final goal of the project, a CNTFET biosensor array will be developed to monitor the different additives at the same time on a real-time basis.

Realizzazione di biosensori flessibili basati su transistori ad effetto di campo con nanotubi di carbonio in grado di rilevare gli additivi tipicamente usati nelle bevande industriali

Questo progetto di tesi di dottorato si propone di fabbricare biosensori flessibili basati su transistori ad effetto di campo con nanotubi di carbonio per rilevare nitrati, solfati, acidi benzoici e aspartame in bevande industriali e vino. Per poter sviluppare biosensori sensibili agli analiti sopra indicate, enzimi specifici e selettivi saranno immobilizzati in modo da poter reagire con gli analiti. Come obiettivo finale del progetto, un array di biosensori basati su transistori con nanotubi di carbonio sarà sviluppato per monitorare additive diversi in tempo reale.

1. State-of-the-Art

The food quality control has become of uttermost importance not only for the consumers, but also for the food industry. Continuous monitoring of adulterants, additives and pesticide residues is a key feature, especially in the beverage industry. Indeed, several food additives, such as sulfites, nitrates, benzoic acids, aspartame, etc. are being used in the beverage industry in order to achieve different purposes, e.g. as preservatives, anti-oxidants, and/or intensive sweeteners. To control the bacterial growth and enzymatic reactions, beverage and wine industries usually apply sulfite for a very long time in different forms like bisulfite, metabisulfite and sulfur dioxide. However, sulfites can provoke serious allergic reaction and fatal asthma attacks, and at the same time they can damage chromosomes and DNA if the maximum limits are exceeded (Wang et al., 2019). Another frequently used additive is nitrate which is not only used as a food additive in the beverage sector, but it can also be found as a natural residue in drinking water. Nitrate may cause infant methemoglobinemia, gastric or bladder cancer and psychological stress if the digestion limit is crossed (Minami et al., 2016). The most common preservatives used in food and beverage industries is benzoic acid which retards the growth of microorganisms and undesirable chemical changes, though, higher than permitted level can result in serious problems to human health et al., 2010). Another common additive, mostly used as an artificial sweetener in diet soda is aspartame (N-L- α -aspartyl-L-phenylalanine methyl ester), which has a zero calorific value. The carcinogenicity and genotoxic risks of aspartame have been topic of debate for a long time. Moreover, methanol originating from aspartame is super toxic and may cause permanent blindness (Balgobind et al., 2016). Hence, to minimize the potential of serious health implications, different methodologies to identify the level of these additives have

been developed from the very beginning. Various conventional methods such as flow injection analysis, capillary electrophoresis, ion chromatography, liquid chromatography-tandem mass spectrometry, spectrophotometry, chemiluminescence, high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) are employed for the detection of sulfite, nitrate, benzoic acid, and aspartame (McGrath et al., 2012). Nevertheless, several drawbacks are connected to the use of these methods especially for what concerns the use of highly expensive, time-consuming, and low sensitivity chemicals and instruments. The detection of these additives in a proper, quick and cost-effective way with high sensitivity and selectivity is therefore of paramount importance.

In this context, biosensors are a promising class of analytical device in alternative to the above-mentioned screening techniques. In fact, biosensors detect chemical substances or analytes by combining biological materials (enzyme, antibody, nucleic acid etc.) with a physicochemical detector (transducer) able to convert the response to the analyte into an electrical signal. Biosensors evolved as a very reliable and promising tool compared to traditional methods mentioned above because of simplicity, sensitivity, selectivity, portability, and miniaturization. A wide range of electrochemical biosensors functionalized with different enzymes has been proved to detect various additives very efficiently. As for example, electrochemical biosensors using enzymes such as sulfite oxidase, nitrate reductase enzyme, polyaniline-polyphenol oxidase and alcohol oxidase have been reported for the successful detection of sulfite, nitrate, benzoic acid and aspartame respectively (Chou, 1996; Li et al., 2010; Minami et al., 2016; Ng et al., 1998). Using field effect transistor (FET)-based biosensors result in further advantages such as low measurement noise, signal amplification, miniaturization, low power consumption, portability, low cost and rapid detection. At the same time, FETs can also be fabricated onto flexible substrates, which are particularly convenient in terms of flexibility, weight, portability, cost, disposability, etc. (Economou et al., 2018). In the case of FET-based biosensors, the quick change of the FET conductance upon binding of charged target biomolecules to receptors is used for real-time detection of analytes et al., 2017). Among the various possible semiconducting materials used in the case of FET-based biosensors, carbon nanotubes (CNTs) offers advantages such as high surface area, good biocompatibility, good chemical and electrochemical stability, as well as high electrical conductivity. CNTs can be employed both in a single or multi-walled structure of which, single-walled CNTs (SWCNTs) are typically a well-suited semiconducting material for FET devices because of their ability to expose every atom to the surface and therefore allowing the detection of small changes (Allen et al., 2007). Different methods can be applied to synthesize CNT such as arc discharge, laser ablation, chemical vapor deposition (CVD), gas phase pyrolysis etc. Upon synthesis, sonication and centrifugation can be employed to disperse SWCNT and therefore solution-process them by drop-casting or spray-coating them. In particular, spray coating is able to provide highly uniform CNT thin film with less irregular agglomeration (Hou et al., 2016). Finally, specific enzymes can be immobilized on the CNT surface to catalyze with specific analytes. Thus, this Ph.D. thesis project will be directed to fabricate four different novel CNT-based FET biosensors on a flexible substrate where different selective enzymes such as sulfite oxidase, nitrate reductase enzyme, polyaniline-polyphenol oxidase and alcohol oxidase to detect sulfite, nitrate, benzoic acid and aspartame respectively will be used to react with specific analytes and finally a FET biosensor array will be developed to monitor the additives altogether on a real-time basis.

2. PhD Thesis Objectives and Milestones

This PhD project can be subdivided into the following activities:

A1) Design and fabrication of FETs: CNTFETs will be designed and fabricated using different deposition techniques and patterning steps.

A2) Characterization of CNTFETs: After fabrication, characterization of material morphology and electrical performance of the CNTFETs will be checked.

A3) Functionalization of CNTFET biosensors: immobilization of specific enzymes (sulfite oxidase, nitrate reductase, polyaniline-polyphenol oxidase and alcohol oxidase) to detect analytes (sulfite, nitrate, benzoic acid and aspartame) during the functionalization process.

A4) Characterization of CNTFET biosensors: the four different CNTFET biosensors will be tested with 4 different analytes.

A5) Development of CNTFET biosensor array: fabrication and characterization of CNTFET biosensor array.

A6) Writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	2	4	6	8	10	12	14	16	18	20	22	24
A1) Design and fabrication of CNTFET												
A2) Characterization of CNTFET												
A3) Functionalization of biosensors												
A4) Characterization of biosensors												
A5) Fabrication and characterization of biosensor array												
A6) Thesis and Paper preparation												

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Probiotic and Technological Characterization of Lactic Acid Bacteria in Fermented Milk Production

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This PhD thesis research project is directed to evaluate the technological and functional characteristics of selected strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, with a particular focus on the exopolysaccharide production in fermented milk.

Caratterizzazione probiotica e tecnologica di batteri lattici per la produzione di lattici fermentati

Questo progetto di tesi di dottorato è finalizzato alla valutazione delle caratteristiche tecnologiche e funzionali di ceppi selezionati di *Lactobacillus bulgaricus* e *Streptococcus thermophilus*, con particolare attenzione alla produzione di esopolisaccaridi in lattici fermentati.

1. State-of-the-Art

Fermented foods has been playing an important role in human evolution, appreciated for sensory properties, shelf life and safety but also for the presence of microbial metabolic products with several biological actions. The metabolic activity of microorganisms also improves the nutritional and functional properties of food with beneficial effects for the human health (Galle et al., 2012, Garcia-Gonzalez et al., 2018). The Yogurt is a fermented food widely consumed and spread globally, and research has improved the knowledge and innovations to the process-product relationships and product innovation satisfying the sensory demands and consumer health benefits. Moreover, it is the only food which EFSA has recognized the probiotic action against the maldigestion of lactose, owing to micro-organisms associated with it (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) (Scientific Opinion EFSA, 2010). These microorganisms can produce and secrete different types of polysaccharides, exopolysaccharides (EPS), with protective function towards environmental stress and matrix and with cellular action recognition and adhesion to surfaces. EPS play a central technological role in fermentation of dairy products and the use of "ropy starter", containing EPS-producing strains in the production of yogurt, is used to improve consistency and reduce the synergy of the product (Korakli et al., 2003). EPS are natural biothickeners, and their *in situ* production by lactic acid bacteria is a viable alternative to artificial stabilizing thickeners/additives (Galle et al., 2012, Korakli et al., 2003). From a microstructural point of view, yogurt is a weak gel: rheological properties and water retention capacity depend on the state of aggregation of milk proteins induced by the fermentative and metabolic activity of the used starter microorganisms. However, if the positive contribution of bacterial EPS is known to improve the rheology of yogurt,

the correlation between viscosity of fermented milk and EPS concentration, as well as their protective role as prebiotics and in the interaction between microorganisms and host with potential beneficial effects for human health can be ascertained.

2. PhD Thesis Objectives and Milestones

In line with the general aim stated above, this PhD thesis project can be categorised into the following specific objectives and activities. This study will take place in the laboratories of Microbiology (MB) and Food Technology (FT) of the Faculty of Biosciences-University of Teramo (UNITE), the R & D department of DALTON Biotechnology and the University of Alberta (Canada, UNICA).

A1) Selection, biotechnological and functional characterization of starter microorganisms (i.e. *S. thermophilus* & *L. delbrueckii* subsp. *bulgaricus*) for the production of yoghurt, with reference to the EPS production capacity and its impact on the quality of the final product. This step includes: (A1.1) identification and molecular typing of a number of strains of micro-organisms producers of EPS with a polyphasic approach, integrating traditional phenotypic and molecular systems (i.e. 16s gene sequencing, RAPD-PCR, MLST). (A1.2) functional characterization of EPS producers (*in vitro* resistance to gastro-intestinal tract, cell adhesion capacity, anti-inflammatory activity). (A1.3) compositional characterization of EPS from different microbial strains and impact of the fermentative activity and production of EPS on the qualitative characteristics of cow's milk yogurt with reference to rheological properties (viscosity, visco-elasticity), color, clot stability, volatile odorous compounds pattern (gas-chromatography, electronic nose etc.), and sensory analysis. (UNITE).

A2) Functional characterization of the EPS production by selected strains with genome sequencing and identifying genes responsible for the production of EPS; creating mutant strains in which the gene EPS will be muted. Study of the role of genes involved in the production of EPS by comparing the metabolism of mutated strains by knocking-out the responsible gene. (UNICA).

A3) Biotechnological and functional characterization (see A1) of knock-out mutated strains to evaluate the role of the production of EPS and its impact on the technological and health aspects of yogurt. (UNITE).

A4) Identification of industrial production process parameters for maintaining the vitality and the activity of the selected probiotic bacterial strains and scaling-up the process. (DALTON).

A5) Biotechnology and functional study of a series of strains selected according to the results of previous A1-A4 in modified milk matrix (reduced or low fat), alternatives (vegetable milks) and/or enriched with functional ingredients (eg. inulin). (UNITE).

A6) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Based on the obtained results from the objective (A1) of first year of PhD project, the next two years of PhD project will be organized according to the Gantt diagram provided in Table 1.

Table 1 Gantt diagram for this PhD thesis project (MB: microbiology; FT: Food Technology labs)

Activity / Months		2nd Year						3rd Year					
		14	16	18	20	22	24	26	28	30	32	34	36
A2)	Genome sequencing, identification and knock-out of EPS producing gene	UNICA											
A3)	Biotechnological and functional characterization of knock-out mutant strains				UNITE (MB)								
					UNITE (FT)								
A4)	Biotechnological parameters for industrial scaling-up							DALTON					
A5)	Biotechnological and functional study in diversified environment for product innovation										UNITE (MB)		
											UNITE (FT)		
A6)	Thesis and Manuscript Preparation												

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Investigating the Biodiversity in Spontaneous Fermentations as a Source of High-Performance Microorganisms

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This PhD project aims to describe the natural microbiota of spontaneously fermented cereals, taking into consideration lactic acid bacteria and yeasts, and their metabolic activities of interest, in order to select suitable strains to be used as starter or adjunct cultures, and evaluate their effectiveness in baked products.

Studio della biodiversità di fermentazioni naturali come fonte di microrganismi ad elevate prestazioni

Questo progetto di dottorato ha lo scopo di descrivere il microbiota naturale di cereali fermentati spontaneamente, prendendo in considerazione batteri lattici e lieviti, e loro attività metaboliche di interesse, in modo da poter selezionare ceppi adatti ad essere utilizzati in colture starter o aggiunte, e valutare la loro efficacia in prodotti da forno.

1. State-of-the-Art

The production of fermented foods is an early practice, as one of the oldest uses of biotechnology, and has served a multitude of purposes, such as food preservation, the improvement of its flavour and texture, the enrichment in nutrients, such as free amino acids and vitamins, as well as the degradation of antinutrients. Fermentation processes were initially based on the development of the natural microbial populations already present on the food matrixes, and on back-slopping, that is, inoculating with small quantities of previous fermentations. The quality of the end products thus obtained results to be variable and dependent on the initial microbial composition of the ingredients and on the selection of the best adapted strains.

Even though natural fermentations are still in use, most industrial fermented foods are produced using specifically designed starter cultures. This allows a better control of the fermentation process and a more standardized final product, but on the other hand a lot of the particular sensory, nutritional and health advantages of the complex native microbial communities are lost, mainly due to the fact that starter cultures are generally designed by taking into consideration a limited number of characteristics, such as the acidification rate, and are comprised by a small number of strains (Brandt, 2014; Navarrete-Bolaños, 2012).

Nowadays there is a renewed interest in selecting starter cultures not only for the rapid acidification, but also based on several metabolic activities of relevance for their functionality. For example, research shows how the variety of aromatic components in sourdough increases with the use of lactic acid bacteria and is even more complex when selected yeast strains are added to the fermentation (Hansen and Schieberle, 2005).

Some of these activities are the production of flavours, the proteolytic activity, the production of compounds with added health and nutritional benefits, such as the release of bound ferulic acid from plant matrixes, which is an antioxidant with anti-inflammatory and antimicrobial activity, the degradation of antinutritional compounds, for example of phytic acid which reduces the bioavailability of minerals in plant based foods, as well as

the ability to inhibit the growth of pathogenic and altering microorganisms (Leroy and De Vuyst, 2004).

Recent studies highlight the importance of cereals and their fermentations as a source of robust bacterial strains, which can be exploited for their metabolic activities (Minervini et al., 2014).

2. PhD Thesis Objectives and Milestones

Towards the achievement of the aforementioned objectives, this project will be divided in 5 main activities, as described in the Gantt diagram (Table 1):

A1) **Strain isolation** from different cereal fermentations (A1.1) and their identification and characterization (A1.2).

A2) **Evaluation of the safety (A2.1) and metabolic activities** of the selected strains (A2.2), both phenotypically and genotypically, with the aim of selecting the highest performing strains to be used as adjunct cultures (A2.3).

A3) **Creation of mixed cultures**, evaluation of their stability, resistance to stressful conditions and interactions in a laboratory scale fermentation (A3.2).

A4) **Assessment of the effectiveness of the created consortia** in improving the shelf life, taste, nutritional value and overall quality of baked products.

A5) **Preparation of the PhD thesis, papers and manuscripts**, bibliographic research.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Strain isolation																									
1) Setup of laboratory scale fermentations																									
2) Identification and genetic characterization																									
A2) Evaluation of safety and metabolic activities																									
1) Genomic study of virulence factors																									
2) Study of metabolic activities of interest																									
3) Selection of highly performing strains																									
A3) Mixed culture fermentations																									
1) Study of the stability of the microbial consortia																									
2) Setup of pilot scale fermentations																									
A4) Assessment of the effectiveness of the consortia																									
1) Evaluation of the quality and shelf life																									
A5) Thesis Preparation																									

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Characterization of Bioactive Compounds in the Skin of Different Hazelnut Varieties Using Mass Spectrometry Analysis

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This project aims to use hazelnut perisperm, considered a waste element in the industrial chain, as a raw material for the creation of a functional food. The first phase involves the evaluation of the nutritional characteristics of the cuticle of two varieties of Tuscia hazelnuts (Tonda di Giffoni; Tonda Gentile Romana); the second purpose of the project is to identify the presence of "functional" compounds and verify their bioavailability after the digestion process. Finally, the cuticle of the variety that showed the best nutritional profile and bioavailability will be used to develop a new healthy food.

Caratterizzazione di Composti Bioattivi nella Cuticola di Diverse Varietà di Nocciole Mediante Analisi di Spettrometria di Massa

Questo progetto ha come obiettivo l'utilizzo del perisperma di nocciola, considerato un elemento di scarto nella filiera industriale, come materia prima per la creazione di un alimento funzionale. La prima fase prevede la valutazione delle caratteristiche nutrizionali della cuticola di due varietà di nocciole della Tuscia (Tonda di Giffoni; Tonda Gentile Romana); la seconda finalità del progetto è di individuare la presenza di composti "funzionali" e verificare la loro biodisponibilità dopo il processo di digestione. In fine la cuticola della varietà che ha mostrato il miglior profilo nutrizionale e biodisponibilità verrà utilizzata per sviluppare un nuovo alimento salutare.

1. State-of-the-Art

Hazelnut (*Corylus avellana* L.), belonging to Betulaceae family, is an important commercial and subsistence crop in many countries (Xu et al., 2007), whose economic value is increasing for its nutritional quality. Italy is the second largest producer of hazelnut (13%) after Turkey. In Latium region are located more than 25% of the national production areas, concentrated in the Viterbo province, a typical hazelnut-growing area. The main variety is the "Tonda Gentile Romana", followed by "Tonda di Giffoni". Hazelnut is an excellent source of manganese, copper, and selenium and also good source of potassium, chromium, iron, magnesium, phosphorus, calcium, and zinc.

Hazelnut contains both fatsoluble vitamins (K, E, and A) and water-soluble vitamins (C, several B vitamins, choline, and betaine). Moreover, it also contains free amino acids, proteins, sugars, organic acids, and proanthocyanidins. Hazelnut may be consumed as natural (with skin) or preferably roasted (without skin). Hazelnut skin is the perisperm of the hazelnut kernel and it represents about 2.5% (skin absorbs oil during roasting) of the total hazelnut weight and is discarded upon roasting (Alasalvar et al., 2009, Lainas et al., 2016). The hazelnut skins, as waste after the roasting process, have a significant portion of polyphenols that can have covalent or strong non-covalent associations with plant proteins and polysaccharides (Le Bourvellec & Renard, 2012). Moreover hazelnut skin is a rich source of dietary fibre as well as of natural antioxidants owing to the presence of phenolic compound. Hence, one of the aims of this project is to identify phenolic profiles in hazelnut samples obtained from two different cultivars, Giffoni and Romana, by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and to assess their antioxidant activities by different in vitro assays. A rapid and sensitive UPLC method

coupled with mass spectrometry is considered a powerful tool for chemical characterization and quantification of phenolic compounds. However, bioactive compounds in hazelnut skin must be bio-available before they can have an effect. In fact many factors influence the transition of metabolites during digestion and there are different mechanisms of absorption of water-soluble and liposoluble molecules. Consequently, the second phase of the project aims to examine the bioavailability of these compounds, mimicking *in vitro* digestion, with the aim of clarifying the effective bioavailability of the hazelnuts skin constituents. Samples obtained from the different digestion steps, which consisted of three-steps to mimic the digestive process in the mouth, stomach (gastric digestion) and small intestine (dynamic duodenal digestion) will be lyophilized and analyzed with chromatographic-mass spectrometry analysis. Finally, according to obtained results, the nuts skin can be potentially utilised as an additive in food production, to obtain a fortified food with high fibre content and antioxidant activity (Zeppa et al., 2015).

2. PhD Thesis Objectives and Milestones

This PhD thesis project can be divided into the following activities (Table 2):

Table 2 Gantt diagram for this PhD thesis project.

Activity / Bi-monthly period		1	2	3	4	5	6	7	8	9	10		11	12	13	14	15
A1)	Nutritional and functional characterization of <i>Hazelnut</i> varieties																
A2)	Examine the bioavailability of these compounds																
A3)	Development of a potential functional food																
A4)	Actual functionality evaluation																
A5)	Thesis and Paper Preparation																

A1) **Nutritional and functional characterization of *Hazelnut "Tonda Gentile Romana"* and *"Tonda di Giffoni"* varieties** by proximate analysis, evaluation of antioxidant compounds, antioxidant activity, and anti-nutrients content of the seeds. Then chemical characterization and quantification of phenolic compounds by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

A2) **Examine the bioavailability of these compounds**, mimicking *in vitro* digestion, with the aim of clarifying the effective bioavailability of the hazelnuts skin constituents.

A3) **Development of a potential functional food.**

A4) **Actual functionality evaluation** of the experimental food by *in vitro* tests

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

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The Study of Meat and Fish Quality Using NMR-Based Metabolomics

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This Ph.D thesis research project is aimed at using ^1H -NMR-based metabolomics techniques to analyze various water-soluble small molecules in meat, providing an important basis for material composition in meat quality evaluation. Simultaneous application of TD-NMR to analyze the moisture distribution of meat and meat products.

Studio della qualità della carne utilizzando la metabolomica basata su NMR

Questo progetto di ricerca di tesi di dottorato ha lo scopo di utilizzare tecniche di metabolomica basate su ^1H -NMR per analizzare varie piccole molecole idrosolubili nella carne, fornendo una base importante per la composizione del materiale nella valutazione della qualità della carne. Applicazione simultanea di TD-NMR per analizzare la distribuzione dell'umidità di carne e prodotti a base di carne.

1. State-of-the-Art

Meat and fish quality studies have taken advantage of nuclear magnetic resonance (NMR) phenomenon to gain pieces of information about water interactions with biopolymers, through time-domain (TD) studies, and about water soluble metabolome, through proton high resolution NMR. Most of the TD-NMR studies have observed the interaction between water and biopolymers by registering proton transverse relaxation time (T_2) weighted signals. From them, information about the compartmentalization of water across meat structures could be obtained. In turn, information about water retention ability of meat could be obtained. For example, Laghi *et al.* observed water located outside the myofibrils, easily lost upon storage or cooking (Laghi *et al.*, 2017). Sánchez-Alonso *et al.* used TD-NMR techniques to study carp at different freezing times, indicating that the T_2 can be used to help assessing the quality of carp upon freezing (Sánchez-Alonso *et al.*, 2014). Interestingly, some works have attempted to establish a direct relationships between proton relaxation time and meat sensory parameters. Hullberg *et al.* focused on T_2 to study the relationship between the moisture diffusion across flue-cured tenderloin and its sensory characteristics (Hullberg *et al.*, 2005). Bianchi *et al.* have found T_2 weighted signals allowed to separately quantify the protons located in the intra- and in the extra-myofibrillar spaces (Bianchi *et al.*, 2004). ^1H -NMR-based metabolomics can systematically characterize low weight metabolites in foodstuff, including amino acids and amines, organic acids, alcohols, nucleotides, sugars and other small molecules. Some of these have been found related to the quality of meat and fish products. For example, it has been found that the molecule mainly determining the umami taste of chicken meat is inosine monophosphate (IMP). Ye *et al.* found that the concentration of IMP in cock muscles is lower than that in hens (Ye *et al.*, 2010). Ciampa, A. *et al.* investigated the release of amino acids in Bogue (Boops boops) fish upon storage, in connection with different storage temperatures (Ciampa *et al.*, 2012).

In the present PhD work I would like to employ NMR in its TD and high-resolution versions to gain information about meat and fish characteristics, as a consequence of technological treatments. As a first step, I was recently involved in an experimental project focusing on the effects of different pressure levels on the shelf life of fish products, in order to establish optimal process parameters. Ramirez-Suarez *et al.* (Ramirez-Suarez

et al., 2006) found that the application of high pressures to meat products leads to a moisture content decrease, probably because high pressures force the hydration of the proteins. To explore this possibility, I decided to follow the changes of moisture content in the samples by TD-NMR, and, in turn, to understand the effect of different pressure treatments on meat quality from a water status point of view. As a next step, as high pressure treatments are expected to accelerate the disruption of cells membranes, I will try to investigate the effects of the pressure treatments also from a metabolomics point of view.

2. PhD Thesis Objectives and Milestones

Current research activities will use metabolomics methods to assess the factors that contribute to changes in meat quality. On the other hand, a TD-NMR experiment will be established for the investigation of the water status of meat.

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature review** about latest research related to the studies of the relationship between food and health through a metabolomics approach.

A2) **Evaluation of different sample preparation systems** in order to identify the method that allows repeatability on a large scale.

A3) **Metabolomics oriented experiments** focusing on the relationship between meat quality and low weight molecules.

A4) **Metabolomics oriented experiments** focusing on the relationship between food processing and food composition.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Literature review and Experimental design																								
1) preliminary studies in metabolomics																								
2) experiment design																								
A2) Evaluation of different sample preparation systems																								
A3) Experiments focusing on meat quality and low weight molecules																								
1) read papers to choose some low weight molecules																								
2) assessments of the relationship between meat quality and low weight molecules																								
A4) Experiments focusing on the food processing																								
1) assessments of the relationship between food processing and composition																								
2)) investigate the relationship through metabonomic approach																								
A5) Thesis and Paper Preparation																								

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The Valorization of Olive Oils Through Innovation and Optimization of Packaging and Storage Conditions Adopted in Order to Increase the Olive Growing Sustainability

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This PhD thesis research project is aimed at setting up a new extraction system in order to enrich the panorama of Italian high nutritional quality oils, and the setting up of a prototype, of a storage tank and a bottling plant able to ensure optimal storage conditions necessary to maintain as long as possible the nutraceutical properties of the olive oils. Moreover, the aim of this study to enhance the value of olive oil coming from the refining of lampante oil using the by-products of some agri-food chains (ex: oil, wine, citrus) in a circular economy perspective should increase the economic sustainability of the olives producers.

La valorizzazione degli oli di oliva attraverso l'innovazione e l'ottimizzazione delle condizioni di confezionamento e conservazione adottate al fine di aumentare la sostenibilità dell'olivicoltura

Questo progetto di tesi di dottorato è finalizzato alla creazione di un nuovo sistema di estrazione al fine di arricchire il panorama degli oli italiani di alta qualità nutrizionale e l'allestimento di un prototipo, di un serbatoio di stoccaggio e di un impianto di imbottigliamento in grado di garantire condizioni di stoccaggio ottimali necessarie a mantenere il più a lungo possibile le proprietà nutraceutiche degli oli d'oliva. Inoltre, l'obiettivo di valorizzare l'olio d'oliva, proveniente dalla raffinazione dell'olio lampante utilizzando i sottoprodotti di alcune filiere agroalimentari (es: olio, vino, agrumi) in una prospettiva di economia circolare, dovrebbe aumentare la sostenibilità economica dell'olivicoltura.

1. State-of-the-Art

Olive oil is a product of excellence that must be enhanced and, in this contest, the current extraction methods do not allow a complete extraction of the oil, so the obtained product is characterized by reduced levels of polyphenols compared to the high content of the olives (Frankel et al. 2013). Nowadays, the available extractive technologies involve a large loss of the phenolic component initially present in the processed fruit and in particular of the water-soluble fraction, which is the one with the highest antioxidant activity. This fraction tends to remain in the vegetation waters and in the pomace, aggravating the pollutant load as the antimicrobial activity exerted by these phenolic components makes its disposal into the environment problematic.

At the end of the production process, the oil must face a more or less long period of storage. This phase is fundamental in order to preserve the product characteristics of the reference category and extend its shelf life (waste reduction); for this reason it is necessary to slow down the natural degradation processes to which the product is subjected during storage time.

Several studies have been carried out about parameters able to influence the kinetic of oil degradation over time. In particular, one of the main factor influencing the oil

evolution over time is light radiation, since it determines its photo-oxidation. The light radiation determines a rise in the secondary oxidation products, corresponding to the increase in the absorbance values at the wavelength of 270 nm and the values of ΔK (Caponio et al. 2005) and a noticeable reduction of tocopherols and carotenoids, while the effect on phenolic compounds seems to be unimportant. Another parameter which can greatly influence the course of lipid oxidation is the storage temperature but its effects are ambivalent: on the one hand it increases the rate of oxidation, favouring the hydroperoxides formation rate and their subsequent decomposition, on the other hand it decreases the solubility of oxygen in the mass of oil (Frankel et al. 2013). Finally, the type of container in which the oil is stored can directly influence its quality, protecting it from two of the main factors that induce oxidative rancidity: light and oxygen (Kanavouras et al. 2004). Among the various plastic containers, the oxygen permeability varies so as to determine the following oil stability classification: PVC (polyvinylchloride) > PET (polyethylene) > PP (polypropylene) > PS (polystyrene). Glass bottles provide to protect the oil from oxidation much better than PET containers even if the best container for the stability of the oil is stainless steel, followed by glass (Dabbou et al. 2011). Nowadays, the degradative kinetics of the oils in the various containers and the interaction of the type of packaging with other operative parameters, such as temperature and composition, of the storage atmosphere are not well known.

So, today there is the need to have an innovative system able to optimize the extraction conditions, packaging and storage of the olive oil in order to increase the extraction yield (amount of oil produced with the same processed olives), the quality of the product for a long period, certainly greater than 12 or 18 months actually required by current laws (D.L. 23 maggio 2016, n. 103) so to obtain "a long-life olive oil" particularly rich in nutraceutical compounds (ex: tocopherols, phenols, carotenes, squalene).

On the other hand, a significant part of the olive oil produced by pressing the olives shows chemical and compositional characteristics that are not suitable for human consumption so it must be refined to become edible. The refining processes involve a drastic reduction of the nutraceutical micro-components. In particular, the phenols are completely lost. Therefore, some research (Venturi et al. 2017) are currently aimed at developing effective methods of extraction and recovery of these substances from natural sources and the replacement of synthetic additives, currently commercially available, for which adverse health effects are reported (Suàrez et al. 2010). The nutraceutical compounds can be obtained from different food matrices (ex: pomace, wash water deriving from the refining process, citrus peels) with a view to recovering waste products from different food chains in order to promote their sustainability.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Identification of the best parameters to be adopted during extraction. In order to reach this goal, it will be used a pilot scale crusher for processing olives (25 kg for each experimental run) using different operative conditions (temperature of olives, composition of atmosphere during extraction, etc.).

A2) Optimization of the conditions for olive oils storage by modification of the main chemical-physical parameters involved in oil degradation (ex: temperature, packaging, storage atmosphere, etc.).

A3) Development of olive oils with a high nutraceutical and health value starting from refined olive oils by enriching some nutraceutical compounds that can be lost in the refining process (ex: squalene and polyphenols). The source of these

compounds could be represented by the waste coming from the physical refining process of the lampante oil or by the waste of different agri-food chains (ex: wine, citrus).

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14 ÷ 22	23	24
A1)	Identification of the best parameters to be adopted during extraction																
	1) Optimization of operative conditions																
	2) Chemical and sensory analysis																
A2)	Optimization of the conditions for the storage																
	1) Modification of the main chemical-physical parameters																
	2) Evaluation of Shelf-life																
A3)	Development of new products																
	1) Chemical-physical characterization																
	2) Sensory characterization																
	3) Evaluation of shelf-life of the product and of the optimum storage conditions																
A4)	Thesis and Paper Preparation																

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Understanding the Association Between Individual Differences in Saliva Characteristics and Oral Responsiveness to Different Sensory Stimuli

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This PhD thesis research project is aimed at understanding the relationship between some physiological characteristics of the oral environment and the complex mechanism of the oral perception. In detail, the salivary fluid is considered and view as an interface between the peripheral sensory system and taste stimuli.

Comprendere l'associazione tra le differenze individuali di caratteristiche salivari e la risposta orale a diversi stimoli sensoriali

Questo progetto di tesi di dottorato mira a comprendere la relazione tra alcune caratteristiche fisiologiche legate all'ambiente orale e il complesso meccanismo della percezione orale. In particolare, viene preso in considerazione il fluido salivare che viene ipotizzato essere un'interfaccia tra il sistema periferico sensoriale e stimoli gustativi.

1. State-of-the-Art

Understanding the underlying mechanisms of individual differences in oral perception is one of the main questions of Sensory Food Science researchers; answering may help to better known individual food behaviours, choices, preferences and consumption. We already know that differences in oral perception are related to environmental, demographics, genetic and physiological factors; these variables all together may influence the oral responsiveness to sensory stimuli and may lead to different food choices (Monteleone et al., 2017).

Food choices and preferences, but also demographics and psychological traits of a population sample can be studied using specific questionnaires: Familiarity Questionnaire and Food Choice Questionnaire can be used to measure, respectively, consumers familiarity with different foods in different eating situations and consumers preferences for different foods chosen within a pair of items in different eating situations. Furthermore, Food Neophobia, Sensitivity to Disgust and Sensitivity to Punishment and Sensitivity to Reward Questionnaire are examples of questionnaires usefull to assess psychological and personality traits.

Regarding genetic and physiological factors, the oral environment plays a primary role in the oral perception; salivary fluid is one of the main variables that need to be considered for its flow rate, its protein content and enzyme activity. Salivary protein content for examples, can be related to individual astringency responsiveness (Dinnella et al., 2009; Fleming et al., 2016); the haze-forming capacity (HFC) is one of the laboratory tests set up to measure saliva reactivity to an astringent stimulus made by a tannic acid solution (Dinnella et al., 2009).

Phenotype markers of taste sensitivity are always measured when studying oral responsiveness to sensory stimuli: the PROP status, the Fungiform Papillae Density (FPD) and the Thermal Taste Status (TTS) are the main indices of oral perceptive acuity. Each of them can describe and characterize the population in terms of phenotype responsiveness. The PROP status describes the different ability to perceive the bitterness

associated to 6-n-propylthiouracil (PROP) compound and it is nowadays considered a general index of perceptive acuity. With PROP status test individuals can be divided in Super-Tasters, Medium-Tasters and Non-Tasters. In addition, the Thermal Taster Status categorises individuals in thermal tasters (TTs) and thermal non-tasters (TnTs) based on their ability to perceive taste solely from temperature stimulation (Skinner et al., 2018). Finally, fungiform papillae are the tongue structures containing the taste buds and the mechanoreceptors that are innervated by both gustatory and trigeminal nerve fibres; for this reason, fungiform papillae are thought to be associated with oral sensitivity, but their number and shape vary across individuals.

Many studies have been devoted to assessing the associations between PROP taste status, Thermal Taste status and Fungiform papillae density and their role in the oral perception. Nevertheless, their relationship remains controversial (Dinnella et al., 2018; Eldeghaidy et al., 2018) and other factors, such as physiological and genetic characteristics, need to be considered. Thus, the aim of this PhD thesis project is the evaluation of individual differences in salivary fluid characteristics and its association with taste responsiveness.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Administration of Questionnaires

Familiarity Questionnaire, Food Choice Questionnaire, Food Neophobia, Sensitivity to Disgust and Sensitivity to Punishment and Sensitivity to Reward Questionnaire will be administered to understand individual differences in food behaviours and choices and their underlying personality and psychological traits.

A2) Collection and analysis of saliva samples: the pilot test and the field study

Saliva samples will be collected under different conditions: first of all, a *resting* saliva will be collected in absence of any kind of mechanical or chemical stimulation, then a *chemically* stimulated saliva will be collected after a citric acid stimulation. These samples will be analysed for flow rate, total protein content, haze-forming capacity and enzyme activity. A pilot study will be organized before starting with the main study that will be made up by almost 150 subjects, balanced for age and gender. The results of these analyses will explain the variation in saliva characteristics considering or not a stimulation, like it happens during the consumption of foods.

A3) Measurements of the indices of oral perceptive acuity and phenotype markers. PROP taste status, Thermal taste status, Fungiform papillae density will be measured and used to categorize the population sample. Relationships between them will be analysed. Individual differences in the perceptive acuity will be studied in terms of intensity perceived after tasting different aqueous solutions characterized for a specific sensory stimulus (sweet, sour, salt, bitter, astringent). gLMS scale will be used to measure intensity rate.

A4) Statistical analysis will be performed to find a relationship between physiological characteristics of saliva samples, markers of perceptive acuity and food behaviours.

A5) Data collection abroad

A6) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Administration of Questionnaires																								
A2)	Collection and analysis of saliva samples																								
	1) Pilot study																								
	2) Big study																								
A3)	Measurements of the indices of perceptive acuity and phenotype markers																								
	1) PROP status																								
	2) Thermal taste status																								
	3) Fungiform Papillae density																								
	4) Solutions intensity rate																								
A4)	Statistical analysis																								
A5)	Data collection abroad																								
A6)	Thesis and Paper Preparation																								

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Valorization of By-Products and Waste from Agri-Food Industries, and their Utilization for Innovative Food Formulation

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This PhD thesis research project aims at valorizing by-products and waste from the agri-food industry rich in bioactive compounds that will be used for the formulation of innovative foods. In the first phase, the extraction methods of bioactive compounds from some by-products and/or agri-food waste, will be set up and tested. After their characterization, the extracts with the best characteristics from the chemical, nutritional and safety standpoints, will be selected. To improve their stability and/or solubility characteristics for their subsequent inclusion in the products to be formulated, different protective systems will be tested. In the last phase, new food products will be formulated, by adding the bioactive extracts or compounds (as such or protected according to the formulation or product) as partial or total substitutes for chemical additives, with the aim of obtaining innovative products that are stable from the oxidative standpoint, with good nutritional, sensory and safety characteristics. The characteristics of the innovative products will be monitored during a shelf-life study.

Valorizzazione di Sottoprodotti e Scarti dell'Industria Agro-Alimentare e loro Utilizzo nella Formulazione di Alimenti Innovativi

Il presente progetto di ricerca consiste nel valorizzare i sottoprodotti e gli scarti di lavorazione dell'industria agro-alimentare ricchi in sostanze bioattive che verranno utilizzati nella formulazione di alimenti innovativi. In una prima fase, si prevede una messa a punto ed applicazione di metodi per l'estrazione di composti bioattivi da alcuni sottoprodotti e/o scarti agro-alimentari. Dopo la loro caratterizzazione, saranno individuati quelli estratti che abbiano le migliori caratteristiche dal punto di vista chimico, nutrizionale, e della sicurezza. Nel caso sia necessario migliorare le loro caratteristiche di stabilità e/o solubilità per la loro successiva inclusione nei prodotti da formulare, saranno testati diversi sistemi protettivi. Nell'ultima fase, saranno formulate nuove tipologie di alimento, aggiungendo gli estratti o composti bioattivi (tal quali o protetti a seconda della formulazione o della tipologia di prodotto) come sostituti parziali o totali di additivi chimici, con lo scopo di ottenere dei prodotti innovativi sicuri che siano stabili dal punto di vista ossidativo, con buone caratteristiche nutrizionali e sensoriali. Le loro caratteristiche saranno monitorate durante uno studio di shelf-life.

1. State-of-the-Art

The increasing quantity of agri-food waste has become a major concern not only at European level, but also worldwide. Their incorrect disposal causes environmental problems and substantial economic losses. For this reason, it is necessary to develop strategies for the re-use and valorization of by-products and waste from the agri-food industry that are not limited to their utilization as fertilizers, compost, biogas material or animal feed, but to exploit them as sources and/or functional ingredients rich in high-biological value compounds (Fritsch et al., 2017) for the formulation of new food. According to the D.L. 152/2006, by-product is defined as any substance or object that meets all of the following requirements: a) the substance or object derives from a production process, of which it is a constituent, and whose primary purpose is not the production of this substance or object; b) the substance or the object will be used, during

the same or a subsequent production process or use, by the producer or third parties; c) the substance or object can be directly used without any further treatment other than normal industrial practices; d) further utilization of such substance or object is legal, i.e. they meet, for the specific use, all the relevant requirements concerning the products and the protection of health and the environment and will not lead to overall negative impacts on the environment or human health. This definition of by-product fits perfectly into the Circular Economy concept, whose principle is the re-use of processing residual materials in successive production cycles, to reduce waste as much as possible. In December 2015, the European Commission presented the new circular economy package, whose action plan provides a deeply integrated approach based on the entire production chain, starting from product design to consumption, aiming at reducing waste production and encouraging recycling of most substances. In February 2016, the Italian Environmental Panel (Legge 221/2015) came into force containing provisions on environmental legislation to promote green economy and sustainable development. This has allowed the principles of the circular economy to become part of the Italian legal system to be applied in a wide range of environment-concerning issues, from waste management to sustainable mobility. Since the European Union is the world's largest producer of some of the most consumed products for both human and animal nutrition, most of the waste derives from the collection and processing of agri-food products, such as wheat, oats, olive oil, tomatoes and potatoes (Fritsch et al., 2017). Extracts from the processing by-products of the aforementioned foods have a potential use as functional ingredients or additives in the food industry thanks to their composition. Most of them are rich in bioactive compounds, such as carotenoids, phenolic compounds, essential oils or β -glucans (Gómez-Caravaca, 2014). Many of these compounds display, in fact, different health properties, antioxidant and antimicrobial activities and some of them can also be used as natural food colorants (Dordevic and Antov, 2016). Other valuable ingredients could be dietary fibers, including polysaccharides and lignin, or proteins that can be used as a functional ingredient, increasing the nutritional value of some food. Some extracts, like phenolic one, could partially or completely replace different synthetic food additives that are known to have a negative impact on human health, such as nitrate salts and nitrites. The latter are added to meat products as preserving agents thanks to their antimicrobial, antioxidant and color-stabilizing properties (Bolger et al., 2017). Regardless of the countless technological advantages, however, the reduction of nitrates and nitrites has become an issue of primary importance for the meat industry. Another very interesting application of the by-products containing molecules with antioxidant and antimicrobial effects is that they can be used for active packaging or coating to prolong their shelf-life. In fact, the residues from cereal processing could be used as a reinforcing agent of bio-composites for the design of new packaging (Berthet et al., 2016).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Bibliographic Research**

A2) **Development of Extraction and Characterization Methods of Bioactive Compounds from Agri-Food Waste**

A3) **Extraction and Physico-Chemical Characterization of Bioactive Compounds**

A4) **Development of Inclusion Systems of Bioactive Compounds**

A5) **Formulation of Innovative Food Products**

A6) **Shelf-life Study of Selected Products**

A7) **Thesis, Paper and Posters Preparation**

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1) Bibliographic Research																		
A2) Development of Extraction and Characterization Methods of Bioactive Compounds from Agri-Food Waste																		
1) Selection of Waste																		
2) Development of Extraction and Characterization Methods																		
A3) Extraction and Physico-Chemical Characterization of Bioactive Compounds																		
A4) Development of Inclusion Systems of Bioactive Compounds																		
1) Study of Iclusion Systems																		
2) Evaluation of Stability of Inclusion Systems																		
A5) Formulation of Innovative Food Products																		
1) Inclusion of Bioactive Compounds and Partial (or Total) Substitution of Some Additives																		
2) Evaluation of Stability and Sensory Characteristics of Innovative Products																		
A6) Shelf Life study of Selected Products																		
A7) Thesis, Paper and Posters Preparation																		

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Novel Biotechnologies Applied to Cereal-Based Food Items

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This PhD thesis research project aims to develop novel biotechnologies to reduce the glycemic index (IG) and salt content in bakery products, to increase the fiber content and antioxidant substances in the same products and to reduce the concentration of any anti-nutritional factors (ANF). The same goals, except for the decrease of salt, will be also pursued in pasta.

Biotechnologie innovative applicate ai prodotti a base di cereali

Il presente progetto di dottorato prevede lo studio e la messa a punto di biotechnologie innovative per ridurre l'indice glicemico (IG) e il contenuto in sale nei prodotti da forno, aumentare negli stessi prodotti il contenuto in fibra e sostanze antiossidanti e ridurre la concentrazione di eventuali fattori anti-nutrizionali (FAN). Gli stessi obiettivi, ad eccezione di quello che attiene al sale, saranno perseguiti anche nella pasta.

1. State-of-the-Art

Cereal-based foods represent very important sources of nutrients and fundamental cultural heritage for several human populations. However some diet-related chronic diseases (e.g., obesity, type II diabetes and hypertension), are correlated to the consumption of wheat-based baked goods. The scientific community and producers have been increasingly interested in the use of vegetable raw matters alternative to wheat to reduce the glycemic index (IG), and fortify or enrich cereal products with compounds/ingredients (e.g., fibers) that increase the nutritional value of such foods (Jenkins et al. 2012). Legumes are an excellent source of proteins, carbohydrates and fiber and are able to provide many essential amino acids, vitamins, minerals, oligosaccharides and phenolic compounds (Roy et al. 2010). Legumes are also considered effective in reducing the risk of cardiovascular disease (Flight and Clifton 2006) and preventing type 2 diabetes and obesity (Jenkins et al. 2012). Minor cereals (e.g., barley and oats) and pseudo-cereals (quinoa, amaranth and buckwheat) are particularly rich in fibers, minerals, vitamins and phenolic compounds (Coda et al. 2014). Milling by-products (mainly wheat bran and wheat germ) are characterized by high content of fibers and functional compounds (α -tocopherol, vitamins, minerals and bioactive compounds), as well as by proteins with high biological value (Rizzello et al. 2010).

However, the use of the above mentioned alternative raw matters is limited due to high presence of anti-nutritional factors (FAN) and barely acceptable organoleptic profile (Campos-Vega et al. 2010).

Such drawbacks may be overcome by fermentation with selected microorganisms. Lactic acid fermentation of these alternative flours can improve both the sensory and bread-making qualities (Coda et al. 2014) and reduce FAN (Coda et al. 2010). The tendency to satisfy the increasing consumers' demand for cereal-based products with a more

balanced nutritional profile has led to considerable efforts in developing products that combine health benefits and good sensory properties.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

Table 1 *Gantt diagram for this PhD thesis project.*

activity/months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	
bibliografic research and study	■																																				
1.1-selection of raw materials and other ingredients	■	■	■																																		
1.2 selection of lactic acid bacteria		■	■	■	■																																
2.1 development of processes in laboratory conditions					■	■	■	■	■	■																											
2.2 development of pilot processes						■	■	■	■	■	■																										
3.1 nutritional and technological evaluation of the novel food														■	■	■	■	■																			
3.2 functional and sensory evaluation of the novel food																				■	■	■	■	■													
4 technologic transfer of innovative processes and food																										■	■	■	■	■							
5.1 approval test in vivo (ethics committee)																													■	■	■	■					
5.2 patients recruitment and dietary intervention																																■	■	■	■		
5.3 evaluation of the effects of the in vivo tests																																			■	■	■

A1) Selection of raw materials and ingredients to obtain foods with better nutritional and functional quality. This step involves the selection of pseudo-cereals (amaranth, buckwheat and quinoa), legumes (chickpea, broad bean and lentil), minor cereals (oats and barley) and milling by-products (wheat bran and wheat germ) to be used, after eventual biotechnological processes (e.g., fermentation), in pasta and baked goods. For all ingredients and raw matters, blends of autochthonous lactic bacteria (LAB) will be selected for their ability to decrease IG and (especially in legumes and pseudo-cereals) FAN concentration and to improve sensorial qualities. Furthermore, for baked goods with reduced salt content, LAB will be selected for their ability to compensate for the lower sapidity by: (i) the production of glutamate and other free amino acids that, directly or indirectly (as precursors of other compounds), positively affect the taste; (ii) the production of balanced concentrations of lactic and acetic acids.

A2) Development of biotechnological processes for novel cereal-based foods. In this step, laboratory protocols will be developed for the ideal combination of LAB, raw matters and other ingredients identified in the A1. Subsequently the biotechnological processes will be tested on a pilot scale plant.

A3) Evaluation of the nutritional and functional characteristics of the different types of novel foods. These activities will be necessary to assess the achievement of nutritional and functional improvement of novel foods: increased fiber content, antihypertensive and anti-oxidant activity, reduced IG and, just for baked goods, reduced salt content. The analyses will be temporally distributed so as to ensure that the results are collected after the development of the biotechnological protocols (A2) and at the end of the industrial scale-up (A4), to guarantee the optimal technological transfer to the GR.A.A.M. s.r.l. company.

A4) Technology transfer of novel processes and foods. The scale-up at the industrial level of biotechnological protocols to obtain the novel cereal-based foods previously developed (A2) will be carried out. In this step, the processes of stabilization, packaging and storage of novel foods with improved nutritional and functional characteristics, compared to their conventional counterpart, will also be tested.

A5) In vivo test. 100 individuals with moderate hypertension will be recruited for a "dietary intervention" lasting 2 months. Individuals will be grouped into two arms: 1-normal diet; 2-diet with novel foods. The effect of the type of diet on the IG, blood pressure and intestinal microbiota will thus be evaluated.

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Effect of Cold Plasma on the Fungal Metabolism and Mycotoxin Inactivation

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The applications of new food processing technologies to reduce fungal contamination and mycotoxins content are one of the most interesting and popular topics, since they are related to food safety. This PhD research is aimed to study the mechanisms of fungal behavior and mycotoxins inactivation after cold plasma treatment, in order to have broader and useful applications in food decontamination.

Effetto del plasma freddo sul metabolismo fungino e inattivazione di micotossine

Le applicazioni di nuove tecnologie di trasformazione alimentare per ridurre la contaminazione da funghi e micotossine sono gli argomenti più interessanti e popolari dal momento che sono legati alla sicurezza alimentare. Questo dottorato è stato progettato per studiare i meccanismi di comportamento dei funghi e l'inattivazione delle micotossine dopo il trattamento al plasma freddo, al fine di avere applicazioni più ampie e utili nella decontaminazione degli alimenti.

1. State of Art

One of the main causes in the deterioration of food is the little investment in infrastructure, packaging and marketing systems in addition to the microbiological deteriorations, that mostly caused by fungal infection during the supply chain. Beside to promote changes in sensory properties of foods fungal infections are leading a depreciation of its market value, the fungal growth could be a concern related to the possibility of mycotoxin formation. Mycotoxins are known for severe toxic effects on vertebrates and which are produced by many important phytopathogenic and food spoilage fungi are classified as carcinogenic, mutagenic and genotoxic, produced such as for *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* species, presently, over 300 mycotoxins have been identified and reported; however, only a few regularly contaminate food and animal feedstuff (Misra, Pankaj, Segat, & Ishikawa, 2016). Processes such as cleaning, milling, brewing, fermentation, cooking, baking, frying, roasting, flaking, alkaline cooking, nixtamalization and extrusion have been able to reduce their content (Karlovsky et al., 2016). Mycotoxins can be partially destroyed or converted to less toxic compounds by chemicals treatments such as acids, bases, hydrogen peroxide, ozone and other oxidizing agent. Recently, the use of inert sorbent materials like aluminium silicates, and of microorganisms to biotransform mycotoxins, have been developed as approaches to mitigate mycotoxins contamination, however they are usually incomplete or inefficient (Upadhaya, Park, & Ha, 2010). In addition, in the last year's cold plasma has been used to decontaminate seeds, cereals, crops and fresh products from the presence of mycotoxins and moulds (Selcuk, Oksuz, & Basaran, 2008, Basaran, Basaran-akgul, & Oksuz, 2008, Zahoranova et al 2016). Cold plasma is a partially ionised gas comprising ions, electrons, ultraviolet photons and reactive neutrals such as radicals, excited and ground-state molecules. Atmospheric cold plasma (ACP) refers to non-equilibrium plasmas generated at environmental temperatures and pressure. The limited studies on the effect of plasma against fungi indicated several damages in cell membrane, DNA chain, metabolic disturbance in biosynthesis, reproduction and repair mechanisms (Sharma, Reddy, & Sethi, 2018). In addition, plasma efficacy depends on the fungal species, microbial load food composition and surface conditions (Selcuk et al., 2008). Therefore, studies on antifungal activities with more diverse strains and species is

required to better understand the complex mechanisms of fungi response to cold plasma. Therefore, deeper studies on the behavior of fungi to this decontamination technology need to be done. On the other hand, the majority of studies have been conducted in controlled environments, using pure cultures on agar/artificial media or artificially inoculated seeds/perishables, thus it is important to perform studies on the naturally occurring mycobiota in more realistic conditions and in particular, in dehydrated and dry fruit, in order to develop procedures for the management of postharvest.

2. PhD Thesis Objectives and Milestones: To study the mechanisms of fungal behavior and mycotoxins inactivation after cold plasma treatment, in order to have broader and useful applications in food decontamination. Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1. Isolation and identification of the strains: The strains will be isolated different culture media (PDA -potate dextrose agar, MEA- malt dextrose agar-, DG18-dichloran-glycerol agar) from dry and dehydrated fruit. The specific identification of the sampled fungi will be performed firstly morphologically and then studied by a multilocus sequence analysis of four independent gene regions (β -tubulin, calmodulin, RNA polymerase II and elongation factor 1 α). The evaluation of the distribution of toxigenic fungi on dry fruits will be focused mainly on *A. flavus*, *A. parasiticus*, *A. carbonarius*, *A. ochraceus*, *A. niger*, *Alternaria alternata*, *A. tenuissima*, and *F. oxysporum*. The occurrence in the genome including both aflatoxigenic and non-aflatoxigenic strains of seven well characterized AFL biosynthetic genes in relation to AFL production will be carried out using two regulatory genes *aflR* and *aflS* and the structural genes *aflD*, *aflM*, *aflO*, *aflP*, and *aflQ* (Scherm et al 2005). The OTA producers will be analyzed using two sets of specific primers designed on the basis of the ITS1 region of rDNA sequence, using quantitative real-time PCR-assay.

A2. Optimization of Cold plasma treatment. The effect of different types of cold plasma sources on the fungal survival, both spores and mycelium of 48 hours old toxigenic and non-toxigenic strains of *A. flavus* will be taken as targets. Growth in solid medium will be daily evaluated for 10 days. Central composite face-centered designs (CCDs) will be used to optimize the operating variables of cold atmospheric plasma treatment (RH, power, and time) and contamination level. The treatment with major efficacy will be used to evaluate the sensitivity of the different species colonizing dry and dehydrate fruits.

A3. Effects on the fungal morphology and metabolism. To determine the changes in morphology of selected treated fungi, Scanning Electron Microscopy images will be obtained. To determine the morphological changes associated with apoptosis-like cell death in the strains, nuclear condensation and plasma membrane integrity will be investigated as early and late apoptotic markers, respectively based on the method describe, using Fluorescence Microscope digital camera and 4',6-diamidino-2-phenylindole filter for the fluorescence images. The mechanism of toxicity by ROS will be investigated using the Ellman's assay. The loss of glutathione will be measured in this method to quantify the ROS generation and of Cell membrane integrity will be examined. For the survival to cold plasma treatment, the evaluation of metabolic activities will be evaluated after 2, 5 and 7 days of incubation and in particular the comparative proteomic profiling of toxigenic and no-toxigenic strains of *Aspergillus*, *Alternaria* and *Fusarium* species and their response to cold plasma will be also evaluated, using bidimensional electrophoresis (with Coomassie or Silver staining). Protein spots will be identified to obtain information about of the changes in fungal metabolism. In addition information on the full volatile metabolome will be performed using headspace-solid phase

microextraction combined with GC× GC with time of flight mass spectrometry detection (HS-SPME/GC× GC-ToFMS). In addition, the production of AFs, and OTA will be evaluated.

A4. Effects of cold plasma on the fungal viability and mycotoxins production in situ during storage. The kinetics of the fungal inactivation and survival after cold plasma exposition will be monitored for 30 days. The mycotoxins detoxifying capability of cold plasma will be assessed adopting the optimized CP treatment. Three approaches will be followed: 1) decontamination of fruits artificially contaminated with selected mycotoxins produced by *Aspergillus*, *Fusarium* and *Alternaria* species, used individually at levels of 10 µg/kg of sample; 2) decontamination of samples inoculated with spores of the myco-pathogenic species and 3) decontamination of natural occurring mycotoxins. In all cases the decay kinetics of mycotoxins exposed to plasma will be monitored. The target mycotoxins will be Aflatoxins (B1, B2, G1, G2), beauvericin, ochratoxin (A and B) for dry and dehydrated fruit. Mycotoxins analysis will be performed by reference validated methods based on liquid chromatography/mass spectrometry.

A5. Statistical analysis.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	25-27	28-31	31-33	34-36
A1	Literature Review												
A1	Isolation and identification of the strains												
A2	Optimization of Cold plasma treatment												
A3	Effects on the fungal morphology and metabolism												
A4	Effects on fungal viability and mycotoxins production during storage												
A5	Statistical analysis												

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Identification and Assessment of Cell Fate Molecular Markers for Food Production Quality and Sustainability

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This PhD project aims to identify molecular markers for assessing and predicting cell fate in response to specific conditions in species of interest in food sciences processes and technologies. We will mainly exploit by bioinformatics approaches the amount of transcriptome data currently available which are related to cell response to bioactive or toxic compounds. Our studies are focused on human cells, as these systems can be used as biosensors to assess food quality. We will also investigate other biological systems: yeast, algae and other microorganisms since monitoring their controlled production and health is of interest in food sciences.

Identificazione e valutazione di markers molecolari del destino cellulare la qualità e la sostenibilità della produzione alimentare

Questo progetto di dottorato mira all'identificazione di marcatori molecolari per valutare e prevedere il destino cellulare in risposta a specifiche condizioni in specie che sono di interesse nei processi e nelle tecnologie delle scienze alimentari. Sfrutteremo principalmente approcci bioinformatici sui dati di trascrittoma attualmente disponibili correlati alla risposta cellulare all'esposizione a composti bioattivi o tossici. I nostri studi sono focalizzati sulle cellule umane, che potranno essere utilizzati come biosensori per valutare la qualità degli alimenti per fini alimentari. Inoltre indagheremo su sistemi biologici come lievito, alghe, microrganismi poichè la loro produzione controllata è di interesse per le scienze alimentari.

1. State-of-the-art

"Omics" technologies are today capable of providing amount of molecular information which characterizes the status of cellular systems. In particular, powerful sequencing technologies, such as those from Next Generation technologies can support the identification of key molecular markers of specific processes depicting cell functionality in response to specific conditions.

This PhD thesis aims to mine gene expression data, mainly from transcriptome sequencing, to identify genes that are differentially expressed during exposure to bioactive compounds. Because of the importance to predict cell fate in terms of survival or death, our initial aim is to analyze current knowledge and available transcriptome data related to these processes. This analysis aims to the set-up of molecular tools for early diagnosis of cell death both in human cells and in microbial species of interest in Food Sciences. Specifically, we aim to employ these approaches to implement tools to exploit human cell cultures as biosensors to validate the toxicity or the bioactivity of specific compounds to be employed for healthy food production and quality assessments. On the other hand, the same approaches will be used to set up dedicated methodologies to predict life quality of microbial species in bioreactors.

Cells from all living kingdoms may die in different ways and can escape death by different processes. Since the initial description of cell death, (Vogt, 1842), cell death has been object of numerous studies in different species (human, *Caenorhabditis elegans*

(Horvitz, 1994), yeasts and prokaryotes) with diagnostic, prognostic and industrial purposes.

The molecular processes depicting the different types of cell death are a set of mechanisms that can related to accidental (ACD) or programmed elimination (PCD) of cells (Kroemer, et al. 2009). Whereas ACD could be triggered by mechanical, physical or chemical stress which lead to instantaneous and catastrophic death of cells, PCD is genetically controlled by a dedicated molecular machinery and could be triggered by development or stress (Galluzzi, et al. 2018).

Cell death has been widely studied since its fundamental role in human development and tissue homeostasis and for its relevance to understand a variety of human diseases, including immunological and developmental disorders, neuro-degeneration, and cancer (Fuchs and Steller, 2015). Moreover, similar studies on yeasts and on prokaryotes have been relevant for different reasons, from medical to environmental applications. The cultural, industrial and biotechnological impact of knowledge on cell death remains unparalleled. Studies on cell death shed light on disease, cancer and development in human, whereas, for yeast and microorganisms, they can offer tools for the optimization of strategies used for food or drug production (eg. fermentation, natural products such as biostimulants, antioxidants, vitamins) or the development of novel biotechnologies, for example enzyme production, as well as the implementation of unconventional species in industrial processes.

This is why a series of transcriptome data related to cell death of survival are today accessible in public molecular databases (such as Sequence Reads Archive (Leinonen, et al. 2010). Starting from these data, we will exploit classical bioinformatics approaches to identify key molecular markers of cell fate and design suitable experiments to test our results.

2. PhD Thesis Objectives and Milestones

In particular this PhD project will be subdivided into the following activities according with Table 1:

- A1) Bibliography and database research.
- A2) Collection and organization of selected data.
- A3) Bioinformatics comparative analysis.
- A4) Identification of molecular markers: selection and validation.
- A5) Thesis and papers preparation.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A1) Bibliography and database research																									
A2) Collection and organization of selected data																									
A3) Bioinformatics comparative analysis																									
A4) Identification of molecular markers: selection, and validation																									
A5) Thesis and Paper Preparation																									

3. Selected references

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Multi-Sensors Reactor to Assess Functional Foods Properties

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This PhD project aims to set-up a multi-sensors reactor system for the study of the chemical changes occurring to functional foods during processing. The device consists of a chemical process analyzer (CPA, reaction calorimeter), which can be combined with different sensors, such as electronic tongue, nose and near infrared probes. The objective of the thesis will be to apply such multi-sensor reactor to assess the functional properties of foods.

Reattore multi-parametrico per valutare le proprietà funzionali degli alimenti

Questo progetto di dottorato ha come scopo l'utilizzo di un reattore multi-parametrico per lo studio di reazioni cinetiche legate alle proprietà funzionali degli alimenti. Si tratta di un reattore calorimetrico (CPA calorimeter) che sarà combinato con diversi sensori quali una lingua elettronica (Electrochemical Coulometric Array Detector), un naso elettronico (PTRM-MS) ed un sensore di spettroscopia nel vicino infrarosso (NIR). Lo scopo finale della tesi sarà quello di fornire una comprensione accurata delle reazioni cinetiche che si sviluppano in alimenti funzionali.

1. State-of-the-Art

Functional foods are products possessing health benefits beyond nutrition. The concept of functional food has aroused the interest of consumers because of the role they play in promoting health (Young, 2000). Functional foods improve the general conditions of the body (e.g. prebiotic and probiotic foods), decrease the risk of some diseases (e.g. cholesterol-lowering products) and cure some illnesses.

Examples of functional foods include antioxidants and fibers. Fibers are believed to enhance the digestive process. Although fibers remain undigested in the human organism and undergo fermentation in the colon, they can sustain the growth of probiotic bacteria (Charalampopoulos et al., 2003; Stanton et al., 2005). A further example of functional foods are antioxidants, which can donate electrons and/or protons to free radicals and neutralize them, thus reducing their capacity to damage cells and tissues (Halliwell, B., 1995). They are efficient in scavenging free radicals, chelating pro-oxidant metal ions or acting as reducing agents (Shahidi, 2012). Foods enriched with antioxidant are a potential strategy to minimize oxidative stress-related diseases.

Nowadays, there are several approaches used to assess and characterize functional foods, i.e. spectrophotometry, chromatography (Ingle et al., 2016). However, none of these analytical techniques can entail a broad range of clear benefits individually. They all suffer from certain limitations.

For instance, near-infrared spectroscopy (NIR) is a technique that uses the near-infrared region of the electromagnetic spectrum (from 780 nm to 2500 nm). NIR is a nondestructive method which can be used for the assessment of liquid and solid samples only. However, there is no absorption of minerals in the NIR spectrum region. Hence, minerals cannot be detected by this technique (Karthek et al., 2011).

The high-performance liquid chromatography (HPLC) is used for detecting and quantifying the majority of food analytes. HPLC has the ability to separate and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations. However, the analysis using HPLC is long, complex and tedious. There is only a limited amount of sample that can be analyzed concurrently. Moreover, the cost associated with the analysis is very high.

Recently, calorimetry has been indicated as a novel technique able to monitor online the reactions occurring in food. Calorimetry is a method used for measuring the heat of physical, chemical and/or microbiological processes. It allows direct measurements of thermal activities (heat flux) involved in different processes. As it is non-specific, it has a broad range of applications and it has been shown to be a valuable methodology to obtain both thermodynamics and kinetics information (Khalef et al., 2016). The instrument used to measure the heat is called “calorimeter”.

In the light of all the advantages and limitations of the above-mentioned technologies, the idea of using a system consisting of a multi-sensors reactor combining different techniques can represent an innovation to alleviate the drawbacks of each technique, while taking advantage of their individual benefits.

Therefore, the present proposal aims to use a novel reactor based multi-sensors device that can study and follow the reaction kinetics occurring in functional food. In the first part of the study, a chemical process analyzer (CPA calorimeter) will be used and combined with different sensors such as electronic tongue (Electrochemical Coulometric Array Detector), electronic nose (PTRM-MS) and near infrared spectroscopy probe (NIR) to follow the reaction kinetics of model food systems. In the second part of the study, functional foods will be produced and their reaction kinetics will be assessed using the device. This study will provide new insights into the understanding of the functional properties of foods.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Preliminary works Literature review on different topics related to this study. Training on the various laboratory apparatuses by performing preliminary experiments on simple compounds.

A2) Development of the novel reactor based multi-sensors device to analyze functional foods. The device will be composed of a chemical process analyzer (CPA, calorimeter) combined with a near infrared spectroscopy probe (NIR), an electronic tongue (Electrochemical Coulometric Array Detector) and an electronic nose (PTR-MS).

A3) Analysis of reactions occurring in a model food using the novel reactor based multi-sensors device. Simple model food compounds such as starch will be analyzed. The results obtained will provide insights into the kinetics and the products resulting from different reactions.

A4) Analysis of reactions occurring in processed food enriched with functional compounds using the developed novel reactor based multi-sensors device. The reaction kinetics occurring in a food enriched with bioactive or functional compounds will be studied with the aim to understand the functionality of the added components.

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Preliminary works																								
1) Literature review																								
2) Training on the laboratory apparatuses																								
A2) Development of the multi-sensor device																								
1) Analysis on individual devices																								
2) Analysis on the combination of the devices																								
A3) Analysis of simple model foods																								
A4) Analysis of functional foods																								
A5) Thesis and Paper Preparation																								

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Microbiome and Resistoma Profile of Foods of Animal Origin from Conventional and Antibiotic-Free Farms

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This PhD thesis research project is aimed at evaluating and comparing the microbiome and resistome in foods derived from animals raised with conventional and antibiotic-free methods, through the application of shotgun metagenomic sequencing and target amplicon sequencing (16S rRNA). Furthermore, it is intended to develop the most suitable sample processing, sequencing and bioinformatics protocols, in order to obtain high quality NGS sequencing data.

Analisi del microbioma e del resistoma di alimenti di origine animale provenienti da allevamenti convenzionali e antibiotic-free.

Questo progetto di tesi di dottorato mira a valutare e comparare la composizione del microbioma e del resistoma in alimenti di origine animale con particolare riferimento alle carni ottenute da animali allevati con metodi convenzionali ed antibiotic-free, tramite l'applicazione di tecniche NGS target e shotgun. Inoltre, si propone di mettere a punto protocolli di processamento del campione, sequenziamento e analisi bioinformatiche più idonei per l'ottenimento di dati di sequenziamento di NGS di alta qualità.

1. State-of-the-Art

The foodborne pathogen responsible for zoonoses is today a relevant public health issue (EFSA & ECDC, 2017). One of the most relevant transmission routes of those agents is represented by consumption of contaminated food of animal origin. Besides the concern on the presence of microorganisms in the foods of animal origin they show growing resistance to different classes of antibiotics mainly due to the massive use of antibiotics in farm animals. This is why poultry farms are trying to activate systems for raising chicken meat that do not involve the use of antibiotics. However, there is still little information on the impact of these rearing methods on the concentration and prevalence of zoonotic bacteria in poultry meat and on the impact of antibiotic resistance genes.

Recent developments in Next Generation Sequencing (NGS) techniques allow to obtain quantities of genetic information unimaginable until few years ago (Diaz-Sanchez et al., 2013). High-throughput sequencing techniques (HTS) consent a culture-independent metagenomic approach that is used to study the resistoma and microbiome, enabling accessing to the complete repertoire of resistance genes and bacteria within a sample (Doster et al., 2018). Moreover, this approach allows the study of food as a possible source of antibiotic resistance genes and then the assessment of the transmission's risk to humans.

In HTS, two different approaches can be used: targeted amplicon sequencing and shotgun sequencing. In the first case, a PCR step is necessary for the selection of the desired target gene. Usually for the microbiological study, the sequences studied are the variable portions of the 16S ribosomal RNA (rRNA) gene (Sharpton et al., 2014). Reads obtained will be aligned to reference sequences in available databases, thereby obtaining the taxonomic composition of the microbiota in a sample. The shotgun-based

approach requires that the entire DNA or RNA, prior to sequencing, be fragmented by enzymatic or mechanical methods. The final output will represent the metagenome of the microbial populations present in the sample. Finally, the presence and abundance of specific genes will be identified through appropriate bioinformatics tools (De Fillippis et al., 2017).

Thus, this PhD thesis project will be directed to characterize and quantify the different microbial species belonging to the microbiome of the food sample. More in particular, the study will verify whether the shotgun results are similar or significantly different from those obtained by the sequencing of the 16S. Furthermore, it will permit to characterize and quantify the food sample resistome, that is the presence and the number of antimicrobial resistance genes. Finally, the similarities and differences between food samples coming from conventional and antibiotic-free farms will be observed, both in terms of microbiome and resistome.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Setting up of more suitable NGS protocols** for DNA extraction, purification and quantification and for library preparation that will be sequenced with various shotgun and 16S methods.

A2) **Metagenomic analysis** using bioinformatics pipelines and software (i.e. MG-RAST) specifically dedicated to the identification of both, microbial populations present in food and antibiotic resistance genes.

A3) **Comparative analysis of metagenomes**, obtained from different foods, for the evaluation of the effect of conventional and antibiotic-free breeding methods on microbial populations and relative resistome.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Setting up of NGS protocols																								
1) DNA extraction and library preparation																								
2) NGS shotgun and 16S sequencings																								
A2) Metagenomic analysis																								
1) Characterization and quantification of microbial population																								
2) Characterization and quantification of antibiotic resistance genes																								
A3) Comparative analysis of metagenomes																								
A4) Thesis and Paper Preparation																								

3. Selected References

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Metagenomics Approach to the Characterization of Microbial Populations in Traditional Persian “Yellow Curd” and Investigate the Probiotic Potential in Food Grade Microorganisms

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Nowadays with increasing the demand for traditional food products plus usage of Lactic Acid Bacteria (LAB) as human probiotics, this project is aiming in not only studying the microbial diversity using metagenomics approach in traditional Persian “yellow curd” but also investigation and characterization of potential probiotic bacteria in typical fermented foods and their relevant health benefits to human body.

Approccio metagenomico alla caratterizzazione di popolazioni microbiche isolate da un prodotto tradizionale iraniano “yellow curd” e studio delle proprietà probiotiche di microrganismi ad uso alimentare.

La richiesta del mercato di derrate tradizionali fermentate con aggiunta di batteri lattici (lab) con attività probiotica è in continuo aumento, per questo motivo lo scopo di questo lavoro non si limita al semplice studio delle diversità del microbioma sfruttando l'approccio metagenomico su un prodotto tradizionale persiano “yellow curd” ma vuole anche analizzare e caratterizzare le potenziali attività probiotiche dei batteri ritrovati in comuni alimenti fermentati e i loro potenziali aspetti positivi per la salute umana.

1. State-of-the-Art

In the last few years, the demand for traditional food products has greatly increased and it poses a contradiction the consumer. Innovation in food production technologies has allowed the production of safer foods with a longer shelf life, and today the risk associated with food ingestion is considerably low. Fermented foods are complex microbial ecosystems, mainly represented by lactic acid bacteria (LAB) and yeasts, whose fermentation confers to the resulting products characteristic features, such as palatability, high sensory quality, structure and texture, stability, nutritional and healthful qualities, and when they are in a living form at the moment of consumption, potential probiotic properties. Since consumers have become more demanding and government requirements more rigorous in the last decades, food producers have faced conflicting challenges. Chemical additives have generally been used to combat specific microorganisms. However, for foods processed with no added synthetic preservatives, natural alternatives for the extension of shelf life of non-fermented foods include microbial inhibitors (e.g. bacteriocins and antifungal compounds) and vegetable by-products (e.g. essential oils and water extracts), and for fermented foods, pro-technological microorganisms that combat undesired (pathogenic/spoilage) agents. All these natural strategies may be used without compromising the naturalness of the traditional products, since they are generally recognized as safe (GRAS) (Settanni & Corsetti, 2008; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008). On the other hand, LAB as the predominant group of microorganism being widely distributed in fermented food and constitutes a notable proportion of human gastrointestinal tract, oral cavity and vaginal microflora (Ahne et al., 1998). Several LAB strains are considered beneficial for

the host because LAB capable of fermenting sugar to produce organic acids (lactic acid and acetic acid) and these acids by lowering the pH can cause growth interference of the surrounding microorganisms. In addition, some bacteria of this group are capable of producing antimicrobial substances such as hydrogen peroxide and bacteriocins that can enhance innate host defense responses. Many studies have been conducted in the past to show the beneficial effects of LAB on human health such as: i) protective ability of LAB against oral infections in humans; ii) protection from vaginal and intestinal infections; iii) role of LAB strains in gut microflora stabilization and competitive elimination of enteric pathogens (Naidu, Bidlack, & Clemens, 1999); iv) role of LAB in the modulation of cell mediated immunity to suppress tumor (Naidu et al., 1999); v) detoxification of carcinogens and decreasing the risk of colon cancer (Naidu et al., 1999); vi) the role of LAB in vitamin synthesis; vii) protection from milk allergy in infants; viii) relief from irritable bowel syndrome (Ducrotté, Sawant, & Jayanthi, 2012); ix) protection from diarrhoea; x) reduction in level of cholesterol (Quilliam et al., 2012); and xi) restoring the ecological balance of mucosa (Maldonado, de Ruiz, Otero, Sesma, & Nader-Macías, 2012).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) Collection and chemical analysis of fermented food samples. Chemical analyses related to nutritional characteristics of the traditional “yellow curd”, namely moisture, ash, protein, fat, Na, Ca, P, K and carbohydrates such as lactose and galactose were also performed.

A2) Assessment of microbial diversity of “yellow curd” using methagenomic approach to study the microbial community of this unknown traditional product. Sample processing and DNA extraction (A2.1). PCR amplification, quantification and sequencing (A2.2). The sequences later will be visualized and evaluated using bioinformatic softwares.

A3) Isolation and identification of newly Lactic Acid Bacteria (LAB) to isolate and identify some new generation of LAB that possess interesting technological and antimicrobial properties. Isolation of LAB using classical microbiology technique (A3.1). Identification of them using both microbiological and molecular techniques (A3.2).

A4) Assessment of probiotic potential of newly selected LAB strains. *In vitro* determination of probiotic properties (A4.1). *In vivo* confirmation using animal model (A4.2).

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Collection and Chemical analysis																									
A2) Methagenomics analysis																									
1) Sample processing and DNA extraction																									
2) PCR amplification, quantification and sequencing																									
A3) Isolation and identification of Lactic Acid Bacteria (LAB)																									
1) Isolation																									
2) Identification																									
A4) Assessment of probiotic potential																									
1) <i>In vitro</i> characterization																									
2) <i>In vivo</i> confirmation																									
A5) Thesis and Paper Preparation																									

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Reducing GHG Emissions and Water Footprint in the EU through Sustainable and Healthy Diets

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The objective of this PhD thesis research project is to achieve a substantial reduction of greenhouse gas emissions and water consumption in the EU food system by engaging citizens in adopting sustainable and healthy diets. Statistical analysis between environmental impacts of foods will be performed through the construction of a water footprint (WF) and carbon footprint (CF) database on the basis of which guidelines meeting both environmental sustainability and healthy dietary recommendations will be identified. Sustainable menus will be proposed during pilot experiments within company and university canteens to address greenhouse gas emissions (GHGe) and virtual water reduction of food choices.

Ridurre le emissioni di gas serra e l'impronta idrica nell'Unione Europea attraverso diete sostenibili e salutari

L'obiettivo di questo progetto di tesi di dottorato mira a ridurre le emissioni di gas serra e il consumo di acqua nell'UE coinvolgendo i cittadini nell'adozione di diete sostenibili e salutari. Verrà costruito un database sull'impronta idrica e carbonica degli alimenti e, sulla base di analisi statistiche condotte per determinare l'impatto ambientale degli alimenti, saranno identificate linee guida che soddisfino sia la sostenibilità ambientale che le raccomandazioni alimentari. Verranno quindi proposti menù sostenibili durante esperimenti pilota condotti all'interno di mense universitarie e private per determinare la riduzione di gas serra e di acqua virtuale delle scelte alimentari.

1. State-of-the-Art

With the Paris Climate Agreement targets have been set as the result of global commitment to reduce GHGe in all sectors in order overcome the Climate Crisis characterizing our era: the Anthropocene. Globally the food chain accounts for 70% of water withdrawals and 30% of GHG emissions besides playing a major role in land use, mineral depletion, desertification, deforestation, eutrophication, acidification, biodiversity loss and genetic erosion (MEA 2005). Meat and dairy production contribute up to 80% of the worlds food-related GHGe with red meat being the most impactful (Gerber *et al.*, 2013). From a health perspective, due to saturate fat and cholesterol content, consumption of meat and animal-derived products above dietary recommendations seem to be related to a higher risk of non-communicable disease compared to plant-based diets which, instead, are worldwide recognized as protective agents (Pan *et al.*, 2012). Moreover in 2015 the International Agency for Research on Cancer, on the basis of more than 800 epidemiological studies, classified consumption of processed meat as "carcinogenic to humans" and consumption of red meat as "probably carcinogenic to humans".

Sustainable diets concept has recently gained increase attention as a possible strategy to face public health related issues, decrease global warming and address food security for the world's growing population.

Table 1 *Most relevant examples of sustainable diets proposed by scientific literature and/or related projects.*

Authors	Diet specifics	Environmental target
Willet <i>et al.</i> 2019	Diversity of plant-based food; low amounts of animal source foods; use unsaturated rather than saturated fats; use limited amounts of refined grains, highly processed foods and added sugars; reduce food loss	GHG, land use, water use, N & P use, biodiversity
Poore <i>et al.</i> 2018	Half meat consumption, in particular beef, lamb, farmed crustaceans; reduce cheese; lowering consumption of more discretionary products (oils, sugar, alcohol, and stimulants), use traced food with land sustainability evidence.	GHG, eutrophication, acidification, water scarcity, land use
Fischer & Garnett. 2016	Food diversity; energy balance; whole grain; field grown vegetables; small quantity of meat; moderate use of dairy; very limited high fat food; limited sugar and salt; oils and fats with Omega 3:6 ratio; tap water.	GHG, land use
Macdiarmid <i>et al.</i> 2011	More vegetables; increase food variety; reduce food waste; moderate meat consumption; increase vegetable proteins; buy certified food; avoid food high in fat, salt, sugar; avoid soft drinks.	GHG, water use, land use

Several analyses have highlighted that adherence to plant-based diets with low meat consumption, such as Mediterranean Diet, or similar dietary patterns, could represent a win-win bottom-up strategy to reduce climate change while promoting public health in developed countries (Willet *et al.*, 2019; Burlingame & Dernini., 2012; Poore & Nemecek, 2018). Despite socio-environmental beneficial evidence, behavioural change is an ambitious challenge thus a multi-dimensional experimental intervention framework aimed at overcoming socio-cultural barriers and emphasize drivers is required to achieve a global shift towards sustainable diets.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) Systematic review on sustainable and healthy diets (A1.1) and collecting data on CF and WF of food items from LCA analysis (A1.2).

A2) Perform statistical analysis between and within food categories (A2.1) and develop a methodological framework for CF and WF assessment on traditional Mediterranean wild edible plant species (A2.2).

A3) Experiments in company and university canteens involving menu's CF and WF quantification (A3.1) and assessment of CF and WF reductions (A3.2).

A4) Defining guidelines for different stakeholders (A4.1) and an implementation design (A4.2) to allow scaling-up activities.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity Months	1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	25-27	28-30
A1) Systematic review										
1) Sustainable and healthy diets										
2) CF and WF for database construction										
A2) Database										
1) Statistical analysis										
2) Performing LCA on wild edible greens										
A3) Experiments in canteens										
1) Quantifying CF and WF of menu's										
2) Asses CF and WF reductions										
A4) Scaling up										
1) Defining guidelines										
2) Implementation design										
A5) Thesis and Paper Preparation										

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Development of Rapid Methods for the Quality Control of Meat and Dairy Products from the Mountain Region

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This Ph.D. research aims to develop rapid methods to evaluate the quality of meat and dairy products from the mountain region by the combination of electronic nose (E-Nose), Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) and Fourier Transform-Near Infrared Spectroscopy (FT-NIR). In the first part of the work, rapid methods based on the aforementioned approaches will be set up and applied to determine the quality of meat and dairy products. In the second part of the work, the methods will be applied to discriminate meat and dairy products coming from extensive vs. intensive production systems and high vs. low altitudes. To validate the results, Gas Chromatography-Flame Injection Detector (GC-FID) and Liquid-Chromatography-Mass-Spectrometry (LC-MS) will also be used. This research falls within the project entitled Heumilch FESR1129, with CUP H36H19000000007, Linea di finanziamento "Investimenti a favore della crescita e dell'occupazione FESR 2014-2020".

Sviluppo di metodi rapidi per il controllo di qualità della carne e dei prodotti lattiero-caseari in aree montane

Questo progetto di tesi di dottorato mira a sviluppare metodi rapidi per valutare la qualità della carne e dei prodotti lattiero-caseari in aree montane combinando il naso elettronico (e-Nose), Spettrometria di massa a reazione di trasferimento del protone (PTR-MS) e la Spettroscopia di Fourier Transform-Near Infrared (FT-NIR). Nella prima parte del lavoro, verranno stabiliti metodi rapidi basati sugli approcci sopra descritti e applicati per determinare la qualità della carne e dei prodotti lattiero-caseari. Nella seconda parte del lavoro, i metodi saranno applicati per discriminare carne e prodotti lattiero-caseari provenienti da agricoltura intensiva rispetto a quella estensiva e da alte a basse altitudini. Per convalidare i risultati, verranno utilizzati anche la gascromatografia con rivelatore a ionizzazione di fiamma (GC-FID) e la cromatografia liquida accoppiata a spettrometria di massa (LC-MS). Questa ricerca rientra nel progetto Heumilch FESR1129, con CUP H36H19000000007, Linea di finanziamento "Investimenti a favore della crescita e dell'occupazione FESR 2014-2020".

1. State-of-the-Art

Meat and dairy consumption has considerably increased in most developed countries (Hernández Adell, Muñoz Pradas, & Pujol-Andreu, 2019; Zheng, Li, Wei, & Peng, 2019). They are major source of proteins, fats, and other nutrients. They are characterized as very heterogeneous product in terms of chemical composition, physical structure, and sensory attributes (Andrés et al., 2007; Park, Lee, Lee, & Lee, 2019). Selecting high-quality meat and dairy products have always been the main concern of consumers (Leroy et al., 2004; Poghosian, Geissler, & Schöning, 2019). In most developed countries, the population expects a certain quality/price ratio. In this context, consumers are willing to pay higher prices to achieve meat and dairy products with higher quality (Prieto, Andrés, Giráldez, Mantecón, & Lavín, 2008; Yanthi, Said, Anggraeni, Damayanti, & ., 2018).

Meat quality is usually defined by chemical composition, palatability, and factors such as appearance, aroma, and texture (Miller, 2014). The most important elements

from a nutritional and processing point of view are the content of nutrients, pH value, color, water holding capacity, tenderness and taste (Lonergan, Topel, & Marple, 2019).

Producing high-quality dairy is a joint effort of dairy farmers and industries. The quality of dairy products depends on various factors such as cows' genotype, feeding materials, feeding environment, feeding methods and also storing, transporting, and sales of the products (Tian & Li, 2019).

The demand for high-quality meat and dairy products has increased because it has great sales potential (Prieto et al., 2008). Compliance with defined quality standards is particularly necessary for the marketing purpose and the success of branded product programs. The final quality of products is determined by the different physico-chemical and sensorial aspects.

A series of conventional and novel techniques are available to assess the physical and chemical quality of meat and dairy products. These methods individually are destructive, time-consuming and expensive. Hence, they cannot meet the requirements of the modern meat industry. Therefore, rapid and accurate methods for predicting the above-mentioned characteristics of products are necessary.

There are pros and cons for each of technique taken individually. To overcome these drawbacks, a method using the combination of different techniques could be used. The main focus of the present research is the development of a rapid method by which the quality of meat and dairy products can be determined rapidly and concisely. The method includes the development of sensors for assessing the aromatic compounds in meat and dairy samples. Further research will deal with the authentication of food using isotope analysis by PTR-MS and FT-NIR. Finally, LC-MS and GC-FID are used to validate the method.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Analysis of autochthonous meat and dairy products by conventional methods and novel technologies Local farmers from the province of South Tyrol will provide samples (A1.1) samples will be characterized in terms of their chemical composition, physical structure, and sensorial attributes (A1.2) E-nose and E-tongue will be used to evaluate product quality (A1.3) PTR-MS will be used to assess the aromatic compounds of samples (A1.4) Fourier Transform-Near Infrared Spectroscopy (FT-NIR) will be used to determine the chemical composition of the samples (A1.5) GC-FID and LC-MS will be used to validate the results (A1.6)

A2) Statistical analysis of data

A3) Development of methods to analyze the quality of meat and dairy products

A4) Paper and thesis preparation

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Analysis of meat and dairy products by conventional and novel methods																								
	1) Sampling																								
	2) Characterization of samples by conventional methods																								
	3) E-nose and E-tongue will be used to evaluate the sensorial attributes of samples																								
	4) PTR-MS will be used to assess the aromatic compounds of samples																								
	5) FT-NIR will be used to determine the chemical composition of the samples																								
	6) GC-FID and LC-MS will be used to validate the results																								
A2)	Data Analysis																								
A3)	Development of rapid methods to analyze the quality of meat and dairy products																								
A4)	Paper and thesis preparation																								

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Development of Functional and Innovative Cereal-based Foods with Reduced Glycaemic Response

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The aim of this PhD research project is to investigate strategies to decrease the glycemic index of cereal based foods and to study the relationship between rheological properties, microstructure and starch digestibility. Innovative and functional systems capable to positively influence the starch metabolism during digestion lowering the glycemic index of food product will be evaluated. Functional systems will be obtained through ingredient combinations (dietary fibre, antioxidant compounds, otherwise addition of pulse proteins and/or other ingredients) and/or technological interventions.

Sviluppo di alimenti funzionali e innovativi a base di cereali con ridotta risposta glicemica

Lo scopo di questo progetto di dottorato di ricerca è di investigare strategie che consentano di ridurre l'indice glicemico di prodotti a base di cereali e di studiare la relazione tra le proprietà reologiche, la microstruttura e la digestione dell'amido. Saranno valutati sistemi innovativi e funzionali in grado di influenzare positivamente il metabolismo dell'amido durante la digestione riducendo l'indice glicemico del prodotto. Tali sistemi funzionali saranno ottenuti attraverso combinazioni di ingredienti (fibre dietetiche, composti antiossidanti, proteine di legumi o altri ingredienti) e/o interventi tecnologici.

1. State-of-the-Art

Cereal-based products, such as bread and pasta, are staple foods in several countries due to high stability, simple formulations and low cost, so they represent a valid candidate for functionalization. Extruded snack formulations are even suitable to deliver good nutritional features to satisfy specific health claim. This prospect is a concrete way to use cereal-based products as a carrier of molecules capable of enhancing the human health by reducing the incidence risk of cardiovascular diseases, obesity, diabetes and reducing the caloric content of the product.

Cereals are the predominant energy-providing food for the global population, and starch is the major energy-providing component in cereals with close to 40% of the global energy intake (Svihus & Hervik, 2016). In terms of digestibility, starch is typically divided into 3 fractions: rapidly digesting starch (RDS), slowly digesting starch (SDS), and resistant starch (RS). RS is not digested in the small intestine and it is fermented by the microbiota in the large intestine. According to Englyst et al. (1992), RDS and SDS are the starch fractions that are hydrolyzed to dextrins by α -amylase within 20 to 120 min after ingestion, respectively. RDS releases a large amount of glucose in the small intestine leading to a rapid increase in glycemic response (Englyst et al., 1992). The rate of starch digestion in the small intestine is a major determinant of the extent that foods raise blood glucose and insulin levels after a meal. This is important as it is generally considered that reducing glycaemic responses can have health beneficial effects (Agustin et al., 2015).

Product formulation and processing can greatly influence starch digestibility due to different proportion of components (proteins, fat and dietary fibres) and food microstructure (Tosh, 2013). Conditions of starch granules influence the inherent

digestibility, as native and partially gelatinised starch granules are less susceptible to α -amylase than starch that has been damaged and fully gelatinized. For cereal food products, competition of ingredients for water and bulk viscosity or elasticity of doughs seem to be crucial for starch granule swelling during gelatinization (Peressini et al., 2015).

In recent years, dietary fibre (DF) has received increasing attention from researchers and industry due to the likely beneficial effects on the reduction of coronary heart-related diseases, diabetes incidence and gut neoplasia. Manufacturing high-DF products has its challenges regarding technological changes, maintenance of desired sensory properties and DF content of food product (Foschia et al., 2017). Recently, some authors observed that functional ingredients in combination gave synergistic or antagonistic effects on the predicted glycaemic response (Brennan et al., 2016; Foschia et al., 2015). Therefore, combination of bioactive compounds could be an interesting strategy to decrease the rate of starch digestion. Moreover, the use of flour from vegetable wastes, source of dietary fibre and antioxidant compounds, and alternative proteins, i.e. insects, reduced starch digestibility in bread and extruded snacks (Azzolini et al., 2018; Plazzotta et al., 2018). Dietary fibre inclusion in food products is responsible of multiple effects. Soluble fibres increase digesta viscosity of the gastrointestinal tract slowing the nutrients digestibility (Grundy et al., 2016). Besides, they compete with starch granules for water, decreasing starch swelling during gelatinization and making it less susceptible to digestive enzymes (Foschia et al., 2015). Wholegrain cereals as well as bran are good source of antioxidants and dietary fibre. Antioxidant compounds inhibit digestive enzymes (α -amylase and α -glucosidase activities) attenuating post-prandial blood glucose after food consumption (Nyambe-Silavwe & Williamson, 2016).

2. PhD Thesis Objectives and Milestones

The main objective of this research program is to study and develop functional and innovative cereal-based foods with reduced glycaemic response. The structuring properties of ingredients and/or technological interventions will be exploited to modulate starch digestibility. With this purpose it will be studied:

- 1) the effect of selected ingredients and/or technological interventions on dough rheological properties and microstructure;
- 2) the effect of structure on starch digestibility;
- 3) the development of food products with health functionality in terms of reduced glycaemic response.

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram (Table 1):

A1) Study and development of formulation: product formulation (pasta, bakery products or extruded snacks) and production; preliminary analysis: mixing properties, microscopy (SEM, CSLM, polarized light microscopy), dough rheology and product texture.

A2) Evaluation of starch digestibility: static *in vitro* gastrointestinal digestion to evaluate starch digestibility and glycaemic response.

A3) Study of nutritional profiles: total dietary fibre and nutritional compound profile.

A4) Sensory analysis: sensory evaluation for customer acceptance.

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Development of formulation																									
A2) Evaluation of starch digestibility																									
A3) Study of nutritional profile																									
A4) Sensory analysis																									
A5) Thesis Preparation																									

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The Dimension of Antibiotic Resistance in the Agri-Food System

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My PhD project deals with the characterization of the antibiotic resistance (AR) dimension in the agri-food system, a worldwide concern for public health, food and environmental security. The perspective is to describe the spread of antibiotic-resistant genes (ARGs) and antibiotic-resistant bacteria (ARB) in the agri-food system by evaluating Horizontal Gene Transfer (HGT) events in: i) lake water, focusing on the role of the crustacean *Daphnia* sp. and ii) plant rhizosphere. Final goal of the project is to enlarge the knowledge of the potential spread of antibiotic resistance determinants in the agri-food system.

Studio della diffusione dell'Antibiotico Resistenza nel sistema agro alimentare

Questo progetto di tesi di dottorato mira alla descrizione della diffusione dei fattori determinanti l'antibiotico resistenza nel sistema agroalimentare, un problema globale per la salute pubblica, la sicurezza alimentare e ambientale. L'obiettivo è quello di descrivere la diffusione, attraverso i meccanismi di trasferimento genico orizzontale, dei geni che conferiscono l'antibiotico resistenza e dei batteri antibiotico resistenti nel sistema agroalimentare; più dettagliatamente verranno studiati in: i) acqua di lago, concentrandosi sul ruolo del crostaceo *Daphnia* sp. e ii) nella rizosfera di piante di interesse agrario. Questo progetto ha lo scopo di descrivere e approfondire le potenziali vie di diffusione dei determinanti dell'antibiotico resistenza nel sistema agroalimentare.

1. State of the Art

Due to the use of antibiotics in medicine, livestock industries and agriculture over the past 80 years, a strong selective pressure has been imposed on microbial communities that have been exposed to a non-lethal concentration of antibiotics allowing the development of AR through mutations and HGT of ARGs in different environments (Smalla et al., 2018).

HGT is crucial for bacterial evolution and there are three main mechanisms that mediate DNA transfer, i.e. i) conjugation, consisting in transfer of DNA between a donor cell and a recipient cell, ii) transformation, consisting in the uptake of exogenous DNA derived from the environment and iii) transduction, in which the transfer of DNA is mediated by bacteriophage.

HGT events, and in particular conjugation, occur in different environments, especially in some specific "hot spots", such as plant rhizosphere and phylloplane. In these hot spots, HGT frequency is high, due to some physical properties such as soil type, moisture, temperature, pH, nutrient amendments and the presence of protozoa, worms or fungi for terrestrial environments; they can be also stimulated by the effects of stresses, such as the presence of metal.

Mobile genetic elements (MGEs), including insertion sequences, transposons, plasmids, integrons, bacteriophages and genomic islands, promote bacterial genome plasticity by the exchange of genetic material between a large number of bacteria (Sorensen et al., 2005). A lot of ARGs have been detected on MGEs and the exchange of these elements between environmental and pathogenic bacteria has been detected. Antibiotics used in different anthropogenic activities such as clinical treatments,

agriculture, or veterinary medicine to promote animal growth in the absence of acute infection i.e. in fish farms, can persist in soil and aquatic environments under sub-lethal concentrations, enhancing the generation of ARB and the spread of ARGs (Allen et al., 2010).

Moreover, recent studies underline that wastewater treatment plants (WWTPs), due to the high concentrations of cells in dense matrices and the compact structure of biofilm, are reservoirs of ARGs that are spread in the environment through their effluents (Petrovich et al., 2018). Due to this evidence it is clear that HGT can confer AR phenotypes to bacteria in the environment, but more studies are required in order to understand which mechanisms of HGT are important, what drives the initial HGT events, the role of selective pressure and the mechanism of capture and dissemination of ARGs in the environment (Smalla et al., 2018).

2. PhD Thesis Objectives and Milestones

The aim of this PhD project is to characterize the spread of ARB and ARGs in the agri-food system, investigating HGT events that may enhance the spread of these AR determinants in different environments related to the agri-food system. I will focus in particular on: i) lake waters, drawing attention to the description of transformation and conjugation events in *Daphnia* sp. *Daphnia* is a keystone species of freshwater ecosystems, a grazer of algae, a primary forage for fish and recent studies have underlined the presence of the resistance gene tet(A) in its bacterial community; ii) lettuce rhizosphere, since it is a major hot spot of HGT events. Moreover, *Enterobacteriaceae* members are main components of the lettuce microbiome and are typically inhabitants of the human and animal gut. To fulfil this objective, activities will be as described below (according to the Gantt diagram given in Table 1):

A1) Study of HGT events in *Daphnia* sp. A1.1) Screening of the possible acquisition of exogenous DNA in environmental conditions by indigenous bacteria isolated from *Daphnia* sp. followed by an in vivo study to verify if *Daphnia* sp. is a hot spot of HGT; transformant cells will be detected by antibiotic selection on proper media. A1.2) Screening of conjugation events by using conjugation systems based on the selection of transconjugant by fluorescent protein. Transconjugants will be detected by flow cytometer, antibiotic selection on proper media or/and by a cell sorter.

A2) Study of HGT events in lettuce rhizosphere. A2.1) Evaluation of HGT events in lettuce rhizosphere using strains already tested for their capability to exchange genetic material in laboratory conditions based on fluorescent protein. Transconjugant and transformant cells will be detected by flow cytometry and antibiotic selection on proper media. A2.2) Evaluation of HGT events in lettuce rhizosphere i) using isolates obtained by lettuce rhizosphere ii) considering the whole bacterial cells associated to lettuce rhizosphere Transconjugant and transformant cells will be detected by flow cytometry, and/or by using a cell sorter.

Table 1 Gantt diagram of this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	HGT in <i>Daphnia</i> Properties																								
	A1.1) Study of transformation in <i>Daphnia</i> sp.																								
	A1.2) Study of conjugation in <i>Daphnia</i> sp.																								
A2)	Study of HGT in lettuce's rhizosphere																								
	A2.1) Study of HGT in lettuce rhizosphere using model strain																								
	A2.2) Study of HGT considering the whole bacterial cells associated to lettuce rhizosphere																								
A3)	Thesis and Paper Preparation																								

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Reinforced Bioplastics Using Added Value-Additives Extracted from Food Wastes

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The aim of this PhD project is to re-consider wastes from the food industry to produce high added-value additives to be incorporated in packaging materials. These additives will be used to improve the overall performance of the most promising bioplastics (PLA, PHAs and BioPBS), which still suffer from a number of drawbacks that limit their wide marketing. Particularly, the attention will be paid both on the production process of these molecules and to their deposition on the surface of the films as coatings.

Bioplastiche migliorate utilizzando additivi ad elevato valore aggiunto ottenuti da scarti alimentari

Lo scopo del presente progetto di dottorato è di riconsiderare i rifiuti provenienti dall'industria alimentare, al fine di produrre additivi ad alto valore aggiunto da incorporare nei materiali di imballaggio. Tali additivi verranno utilizzati al fine di migliorare le performances complessive delle bioplastiche più promettenti (ad es. PLA, PHAs e BioPBS) che, ad oggi, presentano numerose problematiche prestazionali. L'attenzione sarà posta sia sul processo di produzione di queste molecole, sia nella loro deposizione sottoforma di coatings sulla superficie dei film bioplastici.

1. State-of-the-Art

Plastics dumped into the environment pose a serious threat to the environment and to our society. Accordingly, food packaging materials coming from renewable resources are highly sought. In the last 10 years, the demand for innovative, recyclable, biodegradable, and compostable materials has been steadily increasing, as demonstrated not only by the increasing investment by medium and large enterprises in the development of new bio-based materials, but also by the intense financing activity of projects oriented towards these themes, for example by the European Commission (ec.europa.eu/environment/waste/plastic_waste.html). On January 2018, the European Union launched the first ever document to this topic entitled "European Strategy for Plastics in a Circular Economy", which it is deliberately intended to provide guidelines for a better preservation of the environment, reducing marine litter, containing greenhouse gases emission and severely limiting our dependence on fossil resources. In this context, the development of bioplastics is driven by current demands to replace fossil fuel-based polymers. Limitation in fossil fuel resources, price volatility, impact on the environment, and waste disposal problems are some of the main reasons for this shift toward bioplastics. In general, bioplastics are broadly classified as bio-based and/or biodegradable (Siracusa et al., 2008; Song et al., 2009). In the food field, the use of bioplastics for packaging purposes is limited to almost exclusively polylactic acid (PLA) and thermoplastic starch polymers (TPS). Until now, different bio-polymers like polyhydroxyalkanoates (PHAs) and bio-poly-butylene succinate (Bio-PBS) are also used. These materials, although already developed and used on large scale, owns considerable performance limitations, such as poor gas and vapor barrier properties, unsuitable thermal properties, surface recalcitrance, and high cost (Vinka et al., 2003). In order to overcome all of these drawbacks, coating technology (that is, the deposition of thin

polymeric and / or inorganic layers on a substrate having larger thickness) represents a valid approach for the improvement of the "bulk" properties of the base materials (Uysal Unalan et al., 2014). Historically, the coating technology was developed around the '70s with the deposition of very thin inorganic layers through complex and expensive technologies (under high vacuum, sometimes in the presence of a plasma). Subsequently, the food industry has witnessed to the development of "wet" coating technologies, i.e. in the presence of an organic solvent in which one (or more) petroleum-based molecules is dispersed. More recently, supranational regulations have imposed a gradual shift to aqueous solutions / dispersions, in order to limit the amount of volatile organic compounds (VOCs) released into the environment.

In the last years, thanks to the continuous effort to make industrial processes more "green" and "environmentally friendly", the replacement of these synthetic additives of fossil origin with molecules of lower environmental impact has become a priority. According to the circular economy principles, using wastes for the manufacturing of new materials is a target for the next years. Among these molecules, biopolymers obtained from an extraction process of biomass, industrial wastes and by-products, represent an excellent opportunity because of a number of functional properties and their great potential (Chavan et al., 2018). Many biopolymers, such as cellulose nanocrystals, pectins, pullulan, gelatin and chitosan, act as excellent gas barrier (O_2 and CO_2) while having excellent optical and surface properties (Uysal Unalan et al., 2016; Fortunati et al., 2012). For example, cellulose nanocrystals (CNCs), obtained through a top-down approach starting from cellulosic biomass, are one of the today's most promising alternatives as additives, while representing an important field of investigation within numerous research groups due to its attractive properties such as high crystallinity, purity, non-toxicity, dimensional range, and origin (Habibi et al., 2010).

This PhD project will be directed to the selection of the coating's additives molecules (in particular, the attention will be address on cellulosic-rich materials coming from National agri-food wastes, like stalks, beets and graine maizes) as well as to the selection of the most suitable bioplastics materials used for the shelf life extension food products, especially those sensitive to oxygen.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram shown below :

A1) **Additives extraction** (e.g., CNCs) followed by a physicochemical characterization of the molecules (A1.1); extraction process optimization in order to increase the production yields (A1.2); scale-up trials of the extraction process in a pilot plant scale (A1.3).

A2) **Coatings deposition** on bioplastics (PLA, TPS, PHAs and Bio-PBS) and optimization of the coating process conditions (A2.1); characterization of the final coating layer using chemical, optical and mechanical techniques. In particular, the barrier performance against gases and vapors (O_2 , CO_2 and water vapor) will be evaluated (A2.2).

A3) **Packaging characterization** as far as both physicochemical (A3.1) and biodegradability/compostability are concerned (A3.2). A Life Cycle Assessment studies will be also performed in order to verify the sustainability of the new novel final packaging system (A3.3).

A4) **Shelf Life extension studies** on representative food matrices sensitive to oxygen, e.g., condensed milk powdery products aimed to verify the effect of the bio-based packaging system.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Additives extraction																								
	1) Physicochemical characterization																								
	2) Process Optimization																								
	3) Scaling-up of the extraction process																								
A2)	Coatings deposition																								
	1) Process Optimization																								
	2) Physicochem. characterization of coating layer																								
A3)	Packaging characterization																								
	1) Physicochemical characterization																								
	2) Biodegradability and compostability																								
	3) Life Cycle Assessment studies																								
A4)	Shelf Life extension studies																								
A5)	Thesis and Paper Preparation																								

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In Field Application of Biocontrol Agents to Improve the Safety and Quality of Grapevine Products

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This PhD thesis research project aims at evaluating the relation between plant pathogens and yeasts as the biocontrol agents in field and in the laboratory.

Applicazione in campo di agenti di biocontrollo per migliorare la sicurezza e la qualità dei prodotti della vite

Questo progetto di ricerca di dottorato mira a valutare la relazione tra agenti patogeni vegetali e lieviti come agenti di biocontrollo in campo e in laboratorio

1. State-of-the-Art

Fungi is one of the most significant economic losses pre- and post harvest in vineyards worldwide. *Botrytis cinerea* and *Phaeomoniella chlamydospora* are two of important pathogens on grapevine, which are the causal agent of gray mold and Esca disease which causes heavy losses in table and wine grapes (Coertze & Holz, 2002). Fruit maturity and the time of picking and application of the antagonist are the main factors affecting the performance of postharvest biocontrol agents. In addition over-mature fruit is much more susceptible to fungal attack than a fruit picked at optimal maturity (Boonyakiat, Chen, Spotts, & Richardson, 1987). New ways must be found to control them since there is no direct control of these diseases. The use of microbial antagonists for the control of postharvest diseases received special attention, and has been extensively considered. Biological control of postharvest diseases of fruits and vegetables by antagonistic microorganisms seems hopeful in replacing or reducing the use of synthetic fungicides (Lima, Arru, De Curtis, & Arras, 1999). Yeasts have been extensively studied because they possess many structures that make them suitable as biocontrol agents in fruits (Chanchaichaovivat, Ruenwongsa, & Panijpan, 2007). Yeasts are rarely related to occurrences of foodborne gastroenteritis, intoxications or other infections, unlike bacteria and viruses (Fleet & Balia, 2006).

In the first part of my project, I will focus on the relations between plant fungus diseases and yeasts as the biocontrol agents, by relying on laboratory and molecular methods and investigating the role of yeast on increasing the safeties and qualities of grapevine products.

2. PhD Thesis Objectives and Milestones

The present research project aims at evaluating the relation between plant pathogens and yeasts as the biocontrol agents in field and in the laboratory. The doctoral thesis project can be divided into the following tasks, summarized in the Gantt chart shown in table 1:

A1) Literature review about the previous studies based on biological control and using yeast as the biocontrol agents especially on grapevine. In addition, I will search for modern applications in which yeasts were used as biocontrol agents.

A2) Selection of the biocontrol agents in relation to the target pathogens and set up of the protocols to study their interactions in LAB trials.

A3) Set up of the laboratory and field experiments in order to evaluate their potential application

A4) Molecular approaches in order to study the molecular interactions among yeasts, the target pathogens and the host plant and to understand the action mechanisms of the innovative and green strategy to improve grape quality

A5) Analyses using RSM applications to examine the best microorganism as a biocontrol agent for grapevine disease and molecular analyses based on gene sequencing approaches.

A6) Selection of the best candidate for the biocontrol of *Botrytis cinerea* and *Phaeomoniella chlamydospora* in grapevine

A7) Writing and publication of doctoral theses, posters, scientific papers and oral presentations.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	32	36
A1) literature review																		
1) biological control based on yeast																		
2) current applications in grapevine																		
A2) Selection of the biocontrol agents																		
1) set up of the protocols																		
2) study their interactions in LAB trials.																		
A3) Set up of the laboratory and field experiments																		
A4) Molecular approaches																		
1) molecular interactions																		
2) understand the action mechanisms																		
A5) analyses																		
RSM applications																		
molecular analyses																		
A6) Selection of the best candidate for the biocontrol																		
A7) Thesis and articles																		

3. Selected References

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A High-Quality Cappuccino: Evaluation of the Nutritional Quality of Milk and the Consistency of the Foam in Cappuccinos Produced in Different Conditions of Temperature and Steam Used

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This PhD thesis research project is aimed to evaluate the nutritional quality of fresh milk (different types) and cappuccinos prepared by espresso machines produced by the "Simonelli Group" in different conditions of temperature and injected steam in order to find optimal conditions for the production of high-quality cappuccinos and milk-based drinks (Latte, Flat white).

Un cappuccino di alta qualità: valutazione della qualità nutrizionale del latte e della consistenza della schiuma in cappuccini prodotti in differenti condizioni di temperatura e di vapore impiegato

Questo progetto di tesi di dottorato si propone di valutare la qualità nutrizionale del latte fresco (diverse tipologie) e di cappuccini preparati dalle macchine per espresso prodotte dalla "Simonelli Group", in condizioni differenti di temperatura e di vapore iniettato al fine di trovare le condizioni ottimali per la produzione di cappuccini e di bevande a base di latte (Latte, Flat white) di alta qualità.

1. State-of-the-Art

The espresso coffee has reached, during the years, a global notoriety and appreciation. The vast majority of Italian coffee machines are destined abroad, just consider that 3 out of 4 machines are sold in other countries. There is a continuous commitment to improve the quality of the product on the Italian market and at the same time to spread abroad a greater culture of espresso coffee. The same goes for the cappuccino, which is the most consumed beverage both in Italy and in other countries, for which a continuous improvement of the product is required especially regarding the visual aspect, and the foam stability and the organoleptic properties. In particular, for the international market, milk-based drinks represent about 80% of total drinks (Milk; Flat white: with less foam; espresso; cappuccino). Milk and Flat white require a different foam than cappuccino with thinner bubbles and a more liquid final. Furthermore, in the international market, another aspect to consider is the "take-away drinks" which, unlike cappuccino made in a cup, are prepared at higher temperatures (70-75° C) with consequences on the organoleptic characteristics. With this in mind, innovations in espresso machines is still continued, especially in the direction of ergonomics, energy efficiency, design and temperature stability, but also it is important to know the changes occurring at milk during the preparation of cappuccino and/or milk-based drinks. In this sense, my PhD project is inserted, whose main objective is to obtain a high-quality cappuccino. In this purpose, very important is the temperature at which the milk is whipped: it should not exceed 70° C otherwise the milk takes on a "cooked" flavor due to the "caramelization" of lactose, and the proteins present in the milk undergo a process of denaturation. Denatured proteins bind to the tannic acid present in the espresso forming the "casein tannate", a compound that becomes difficult to digest. In addition, in a perfect

cappuccino, the foam should be compact and creamy, with a fine texture and shiny surface. The foam of the cappuccino is a colloidal dispersion of a gas in a liquid, the liquid is the milk used for cappuccino, while the gas consists of the air and water vapor used to whip the milk [Jimenez-Junca et al., 2015]. The air droplets can form a colloidal dispersion in the milk thanks to the presence in it of surfactants that in the milk is represented by proteins (mainly caseins) and phospholipids. The stability of the foam depends on the relative concentration of surfactants. The higher it is, the more stable the foam, as the gas droplets are small and well separated from each other [Hatakeyama et al., 2018]. Therefore, if the concentration of fats like triglycerides (which are not surfactants) is low, the foam of the cappuccino is more persistent. In whole milk the percentage of fat is around 3.6% and therefore to obtain a creamy and above all stable foam it is important to be able to disperse as much gas as possible during the heating time due to the steam blown into the milk. This is obtained starting from a milk temperature of about 5°C (refrigerator temperature), as the temperature rises (to reach the temperature of 60°C), the milk proteins are partially denatured, contributing to the stabilization of the bubbles in the cappuccino foam [Zayas, 1997]. Finally, in a perfect cappuccino, the cream should have a thickness of about one centimeter in the cup, this requires a greater injection of air and steam to be incorporated in the milk in the subsequent phase of micronization of the bubbles form at the beginning.

2. PhD Thesis Objectives and Milestones

The objectives of this PhD thesis project, are: 1) Determine the content and thermal stability of milk proteins (whey proteins and caseins), fundamental for the foam stability; 3) Determine the lipid content: even if fats tend to destabilize the cappuccino froth, they are essential to give a full taste and a creamy cappuccino taste; 4) Optimization of the air/steam mixture in the milk in order to obtain a better micronization of the larger bubbles that initially form in the cappuccino froth; 5) Consistency and texture of the foam obtained in the cappuccino and in the "flat white" following the changes made; 6) Temperature optimization for the preparation of "take away" products in order to get drinks of greater sensorial quality.

All analysis will be performed on milk samples (different types) and on freshly prepared cappuccinos and milk-based drinks. In cappuccinos and flat white, both milk and foam will be analyzed, and the values will be compared with those obtained from the same batch of milk before making cappuccino

Listed the objectives of this PhD thesis project, the activities in the 2 years are divided as follows (see table 1):

A1) Thermal stability and self-assembling properties of proteins: study of proteins contained in the milk and in cappuccinos prepared under different temperature and injected steam conditions. In particular, the stability of the casein fraction will be taken into consideration especially in relation to the possibility of the same to form the "casein tannate" in the cappuccino. The content, thermal stability and self-assembling properties of milk proteins (whey proteins and caseins) will be evaluated by electrophoretic, chromatographic (Reversed-Phase HPLC), microcalorimetric techniques (micro-Differential Scanning Calorimetry, micro-DSC), Dynamic Light-Scattering (DLS), fluorimetry.

A2) Nutritional characterization of milk in cappuccino preparations: analysis of the nutritional characteristics of the milk used and the cappuccinos produced, with particular regard to the vitamin and lipid content. The vitamin content will be evaluated

using HPLC and by spectrofluorimetric techniques, while the lipid content will be evaluated in gas chromatography.

A3) **Milk fat globules analysis**: study on the milk fat globules composition in milk and in freshly prepared cappuccinos and milk-based drinks.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Months																									
A1) <i>Thermal stability and self-assembling properties of proteins</i>																									
1) <u>Standardize procedures</u>																									
2) <u>Choice of milk</u>																									
3) <u>Milk protein analysis</u>																									
4) <u>Data processing</u>																									
A2) <i>Nutritional characterization of milk in cappuccino preparations</i>																									
1) <u>Vitamin content determination</u>																									
2) <u>Lipid content determination</u>																									
4) <u>Data processing</u>																									
A3) <i>Milk fat globules analysis</i>																									
1) <u>Milk fat globules analysis</u>																									
3) <u>Data processing</u>																									
A4) <i>Writing and Editing</i>																									

Expected impact: Production of a “high quality” cappuccinos, in which the milk keeps as much as possible unaltered its nutritional and organoleptic qualities.

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Innovative Strategies for the Mitigation of Acrylamide and Other Toxicants Content in Different Food Products

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The aim of this PhD research project is to identify, characterize and optimize traditional and innovative technological solutions for the mitigation of acrylamide and other toxicants in different cooked food products, such as: fried potatoes, bakery products and coffee.

Strategie innovative per la mitigazione del contenuto di acrilammide e altre sostanze tossiche in diverse matrici alimentari

Questo progetto di dottorato mira a identificare, caratterizzare e ottimizzare tecnologie tradizionali e innovative per la mitigazione di acrilammide e altre sostanze tossiche in diversi prodotti alimentari, quali: patate fritte, prodotti da forno e caffè.

1. State-of-the-Art

Food heat treatments are extremely important for the transformation and sanitation of many raw materials, semi-finished and formulated products which, through these processes, reach the desired levels of shelf-life and sensorial properties. Nevertheless, the same treatments (cooking, frying, roasting, etc.) are often responsible for the reduction of nutritional properties and the formation of toxic and potentially carcinogenic compounds (Rannou et al., 2016). Among them, the neurotoxic and genotoxic effects of acrylamide (AA) have been extensively highlighted. AA is formed mainly during the Maillard reactions, starting from a condensation reaction between the amino group of an amino acid (i.e. asparagine) and the carbonyl group of a reducing sugar (i.e. glucose) (Hedegaard et al., 2008).

According to the new EU Regulation (2017/2158), new “mitigation measures and benchmark levels for the reduction of AA in foods” have been established. The legislation is always more restrictive concerning the levels of AA and other toxicants allowed in food products, so interventions aimed at preventing its formation or reduction in different food matrices are an important goal, even if not always easy to reach, for the food industries.

The categories of foods in which the highest levels of this toxic compound have been found are: potato products (baked, fried), baked products (bread, biscuits, breakfast cereals, snacks) and roasted coffee (Konings et al., 2007; Rannou et al., 2016; Baskar and Aiswarya, 2018). The mechanisms and kinetics linked to the formation of AA are rather complex and influenced by numerous factors such as the concentration of precursors, pH, the content and activity of water in the matrix, process parameters, such as time, temperature, moisture and mode of heat transfer (Romani et al., 2009; Anese et al., 2013).

Depending on the type of food and process used, there are different strategies for controlling the content of AA, most of which are aimed at reducing its precursors, in order to decrease the intensity of Maillard reactions, or control of process parameters (Anese et al., 2013; Pedreschi et al., 2014; Rannou et al., 2016). However, the strategies designed for the AA reduction are still to be clarified and investigated because they are

not always effective on all products, not always easy to apply on an industrial scale and many of them negatively influence the sensory proprieties in the finished product (Pedreschi et al., 2014).

The application of biotechnologies and emerging non-thermal technologies can significantly contribute to controlling and reducing the formation of AA in many foods, bringing various advantages, including the maintenance of organoleptic characteristics. For example, pre-treatments with pulsed electric fields (PEF) can contribute to increase the mass transfer processes and therefore the leaching of Maillard reaction substrates reducing the AA formation in the subsequent cooking step (Lebovka and Vorobiev, 2010). Moreover, the application of yeast pre-treatments can deplete the AA precursors due to yeast activity (Di Francesco et al., 2019). The studies addressing the application of these technologies for the elimination/reduction of AA in food are very limited or even absent, therefore in order to optimize their application an in-depth study is needed.

The healthiness and quality of food products are indispensable requisites and for this it is necessary to adopt strategies able to reduce the presence of toxic compounds in the finished products.

2. PhD Thesis Objectives and Milestones

Overall, the present PhD thesis project aims to:

- provide a more in-depth knowledge of the aspects still poorly studied on the application of traditional and innovative technological treatments or interventions for the control of the AA formation and reduction in the most interesting foods;
- verify the synergistic efficacy of traditional and innovative mitigation treatments;
- select, based on the results obtained, the best strategies in the different food groups due to low AA levels and peculiar qualitative characteristics (color, taste, aroma, texture);
- optimize the use of the most promising treatments studied, through the definition of process parameters suitable for industrial applications.

This PhD project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Bibliographic research.

A2) **Study of innovative treatments for the mitigation of AA in fried potato products**, such as: pre-treatment with yeasts and pulsed electric fields.

A3) **Study of traditional and innovative technological interventions for the mitigation of AA in roasted coffee**, such as: selection of species and varieties, control of roasting conditions and brew preparations.

A4) **Study of traditional and innovative technological interventions for the mitigation of AA in bakery products**, such as: modification of formulation, control of fermentation, control of heat parameters and application of innovative cooking (i.e. steam cooking).

A5) Scientific posters, papers and thesis preparation.

Table 1 Gantt diagram for this PhD research project.

Activity /	Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1) Bibliographic research																			
A2) Study of innovative treatments for the mitigation of AA in fried potato products																			
1) Selection and optimization of process parameters																			
2) Evaluation of the effect of treatments on the characteristics of the food																			
A3) Study of traditional and innovative technological interventions for the mitigation of AA in roasted coffee																			
1) Selection and optimization of process parameters																			
2) Evaluation of the effect of treatments on the characteristics of the beverage																			
A4) Study of traditional and innovative technological interventions for the mitigation of AA in bakery products																			
1) Selection and optimization of process parameters																			
2) Evaluation of the effect of treatments on the characteristics of the food																			
A5) Scientific posters, papers and thesis preparation																			

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The Role of Emotions, Personality traits and Sensory Sensitivity in Food Preferences in Preadolescents

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This PhD thesis research project is aimed at investigating preadolescents' food preferences by examining the role of emotions, personality traits and sensory sensitivity. This PhD thesis is part of the project Edulia, which received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 764985.

Il ruolo delle emozioni, dei tratti della personalità e della sensibilità ai gusti nel determinare le preferenze alimentari dei bambini (preadolescenti)

Questo progetto di tesi di dottorato ha lo scopo di indagare il ruolo delle emozioni, dei tratti della personalità e della sensibilità ai gusti nelle preferenze alimentari dei preadolescenti con particolare attenzione alle differenze individuali. Questo progetto di tesi di dottorato fa parte del progetto Edulia, che ha ricevuto finanziamenti dal programma di ricerca e innovazione Horizon 2020 dell'Unione europea nell'ambito della convenzione di sovvenzione Marie Skłodowska-Curie n. 764985.

1. State-of-the-Art

Within the last years, children were recognized as an important consumer group that influences household decisions including food purchases. To develop and maintain healthy eating habits, it is important to understand influencing factors and the main drivers of children's food preferences. Traditional methods to determine children's food preferences mainly focused on the evaluation of hedonic measurements of food products. But more recently, emotions have been shown to play an important role in children's food preferences as they can help to discriminate between food products compared to the sole use of liking measurements and to better predict actual food choices. Until now, only a limited number of studies investigated children's food-elicited emotions (Table 1) (De Pelsmaeker, Schouteten, & Gellynck, 2013; Gallo, Swaney-Stueve, & Chambers, 2017; Schouteten, Verwaeren, Gellynck, & Almlí, 2019; Schouteten, Verwaeren, Lagast, Gellynck, & De Steur, 2018; Swaney-Stueve, Jepsen, & Deubler, 2018).

Self-reported questionnaires have been the most common method for the evaluation of emotional responses to food products (De Pelsmaeker et al., 2013; Gallo et al., 2017; Jervis, Jervis, Guthrie, & Drake, 2014; Schouteten et al., 2019, 2018). However, researchers expressed the urgent need for the improvement of emotion measurements and the development of new age-appropriate emotion measurement tools. Recently, emojis were suggested to describe emotional profiles of food products and were shown to be able to discriminate between food products in preadolescents (Gallo et al., 2017; Schouteten et al., 2019, 2018; Swaney-Stueve et al., 2018) and to predict children's food choices opposed to only measuring overall liking (Schouteten et al., 2018). The use of emojis seems to be an easy and intuitive tool, which is especially useful in children with reduced verbal skills and limited vocabulary. Emojis imply a game-like situation and can also be an advantage for shy children.

Although emojis are already commonly used on several social media platforms among children, their meaning still needs to be clarified and it should be demonstrated if they can measure emotions or broader dimensions of product experience (Gallo et al., 2017; Schouteten et al., 2019, 2018; Swaney-Stueve et al., 2018).

Table 1 Summary of methods used for studying food-related emotions in preadolescents

Method		References
Emotion words (CATA)	9 positive, 9 negative, 2 neutral	De Pelsmaecker et al. 2013
Emotion words (CATA)	12 emotion words	Jervis et al. 2014
Emotion words and emojis (CATA)	28 words and 28 emojis	Gallo et al. 2017
Peryam and Kroll scale vs emojis	P&K (7 items) and 7 emojis	Swaney-Stuewe et al. 2018
Emojis (CATA)	33 emojis (positive, negative, neutral and unclassified)	Schouteten et al. 2018
Emojis (CATA)	Standardized (38 emojis) vs product-specific (29 emojis) method	Schouteten et al. 2019

CATA: Check-All-That-Apply; P&K: Peryam and Kroll scale

2. PhD Thesis Objectives and Milestones

The PhD thesis project focuses on preadolescents (9-13 years) collecting data from schools in Italy and Norway for a cross-cultural comparison with 200 preadolescents in each country. Within the overall objective mentioned above, the project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) Development of a food-related emotion lexicon using emojis and emotion words to (1) identify food-related emotions in different food contexts and to (2) explore the meaning of food-related emojis (and words) by using projective mapping techniques, word association and a modified version of Repertory Grid Method interviews (EmoSemio, Spinelli et al. 2014).

A2) Testing the emotion measurement tool in a real food-context to (1) test its ability to discriminate between food products in a specific food context. (2) Also, food-related individual differences will be investigated to understand their role in emotions by measuring personality traits (food neophobia, sensation seeking and sensitivity to reward and punishment) and sensory sensitivity (fungiform papillae density and *6-n-Propylthiouracil*).

A3) Academic research project (6 months) at Nofima (Ås, Norway) to conduct a cross-cultural study on the development of a similar emotion measurement tool to test if Norwegian preadolescents interpret and use food-related emotions similarly.

A4) Applied research project with Sammontana (3 months), directed to the exploration of emotion measurement approaches in connection to a product development project (e.g. reduced sugar, healthier ice-cream).

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months	1	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1) Development of an emotion measurement tool																			
1) Selection of food-related emoji and emotion words																			
2) Exploring the meaning of emojis and emotion words																			
A2) Testing the emotion measurement tool in a real food-context																			
1) Testing on real food products in a specific food context																			
2) Testing individual differences (personality traits and sensory sensitivity)																			
A3) Academic research project at Nofima, Norway																			
A4) Applied research project with Sammontana																			
A5) Thesis and Paper Preparation																			

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Development of Integrated, Multidimensional Approaches for Food Safety Assessment, with Special Focus on Contaminants from Food Packaging

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This PhD thesis research project is aimed on the development of integrated and multidimensional analytical strategies for assessing targeted food contaminants, among which mineral oils and other hydrocarbon contaminants such as oligomers from polyolefins, whose presence in food is mainly due to migration from food packaging.

Sviluppo di approcci integrati e multidimensionali per la valutazione della sicurezza alimentare, con particolare attenzione ai contaminanti provenienti dagli imballaggi alimentari

Questo progetto di ricerca di tesi di dottorato è finalizzato allo sviluppo di strategie analitiche integrate e multidimensionali per la valutazione di contaminanti alimentari specifici, tra i quali oli minerali e oligomeri delle poliolefine, la cui presenza negli alimenti è dovuta principalmente alla migrazione dagli imballaggi alimentari.

1. State-of-the-Art

Food contamination can occur at any stage of food production, such as in field (environmental contamination), during farming practices, food transformation processes (industrial and domestic), and migration from packaging materials.

Food contact materials (FCMs) comprise a variety of materials and articles intended to come into contact with food during its manufacture, storage, preparation and consumption. According to EU Regulation (1935/2004/CE), any material or article intended to come into contact with food must not endanger human health, that means that it must not transfer components that may impact consumer health or cause unacceptable changes of the composition or organoleptic deterioration.

Especially when analyzing contaminants in traces, the analytical determination must be preceded by appropriate sample preparation to isolate the analytes of interest and eliminate any interference. Furthermore, a great attention has to be posed to avoid potential cross-contamination, during the analytical steps. In recent years efficient and rapid extraction techniques involving microwave heating or heating at high temperature and pressure have been successfully applied (Moret et al., 2014; Moret et al., 2016; Purcaro et al., 2009).

The contamination of food with mineral oil hydrocarbons (MOH) is a very long-running concern, and from an analytical point of view, reliable quantification of these contaminants is very hard.

An important source of MOH in foods is migration from food packaging made of recycled board. MOH are very complex mixture of isomers mainly related to two classes of compounds, namely mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH), with different toxicological relevance: the former are of concern due to their bioaccumulation potential, while the latter may include suspected carcinogens and genotoxic substances (EFSA, 2012).

When there is a doubt of interference by matrix compounds or by hydrocarbons of synthetic origin (POH, PAO), more complex multidimensional chromatographic techniques able to distinguish MOH from other hydrocarbon compounds, are required. Presently, the most powerful approach for MOH verification and characterization is comprehensive two-dimensional GC (GCxGC) (Biedermann & Grob, 2015). Packaged food can be also contaminated with hydrocarbons of synthetic origin such as poly-alpha-olefins (PAO), mainly from hot melts adhesives and synthetic lubricants, and polyolefin oligomeric hydrocarbons (POH) from PP and PE materials used as primary packaging to direct contact with the food (Barp et al., 2015; Biedermann-Brem et al., 2012; Lommatzsch et al., 2016).

According to the objectives mentioned above, this PhD thesis can be divided into the following activities and periods, reported also in Gantt diagram presented in Table 1.

A8) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1) Bibliographical research																			
A2) Choice of different samples to be analyzed																			
A3) Develop and validate matrix-tailored sample preparation protocols																			
A4) Develop and validate integrated and multidimensional analytical strategies																			
A5) Obtain occurrence data on the presence of targeted contaminants in food																			
A6) Investigate in deep the 'MOAH fraction'																			
A7) Perform an in-depth characterization of polyolefins intended for contact at high temperature																			
A8) Thesis and Papers preparation																			

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The Effect of Formulation and Processing on Pasta Quality

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Pasta industry is moving towards the implementation of legumes in pasta formulation to increase nutritive value and health qualities of pasta. The proper choice of ingredients and processing conditions may increase the nutritional value of legume containing pasta. This PhD thesis research project will address the effect of legumes flour characteristic (germinated/non germinated; particle size; cell intactness; high hydrostatic pressure treated) and processing (pasta shape) on pasta's chemical composition, physical and nutritional properties and sensory features. Both gluten containing and gluten free pasta will be considered.

Effetto della formulazione e del processo produttivo sulla qualità della pasta

L'industria della pasta si sta orientando verso l'aggiunta di legumi nella formulazione della pasta per aumentarne il valore nutritivo e le qualità salutistiche. La corretta scelta degli ingredienti e del processo produttivo possono essere ottimizzati per aumentare il valore nutrizionale della pasta contenente legumi. Questo progetto di ricerca di dottorato riguarderà l'effetto delle caratteristiche della farina di legumi (germinata/non germinata, granulometria, integrità cellulare; pretrattata con alta pressione idrostatica) e del processo (formato della pasta) sulla composizione chimica, proprietà fisiche e nutrizionali e caratteristiche sensoriali del prodotto. Saranno considerate paste con e senza glutine.

1. State-of-the-Art

Pasta industry is undergoing important innovation with a wide variety of new formulations aiming at increasing protein, fiber, and micronutrients content and, in the gluten-free niche, also to reduce the glycemic impact of the product. Large attention has been devoted to the implementation of legumes in the diet, because they are rich in protein, resistant starch, dietary fiber, vitamins, minerals, and phytochemicals which contribute to stimulating bacteria beneficial to human organism (Raigond et al., 2015) and they are considered a sustainable crop as they have low water, soil, and fertilizers footprint (Boukid et al, 2019). By adding legume flour, the lightness and lysine content of pasta decreased, and about +38% and +48% increase in firmness is observed in 35% faba or split pea of fortified pasta (Petitot, 2010). What's more, it has been proved that adding legume flour increases the protein, dietary fiber content and decreased the available carbohydrate contents and moisture content of pasta. With adding legume flour in different proportions, the in-vitro protein digestion, resilience, and cooking loss are improved or decreased depending on the proportion of legume. Potentially available starch and its hydrolysis index (HI) decreased and resistant starch (RS) increased compared to control pasta. The predicted glycemic index is lower in spaghetti added with chickpea flour (Osorio-Díaz et al., 2008).

Different strategies may be used to increase the quality of legume containing pasta. Sprouting may further improve nutritional attributes of legumes, depending on the germination conditions, that affects the content of nutrients (micro- and macro-components, such as phytic acid, dietary fiber and antinutrient factors like trypsin and chymotrypsin inhibitors (Frias et al., 1995). Particle size and cell intactness of legumes' flour used in the formulation may have an important impact in modulating nutrients

availability as, for example, in modifying starch digestibility (Capuano and Pellegrini 2019; Boukid et al., 2019). High hydrostatic pressure was shown to be an effective treatment in reducing anti-nutrients (β -ODAP and IP6) content in *Lathyrus sativus* (Buta et al., 2019). For the analysis of pasta characteristics, the texture, color and cooking quality will be evaluated following Carini et al., (2014). Sensory evaluation of cooked pasta will be carried out using both an acceptability test and a rapid profiling method check-all-that-apply (CATA) test. The protocol described by Englyst et al. (2000) will be used for starch digestion and resistant starch assay. All data will be analyzed and elaborated with statistical analysis. The nutritive value and bioactive compounds will be measured following (Laleg, K. et al., 2016).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Experimental set up In this stage, the proper characterization methods on pasta are identified including the determination methods of pasta physical characteristics, nutrients analysis, digestion ability, and sensory tests (A1.2). Study characteristics of series Italian commercial pasta with different legumes (A1.3).

A2) Effect of pasta shape In the second stage of this work, the effect of different shapes of commercial pasta with the same legume proportion on sensory acceptability, physicochemical, and nutritional properties will be identified (A2.1).

A3) Effect of sprouting and flour particle size In this stage, we are going to look for suitable legume seed for germination (A3.1). Further more, the effect of different sprouting conditions on all components will be determined for future optimization of germination condition (A3.1). To assess whether and how the different legume flour particle size affects pasta quality to optimize milling/classification method (A3.2).

A4) Effect of high-pressure processing (HPP) on anti-nutrients content and pasta properties made with HPP legume flour.

A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Experimental set up																								
	1) Literature review																								
	2) Identification, set up and validation of analytical methods																								
	3) Study of the Italian pasta with legumes market																								
A2)	Effect of pasta shape																								
	1) Sensory, physico-chemical, nutritional analysis																								
A3)	Effect of sprouting and flour particle size																								
	1) Determine the suitable legume seed and optimal germination processes																								
	2) Develop different flour particle size																								
	3) Sensory, physico-chemical, nutritional analysis																								
A4)	Effect of HPP																								
	1) HPP treatment of legume flours and their characterization																								
	2) Sensory, physico-chemical, nutritional analysis																								
A5)	Thesis and Paper Preparation																								

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Food Digestibility: a Physiological Approach for New Product Development

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Gastro-intestinal discomforts (GID) induced by some foods, such as milk or gluten containing products, are widely distributed among general population. GID often lead sensitive people to self-diagnosis of allergy/intolerance and exclusion from the diet of those foods perceived as “few digestible”. That behaviour causes incorrect dietary choices and increase the individual risk of unbalanced intake of several nutrients and related metabolic diseases. The aim of the present PhD project is to identify physiological mechanisms eliciting GID onset in healthy people in order to develop food tailored on sensitive population to reduce and/or resolve the GID and prevent metabolic disease.

Digeribilità degli alimenti: un approccio fisiologico per lo sviluppo di nuovi prodotti.

I disturbi gastro-intestinali (GID) indotti da alcuni alimenti, come prodotti contenenti latte o glutine, sono molto frequenti nella popolazione generale. I GID spesso inducono la popolazione suscettibile ad un'auto-diagnosi di allergia/intolleranza e all'esclusione dalla dieta di quegli alimenti percepiti come “poco digeribili”. Questo atteggiamento comporta un'alimentazione non corretta, incrementando il rischio individuale di un introito sbilanciato dei diversi nutrienti e di disturbi metabolici associati. Lo scopo di questo progetto di dottorato è l'identificazione dei meccanismi fisiologici implicati nell'insorgenza dei disturbi gastro-intestinali nella popolazione sana, per lo sviluppo di prodotti *ad hoc* che possano ridurre/risolvere tali disturbi e prevenire malattie metaboliche.

1. State-of-the-Art

Nutrition plays a key role in the development of chronic diseases, in fact an incorrect dietary pattern is associated with the majority of chronic diseases such as coronary heart disease, stroke and type 2 diabetes.

Functional dyspepsia may be a possible cofactor for the onset of metabolic and chronic diseases. It is defined as a debilitating gastro-intestinal disorder characterised by early satiety, post-prandial fullness or epi-gastric pain, generally related to food intake (Duncanson et al., 2018). This condition affects 10-40% of Western population (Mahadeva and Ford, 2016) and influences dramatically the quality of life, anxiety and stress of sensitive people. In absence of a diagnosis many people suffering from gastro-intestinal discomforts (GID) after consuming some food, believes it is “few digestible” and in order to relief from those discomforts, often reduces or even excludes from the diet that food. This behaviour increases the risk of deficiency of some nutrients and/or excess of others thus causing unbalanced nutrition that may increase the risk of metabolic and chronic diseases.

For instance, GID induced by milk or cereal consumption are very frequent among population and they are often associated with self-diagnosis of lactose intolerance or gluten sensitivity. The exclusion of milk, dairy products and gluten-containing food from the diet may induce Calcium, Vitamin D, some B vitamins deficiency and, consequently, related pathologies. Although GID are widespread in healthy people, they are still under-investigated.

Recent evidence indicated that milk-induced GID may be due to a group of milk peptides deriving from the proteolysis of A1 β -casein (but not of A2 β -casein), particularly β -casomorphin-7 (BCM-7) (Jianquin et al, 2015; Deth et al., 2015, He et al., 2017). BCM-7 is able to bind μ -opioid receptors, which are widely expressed in the gastrointestinal (GI) tract, regulating motility, mucus production and hormone production (Zoghby et al., 2006). On the other hand, gut permeability, together with the activity of the brush border enzymes, influences what and how much of dietary nutrients, such as lactose and peptides, cross the intestinal mucosal barrier. Once absorbed some peptides can influence the intestinal transit time and, in turn, digestive processes and inflammatory responses (Teschemacher, 2003; Pimentel et al. 2017); this is perfectly in line with the known implications of gut permeability on the genesis of some metabolic disease caused by intestinal dysbiosis (Cani, 2016).

Mounting evidence indicates that also Endocannabinoids (ECs) and N-acyl ethanolamines (NAEs) can be involved in physiological mechanisms underpinning GID. Indeed, these lipid mediators have a pleiotropic activity in the body and modulate several biological pathways including appetite cues, food intake, macronutrient metabolism, pain sensation and immunity (Witkamp, 2018). Interestingly, in our laboratory, 43 food products were analysed and they were demonstrated to be, at a different extent, sources of ECs, NAEs and NAEs (De Luca et al., 2019_revision submitted to Food Chem).

The aim of this PhD project is to clarify the physiological mechanisms underpinning GID in order to develop tailored food products or diets that may ameliorate GI and overall wellbeing of sensitive people. To achieve the objectives, the project will focus on food protein digestibility, gut permeability and gut microbiota composition/activity, as well as, on ECs and ECs-like molecules.

2. PhD Thesis Objectives and Milestones

The overall objective will be achieved through the intermediate objectives and activities listed below and according to the Gantt diagram given in Table 1:

- A1) **Study of milk digestibility**, in GID sensitive and healthy subjects.
- A2) **Study of NAEs/ECs bioavailability in different categories of people** following different diets.
- A3) **Design and development of food products tailored on GID-sensitive people.**
- A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) <i>In vivo</i> milk digestibility																									
A2) <i>In vivo</i> NAEs/ECs bioavailability in different categories of people																									
A3) Design and development of highly digestible food products																									
A4) Writing and Editing																									

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Feasibility Studies and Engineering of Optical Simplified and Stand-alone Instruments for Agro-Food Applications

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The goal of this PhD thesis is the development of simplified, customized and stand-alone Visible and Near-Infrared (vis/NIR) instruments for applications in agro-food chain. These tools will be used to satisfy the needs of the companies to monitor (in-line) in real time (analyses directly in field) in a non-destructive way the ripening of horticultural products (e.g. grapes) and the transformation during the production process in a view of Industry 4.0.

Studi di fattibilità e ingegnerizzazione di strumentazione ottica semplificata e autonoma per applicazioni agroalimentari

Lo scopo di questa tesi di dottorato verte allo sviluppo di sistemi ottici semplificati per specifiche applicazioni in ambito agro-alimentare. Pertanto, verranno utilizzati strumenti ottici che lavorano nel range del visibile e vicino infrarosso (vis/NIR) in modo da soddisfare la necessità delle singole aziende, di un monitoraggio (in-line) direttamente in campo e in linea produttiva per ottenere informazioni, in tempo reale e in modo non distruttivo, sull'andamento del processo di maturazione di prodotti orticoli (es. uva) e per quanto riguarda processo di trasformazione in azienda in un'ottica di Industria 4.0.

1. State-of-the-Art

The vis/NIR radiation covers the range of the electromagnetic spectrum between 400 and 2500 nm. In vis/NIR spectroscopy the product is irradiated with light and the reflected, transmitted or absorbed light is measured (Nicolaï et al., 2007). While the radiation penetrates through the product, its spectral characteristics change due to scattering and absorption processes related to the chemical composition of the product.

The processing of spectral data obtained with vis/NIR devices requires a multivariate statistical approach for extracting useful information from the acquired signals, this is necessary because wavelength-dependent scattering effects, instrumental noise, ambient effects, and other sources of variability may affect the spectra. In order to relate the spectra (independent variables) to specific quality parameters (dependent variables) a regression method is required (Malegori et al., 2017).

The vis/NIR spectroscopy, thanks to its application versatility, has been used in different fields (agriculture, food and feed, chemistry, polymers, petrochemistry, pharmaceuticals, medicine, environment, ecology, geology, forensic analysis etc.). During the harvest, post-harvest and food processing this technique is well used to predict crucial quality parameters (Table 1).

Spectra of intact food samples can be measured in few seconds without any sample preparation and expert personnel trained. Compared to this technology, chemical techniques are time-consuming, require sample preparation and the use of chemical reagents which, often, are not sustainable for the environment (Casson et al., 2019).

However, especially in the agro-food sector, the limited adoption of NIR technology can be attributed to the costs and technical limitations (Magwaza et al., 2012). Therefore, in order to support the small companies, the research is moving towards simplified, easy

to use and low-cost systems for real-time assessment of the qualitative parameters even directly in the field with stand-alone instruments.

Thus, this PhD thesis project will aim to design, build and test miniaturised low-cost and user-friendly devices to support small-scale growers in determining the optimal moment of harvest and assess the qualitative parameters of food products in a view of Industry 4.0 completely inter-connected.

Table 1 Some applications of vis/NIR and NIR spectroscopy in harvest, post-harvest and food processing.

Application	Matrix	Parameter	Wavelength (nm)	R ² / Accuracy (%)	RMSE	Reference
Harvest	<i>Agaricus bisporus</i>	Hardness	400-1,000	R ² =0.78	8.87	(Giovenzana et al, 2019)
		Moisture		R ² =0.78	2.14	
		Solid Soluble		R ² =0.65	1.32	
Post-Harvest	Red Must White Must	Infected/Not infected	430-1,650	Accuracy=81.3		(Giovenzana et al, 2018)
				Accuracy=87.5		
Food Processing	Roasted Coffee	Moisture	950-1,650	R ² =0.87	0.09	(Tugnolo et al, 2019)
	Ground Coffee	Moisture		R ² =0.98	0.02	
		Tap Density		R ² =0.58	2	
		Granulometry		R ² =0.55	0.9	

R²: coefficient of determination; RMSE: root mean square error

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) Identification of the food matrix and the relative quality parameters to be assessed in order to meet the real needs of the market in a view of Industry 4.0.

A2) Experimental phase with optical measurements (with a vis/NIR spectrophotometer) and chemical analyses of the most important quality parameters (A2.1). Subsequently, the data will be processed in order to build predictive or classification models and to select the most relevant wavelengths (A2.2).

A3) Definition of component specs from the specific LEDs, photodiodes and filters (A3.1) to the identification of the materials to be used and the optoelectronic components to be implemented for the construction of the prototype (A3.2).

A4) Experimental phase with optical measurements (with a vis/NIR spectrophotometer and prototype) and chemical analyses in order to obtain a wide dataset to be used for the phase of calibration and validation of the models (A4.1). Moreover, a phase of comparison between the commercial vis/NIR spectrophotometer and the prototype (A4.2) will follow.

A5) Writing and Editing of scientific papers (A5.1), oral and/or poster communications at conferences and PhD workshops (A5.2) and the PhD thesis (A5.3).

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	Matrix and quality parameters identification																								
A2	Experimental phase																								
1)	vis/NIR and chemical analyses																								
2)	models building and wavelengths selection																								
A3	Component spec.																								
1)	optical components																								
2)	electronic and materials definitions																								
A4	Experimental phase																								
1)	optical and chemical analyses																								
2)	performance comparison																								
A5	Writing and Editing																								
1)	scientific papers																								
2)	communications																								
3)	PhD thesis																								

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Interaction Studies of Closure System for Winery Products

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Companies producing closures for winery products have to be certain these closures meet the requirements of the European Union framework directive on materials and articles intended to come into contact with food, i.e. EU regulations n. 1935/2004 and n. 10/2011. Thus, the aim of this PhD project is to evaluate the effect of different closure types over storage on the volatile and non-volatile composition of grappa, a byproduct of wine production obtained by distillation of fermented grape pomace. In addition, the sensory quality of grappa will be evaluated by a trained panel. Most of the scientific literature reports the data on the effect of stoppers on wine, but results related to distilled beverages are scarce.

Studi sull'interazione di sistemi di chiusura per prodotti della cantina

Le aziende che producono tappi per i prodotti della cantina devono assicurare che questi tappi soddisfino i requisiti dell'Unione europea sui materiali e gli oggetti destinati a venire a contatto con i prodotti alimentari, in particolare i regolamenti UE n. 1935/2004 e n. 10/2011. Pertanto, il progetto di questa tesi di dottorato di ricerca, prevede lo studio dell'effetto del tipo di chiusura e della conservazione sulla composizione in sostanze volatili e non volatili in un distillato come la grappa, un prodotto collaterale della produzione del vino ottenuto dalla distillazione delle vinacce. Inoltre, la qualità sensoriale della grappa verrà valutata da un panel esperto. Esistono infatti molti dati scientifici sull'effetto dei tappi sul vino, ma pochissimi sui distillati alcolici.

1. State-of-the-Art

Closure systems ensure the sealing and a proper conservation of winery products. During the storage, a gradual evolution of the alcoholic beverage into the bottle occurs in an environment with a very low oxygen content. If storage is prolonged over time, it can promote numerous physical and chemical processes between the components of the distilled beverage which could be influenced by the quality of the closure system (Varea et al., 2001; Skouroumounis et al., 2005; González-Adrados et al., 2008; Azevedo et al., 2014). For instance, condensation between organic acids and alcohols lead to esters with fruity and floral aromas, which render the 'bouquet' less rough and more smooth; or oxidation when the closure is too permeable to air.

The hermetic quality ensured by a stopper is not only indispensable for the maturation of the alcoholic beverage but is also necessary for winery products that will be consumed more quickly. Natural cork stoppers enable a good conservation of winery products if the molecules responsible for the cork taint do not contaminate them; in addition, they can be subjected to dehydration and thus loss of their sealing properties in the specific case of distilled beverages. On the other hand, technical stoppers are claimed to be chemically stable, mechanically resistant and to behave very well under the torsion to which they are submitted when bottling and uncorking. In addition, these stoppers have proven to be excellent over time (Godden et al., 2001; Silva et al., 2003; Marin et al., 2007), preventing the development of unpleasant aromas. Therefore, the choice of

closure type is crucial for the quality preservation of distilled beverages from the winery. There are several studies evaluating the impact of different closures and storage conditions on the development of wine (Chatonnet et al. 2000, Mas et al. 2002). However, only very few studies are available in literature concerning the effect of (cork or technical) stoppers on distillates, and practically no studies on grappa.

This PhD project aims to determine the effects of the closure type (microgranulated cork, two different synthetic stoppers, a blend of sanitized micro-granules of natural cork and polymers with no glue) on the development of volatile and non-volatile compounds and sensory profile during the storage of the bottles of grappa. In addition, the focus will be the elucidation of the compositional and sensory quality between grappa sealed under different closures and shipped abroad to evaluate the effect of temperature changes and mechanical acceleration on the chemical and sensory evolution of the product. The study is an ongoing collaboration with a local grappa producer (Distillerie Roner, Termeno, BZ) and a company producing the stoppers for commercial alcoholic beverages (Supercap s.r.l., Mombaroccio, PU).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Definition of the subject and **literature review** to achieve deeper knowledge about the PhD topic.

A2) **Instrumental** and **software training** based on the main analysis techniques.

A3) **Enrolling courses** at the University of Bolzano during the first year to obtain a certain amount of credits (mandatory and optional courses as part of the PhD program).

A4) **Attending seminars, workshops, summer/winter schools, national and international conferences** (preparation for poster presentations and oral communications).

A5) **Research activity** (Laboratory experiment, sensory analysis and statistical analysis).

A6) **Period abroad for 6 months** (the exact period will be decided and will take place after reaching a good level of technical skills at the UNIBZ and NOI TECHPARK laboratories).

A7) **Writing and editing scientific papers.**

A8) **Writing PhD thesis.**

Table 1 *Gantt diagram for this PhD thesis project.*

Activity /	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Literature review																									
A2) Instrumental training																									
A3) Courses																									
A4) Seminars and schools																									
A5) Research																									
A6) Period abroad																									
A7) Writing scientific papers																									
A8) Writing PhD thesis																									

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Harmonized Procedures of Analysis of Medical, Herbal, Food and Industrial Cannabis: Development and Validation of Cannabinoids Quality Control Methods and of Extraction and Preparation of Derivatives from the Plant Raw Material

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This PhD thesis research project is aimed at developing analytical methods for the qualitative and quantitative determination of bioactive compounds naturally present in *Cannabis sativa* L., in particular cannabinoids and terpenes. The research project also aims at validating and starting the certification procedure of at least one analytical method related to the determination of cannabinoids in cannabis. Furthermore, another goal of this PhD research project will be the development of extraction techniques and preparations of derivatives from cannabis raw material, according to product destination and given the high potential in different industrial sectors.

Metodiche di analisi di derivati della cannabis ad uso alimentare, fitoterapico, farmaceutico ed industriale: messa a punto e validazione di metodi per il controllo di qualità e sviluppo di tecniche estrattive e preparative di derivati, secondo destinazione merceologica

Questo progetto di tesi di dottorato è finalizzato allo sviluppo di metodi analitici per la determinazione qualitativa e quantitativa dei composti bioattivi naturalmente presenti in *Cannabis sativa* L., in particolare cannabinoidi e terpeni. Il progetto di ricerca mira anche a validare ed avviare la procedura di certificazione di almeno un metodo analitico relativo alla determinazione dei cannabinoidi nella cannabis. Inoltre, ulteriore obiettivo di questo progetto di ricerca di dottorato sarà lo sviluppo di tecniche di estrazione e di preparazione di derivati della cannabis, in base alla destinazione merceologica, visto il potenziale in differenti settori industriali.

1. State-of-the-Art

Recently, in Italy the interest in *Cannabis sativa* L. has increased mainly due to the latest legislation (Legge n. 242 del 2 dicembre 2016). According to this law, the Italian regulatory framework classified two types of *Cannabis sativa* L. depending on the content of Δ^9 -tetrahydrocannabinol (Δ^9 -THC). In particular, fiber-type plants of *Cannabis sativa* L., also called “hemp”, are characterized by a low content of Δ^9 -THC ($<0.2\%$ w/w). If the content of Δ^9 -THC is $>0.6\%$ w/w it is considered as drug-type, also called “therapeutic” or “marijuana”. As a consequence, there is a request to develop cost effective and easy to use quantitative and qualitative methods for analysis of cannabinoids.

To date, more than 500 different chemical components have been reportedly found in cannabis, 100 of which were identified as cannabinoids, the main bioactive principles of the plant and, as regards some cultivars, up to 100 can be terpenes and terpenoids (Leghissa et al., 2018a). Although, until now, there is not yet a complete list of terpenic compounds in cannabis (Leghissa et al., 2018a), they are found in the volatile component of the plant and have been shown to have a synergistic action with cannabinoids (Citti et al., 2018).

Therefore, the development of methods able to determine the greatest number of cannabinoids and of methods that can detect terpenes is even more important. In fact, as a consequence of the increased development of medicinal preparations based on cannabis, there is also a growing demand for the development of qualitative and quantitative methods for the analysis of the bioactive components of this plant (Citti et al., 2018).

Gas chromatography has demonstrated a good sensitivity in the determination of total cannabinoids, both neutral and the related acid precursors (Cardenia et al., 2018; Leghissa et al., 2018b), although derivatization is needed to properly quantify acid cannabinoids (Radwan et al., 2017). The HPLC analysis does not require the step of derivatization, but it has the disadvantage of not always having a resolution suitable for the identification of the different cannabinoids (Radwan et al., 2017); moreover, tests were made in relation to the different detectors (Citti et al., 2018; Leghissa et al., 2018a). In order to separate, identify and quantify the compounds of interest from the plant material and obtain a high recovery of these components, the extraction technique applied in the preparation of the sample is also fundamental (Brighenti et al., 2017). Therefore, in addition to the development of robust and sensitive chromatographic methods capable of analyzing this matrix, it is necessary to develop efficient methods to extract the main compounds and those present in traces. The plant of *Cannabis sativa* L. is, in fact, characterized by the presence of many different compounds that make it a very complex matrix, considering also the many varieties of species and all the hybridized strains. Also, for this reason, the standardization and validation of protocols for both the analysis and the production of extracts and other derivatives from this plant is very challenging (Aizpurua-Olaizola et al., 2014).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) Bibliographic research

A2) Development of analytical methods for the determination of cannabinoids, in particular at least two analytical protocols for the determination of cannabinoids, one of which fast, cost-efficiently and applicable at industrial level, and the other one able to determine the presence of at least 10 cannabinoids. Furthermore, one of these two methods will be validated, and the certification procedure will be started.

A3) Development of preparative and extractive procedures for the production of derivatives, depending on the product destination, given the potential in the phytotherapeutic, food, pharmaceutical, cosmetic and, more in general, in the industrial fields. For example, the cold extraction of hemp seed oil or the co-milling of hemp seed and olive, and the related characterization and stability studies.

A4) Development of analytical methods for the determination of minor compounds naturally present in *Cannabis sativa* L., in particular for the qualitative and quantitative determination of terpenic compounds.

A5) Writing and editing of the PhD thesis, scientific papers, oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	Bibliographic research																		
A2)	Development of methods for the determination of cannabinoids																		
	1) Sample selection																		
	2) Development of two analytical methods																		
	3) Validation and start of the certification procedure																		
A3)	Development of preparative and extractive procedures																		
	1) Sample selection																		
A4)	Development of analytical methods for the determination of minor compounds																		
	1) Sample selection																		
	2) Development of a method for the determination of terpenic compounds																		
A5)	Preparation of the thesis and other scientific contributions																		

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POSTER COMMUNICATIONS

Mixed Starter Yeasts as Biotechnological Tool to Produce Wine with Low Alcoholic Content

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The aim of this PhD project is the selection of a mixed starter culture able to produce wine with lower ethanol content and enhanced aromatic characteristics. At this aim twenty-nine strains of non-*Saccharomyces* species were tested for physiological traits, such as resistance to antimicrobial compounds, growth capacity in presence of ethanol and high sugar concentration, β -glucosidase activity, resistance to oxidative stress. Strains possessing the best combination of these traits will be selected for further characterization.

Lieviti starter misti come strumento biotecnologico per la produzione di vino a basso tenore di alcol

Lo scopo di questo progetto di tesi di dottorato è la selezione di uno starter misto per la produzione di vini a ridotto tenore alcolico, ma con caratteristiche aromatiche complesse. A questo scopo, 29 ceppi di lieviti non-*Saccharomyces*, sono stati saggiati per caratteri fisiologici, come resistenza a composti antimicrobici, capacità di crescita in presenza di etanolo e alte concentrazioni di zuccheri, attività β -glucosidasi, resistenza allo stress ossidativo. I ceppi in possesso di della migliore combinazione di queste caratteristiche saranno selezionati per le prove di caratterizzazione successiva al fine di individuare i ceppi con migliori proprietà di interesse enologico e potenzialmente idonei ad essere impiegati in colture miste per la produzione di vino a ridotto tenore alcolico.

Key words: Oenological traits, non-*Saccharomyces* yeasts, oxidative fermentative metabolism, ethanol reduction.

1. Introduction

According to PhD thesis project previously described (Alberico, 2018), this poster reports the main results of the first research phase (A1), aimed to select the most suitable strains to be used as mixed starter. The main activities were:

A1), A2) Screening of non-*Saccharomyces* yeasts (belonging to *Hanseniaspora guilliermondii/osmophila*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Pichia fermentans*, *Saccharomycodes ludwigii* species) for resistance to antimicrobial compounds, tolerance to high ethanol and sugar concentration, β -glucosidase activity;

A3) Microfermentation in natural grape must as pure culture to evaluate strain fermentative aptitude.

2. Materials and Methods

2.1 Resistance to SO₂ and CuSO₄, and H₂S production

The resistance to sulphur dioxide and copper was tested on agarized substrate, added by increasing doses of SO₂ and CuSO₄, following the protocol reported by

Mauriello et al. (2009). The production of H₂S was evaluated on BIGGY agar in function of yeast colony browning by an arbitrary scale from 0 (white colour = no production) until 3 (coffee = high production).

2.2 Growth in Ethanol and high sugars concentration

The growth test in ethanol and high sugar concentration was performed in microplates following the method by Eglezos et al. (2015), with some changes. The strain growth was evaluated in YNB with amino acids, added with different ethanol (8, 12, 14 % v/v) and sugars (2, 20, 40%) concentrations. The cell growth, measured as optical density at 630 nm, was calculated as ratio between strain growth in the medium with and without ethanol or sugars addition.

2.3 β -glucosidase activity

This enzymatic activity was evaluated both by qualitative and quantitative methods. The qualitative β -glucosidase activity was determined on a synthetic medium containing arbutin as carbon source. The plates were incubated at 25°C for 5 days. The presence of β -glucosidase activity was related to the browning of strain colony. The quantitative β -glucosidase activity was measured following the method previously described Manzanares et al. (2000).

2.4 Yeast respiratory activity

The oxidative fermentative metabolism of the strains was evaluated on the basis of cytochrome C oxidase activity, oxidative stress tolerance and catalase activity, following the protocol described by Furlani et al. (2017).

2.5 Microfermentations

Strain fermentative aptitude was tested in natural grape must. The process was monitored by weight loss and °Brix measurement and the fermentations were stopped when these parameters were constant for three consecutive days.

3. Results and Discussion

The results of preliminary screening showed that the non-*Saccharomyces* strains exhibited a high variability for all the tested parameters. All twenty-nine non-*Saccharomyces* strains are able to grow in presence of 100 mg/L of SO₂ and *T. delbrueckii* strains exhibited the higher resistance (200 mg/L SO₂). As regards copper resistance, the highest tolerance level was found among *M. pulcherrima*, with 9 strains tolerating 300 μ M of CuSO₄.

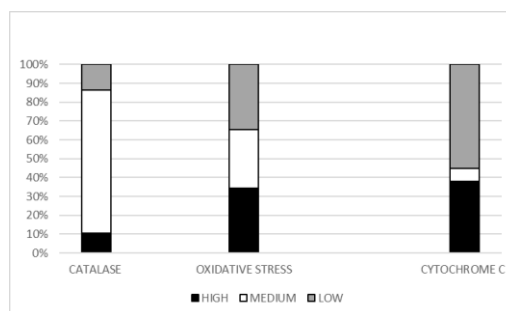
Table 1 Results of technological characterization among non-*Saccharomyces* strains.

Specie	SO ₂ Resistance (mg/L)	CuSO ₄ Resistance (μ M)	H ₂ S production
<i>H. guilliermondii/osmophila</i>	100-200	100-200	Medium
<i>T. delbrueckii</i>	200	50	High
<i>M. pulcherrima</i>	150-175	200-300	Medium
<i>P. fermentans</i>	150-175	200	High
<i>S. codes ludwigii</i>	100-150	100	Medium

Regarding the growth in ethanol and sugar concentrations, the strains exhibited significant differences only in presence of the highest ethanol dose (14% v/v) and sugar concentration (40%). About half of the strains showed a good cell growth in high sugar/ethanol concentration, demonstrating their potential ability to grow in substrate as must or wine. As regards β -glucosidase activity, the strains showed a different level of this activity and the highest level was found in six strains, belonging to *H. guilliermondii*, *S. codes ludwigii*, *M. pulcherrima* and *H. osmophila*. These six strains could potentially be used in mixed fermentation to improve the flavor of wines.

Evaluation of catalase, cytochrome C and oxidative stress tolerance were used as an indirectly measurement of oxidative activity by the strains. In particular, all the strains showed good catalase activity and variable behaviour in oxidative stress resistance test. The best result for resistance to hydrogen peroxide was exhibited by *M. pulcherrima* strains.

Figure 1 Respiratory activity of 29 non-Saccharomyces strains based on evaluation of catalase, cytochrome C and oxidative stress tolerance



As regards the evaluation of strain fermentative aptitude, all the strains were not able to complete the process, although some differences among the strains for sugar consumption/ethanol production ratio were found. The strains exhibiting high values of this ratio might be considered as useful starter candidate to obtain alcohol reduction in wine. Preliminary results obtained in this study will allow to select yeast strains for further characterization in fermentations at different levels that will lead to the identification of strains to be used as mixed starter at pilot scale.

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Ionization Technology to Control Microbial Contamination in Food Storage Equipment

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The first activities of the PhD thesis project are described. Operative parameters of different ionizers A and B were determined. Firstly, three treatments times were tested at room temperature on *Escherichia coli* 8048 and *Pseudomonas fluorescens* L22 with ionizer A. Since scarce repeatability of data were resulted, ionizer B was used to carry out the same treatments. Thanks to the best performances of this device, *Penicillium roqueforti* PR N was included in the experimentation and further treatment conditions of time and temperature were tested.

Controllo della contaminazione microbica tramite ionizzazione in attrezzature deputate alla conservazione degli alimenti

Vengono descritte le prime attività del progetto di tesi di dottorato. Sono stati determinati i parametri operativi di due diversi ionizzatori A e B. Inizialmente, con lo ionizzatore A sono stati condotti su *Escherichia coli* 8048 e *Pseudomonas fluorescens* L22 tre trattamenti a tempi diversi a temperatura ambiente. A causa della scarsa ripetibilità dei dati, si è provato a condurre gli stessi trattamenti con lo ionizzatore B. Grazie alle migliori prestazioni di questo dispositivo, *Penicillium roqueforti* PR N è stato incluso nella sperimentazione e ulteriori condizioni di tempo e temperatura sono state valutate in aggiunta a quelle precedenti.

Key words: ionization technology, microbial analysis, *Escherichia coli*, *Pseudomonas fluorescens*, *Penicillium roqueforti*, microbial reduction

1. Introduction

In the domestic environment, food spoilage could be reduced using eco-friendly equipment with active systems inside it, ionization technology in the specific case. In this PhD thesis project, the application of ionization was performed in order to reduce microbial load of *E. coli* 8048, *Ps. fluorescens* L22 and *P. roqueforti* PR N.

This poster reports the main results of the first activities concerning:

A1) the evaluation of microbial reduction of *E. coli* 8048 and *Ps. fluorescens* L22 after treatments of 1.5, 3 and 6 h at room temperature with ionizer A;

A2) the evaluation of microbial reduction of *E. coli* 8048, *Ps. fluorescens* L22 and *P. roqueforti* PR N after treatments of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 6 h at room and refrigeration temperatures with ionizer B.

2. Materials and Methods

The determination of ionizers operative parameters was carried out. Ion current was measured (90 %RH, 4 and 23 °C) by a setup created with two multimeters (Fluke 179 True RMS multimeter, Fluke 189 True RMS multimeter), ion concentration by an ion counter (Air Ion Counter Model AIC2) and ozone amount by an ozone analyzer (Ozone Analyzer UV-100 Serial #160, Eco Sensors). Microbial suspensions of *E. coli* 8048 and

Ps. fluorescens L22 were prepared in Brain Heart Infusion (BHI, Oxoid, Milano), that of *P. roqueforti* PR N in Malt Extract broth (ME, Oxoid, Milano). Serial dilutions of suspensions were done in Maximum Recovery Diluent (MRD, Oxoid, Milano) in order to set up the samples according to the drop plate method (Herigstad et al., 2001) using Plate Count Agar (PCA; Oxoid, Milano) for *E. coli* 8048 and *Ps. fluorescens* L22 and ME Agar for *P. roqueforti* PR N. Plates were put inside a closed box equipped with ionizer, fan (NMB 3610RL-04W-B10, Minebea Co., Ltd) and temperature/RH sensor (AMS CCS811). Thus, plates were exposed to ionization for 1.5, 3 and 6 h at room temperature with ionizer A and for 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 6 h at room and refrigeration temperatures with ionizer B. After the treatment, plates were incubated at specific conditions (*P. roqueforti* PR N at 22°C, *Ps. fluorescens* L22 at 30°C, *E. coli* 8048 at 37°C). Difference between non-treated samples (control, N_0) and treated samples (N) were calculated; results were expressed as Log (N/N_0).

3. Results and Discussion

3.1 Determination of the operative parameters of ionizers A and B

Operative parameters were investigated in order to evaluate devices functionality. Differences between two ionizers were finally found: ionizer A was resulted very sensitive to performance change over AC input voltage variation. This instability probably reflected in the lower ion current (data not shown) and therefore in the poor generation of ions ($<10^6$ ions/cm³) and ozone (data not shown) compared to ionizer B. In contrast, this one demonstrated to generate continuously an ion concentration $>10^6$ ions/cm³, that is the value for which is demonstrated to obtain an effect on microbial cells (Fan et al., 2002; Tyagi et al., 2008).

3.2 Ionization treatments of *E. coli* 8048, *Ps. fluorescens* L22 and *P. roqueforti* PR N

In the first part of experimentation, ionizer A was used. Table 1 shows the results of the treatment of *E. coli* 8048 and *Ps. fluorescens* L22 after 1.5, 3 and 6 h at room temperature (22.63 °C $\pm$ 0.98 °C; 96.47 %RH $\pm$ 3.54 %RH). Data were not as promising as expected for both microorganisms (Tyagi et al., 2008) as evidenced by the poor Log (N/N_0) generated following the treatment. In addition, the standard deviations of treatments were particularly wide pointing out that the device A had a variability during the working.

Table 1 Microbial reductions (Log (N/N_0); mean $\pm$ st.dev.; n=3) of *E. coli* 8048 and *Ps. fluorescens* L22 after 1.5, 3 and 6 h of ionization treatment with ionizer A.

Treatment time	<i>E. coli</i> 8048	<i>Ps. fluorescens</i> L22
h	Log (N/N_0)	Log (N/N_0)
1.5	0.00 $\pm$ 0.07	-0.26 $\pm$ 0.21
3	-2.65 $\pm$ 0.56	-0.48 $\pm$ 0.52
6	-2.15 $\pm$ 1.93	-0.95 $\pm$ 0.35

Subsequently, ionizer B was used to carry out the same tests. Thanks to the best results obtained, other microorganisms were included in the experimentation and among these *P. roqueforti* PR N. Moreover, further treatment conditions were evaluated. Firstly, additional times (0.5, 1, 2, 2.5, 4 and 5 h) were tested at room temperature focusing on shorter times in order to understand if the same effect could be obtained shortening the treatment time. Once the effectiveness was verified, all the tests were also done at

refrigeration temperature ($5.29 \text{ }^{\circ}\text{C} \pm 0.64 \text{ }^{\circ}\text{C}$; $97.85 \text{ \%RH} \pm 1.01 \text{ \%RH}$), since the aim is to control microbial growth in food storage equipment.

Figure 1 shows the trends of *E. coli* 8048, *Ps. fluorescens* L22 and *P. roqueforti* PR N after 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 6 h at room and refrigeration temperatures. Generally, all the trends were similar, showing a decrease in the first three hours, less efficiency up to 5 h and final decrease at 6 h ($-5.28 \pm 0.89 \text{ Log CFU/mL}$ for *E. coli* 8048, $-4.52 \pm 0.07 \text{ Log CFU/mL}$ for *Ps. fluorescens* L22 and $-3.89 \pm 0.21 \text{ Log CFU/mL}$ for *P. roqueforti* PR N at refrigeration temperature). Only for *Ps. fluorescens* L22 at refrigeration temperature the initial decrease was not observed probably due to its psychrotrophic nature. These results were only partially comparable with previous works (Fan et al., 2002; Fletcher et al., 2007; Park et al., 2016; Tyagi et al., 2008) because of the different setups of experiments and different treatments times. However, the efficacy of this technology was confirmed, and further studies (involving other microbial species or treatment conditions) might be carried out in order to make it a promising device for the real application in storage equipment.

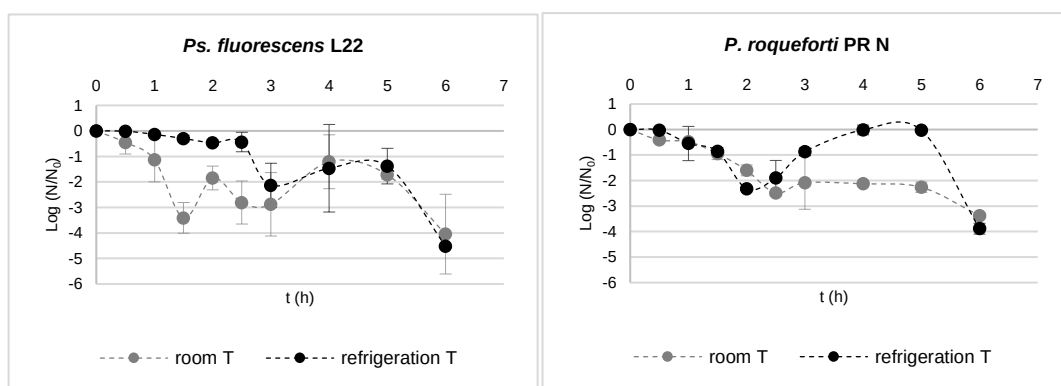


Figure 1 Trends of treatments at room and refrigeration temperature of *E. coli* 8048, *Ps. fluorescens* L22 and *P. roqueforti* PR N after 0.5, 1, 1.5, 2, 2.5, 3, 4, 5 and 6 h.

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Formulation of Nanoemulsion Systems for the Encapsulation of Bioactive Compounds

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This work aims to develop oil-in-water nanoemulsion systems prepared by high pressure homogenization for the stable encapsulation and bioavailability of bioactives, such as Vitamin A. In the first part of the work, model oil-in-water nanoemulsions will be prepared in an experimental design, by varying pressure of the homogenizer and the chemical composition of the formulations. In the second and third part of the work, the chemical and physical properties of the nanoemulsions will be studied, including the stability and bioavailability of Vitamin A.

Formulazione di nanoemulsioni per l'incapsulamento di composti bioattivi

Lo scopo del lavoro è lo sviluppo di nanoemulsioni olio in acqua mediante omogeneizzazione ad alte pressioni per l'incapsulazione di bioattivi, quali la Vitamina A, che siano stabili e biodisponibili. Nella prima parte del lavoro, nanoemulsioni olio in acqua saranno preparate all'interno di un disegno sperimentale facendo variare la pressione dell'omogeneizzatore e la composizione chimica delle formulazioni. Nella seconda e terza parte del lavoro, le proprietà chimiche e fisiche delle nanoemulsioni saranno studiate, includendo la stabilità e la biodisponibilità della Vitamina A.

Key words: Encapsulation, nanoemulsion, vitamin A, whey protein isolate, degradation, stability.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the three main activities concerning:

A1) Preparation of nanoemulsions by high pressure microfluidizer. The effect of pressure, the type of emulsifier, the ratio of oil and water, and the concentration of the bioactive (Vitamin A) and antioxidants (α -tocopherol) will be studied in terms of physical parameters, such as particle size, zeta-potential, color and viscosity. (Sharif et al., 2017).

A2) The chemical changes occurring to the nanoemulsions and, in particular, to Vitamin A, will be studied by UHPLC coupled by DAD and MS/MS (Gatti et al., 1999).

A3) The physical changes of nanoemulsions, including their stability, will be performed by particle laser scattering (Mastersizer), zeta potential (Zetasizer), optical analyzers (Turbiscan) and scanning electron microscopy (SEM).

2. Materials and Methods

Oil-in-water nanoemulsions were prepared by homogenising 10% (w/w) oil phase with 90% (w/w) aqueous phase at room temperature (25 °C) (Qian et al., 2012). coarse emulsion was prepared using an Ultra-Turrax homogenizer operating at 18,000 rpm for 2 min, which was then passed through a high pressure microfluidizer (Model 101,

Microfluidics, Newton, MA) three times at four different pressures (100, 500, 1000 and 2000 bar) (Qian et al., 2011). Oil phase was prepared by adding α -tocopherol in three different concentrations (2500 ppm, 5000 ppm and 10000 ppm). Aqueous phase was prepared by dispersing 2% (w/w) whey protein isolate (WPI) in aqueous buffer solution. Emulsion stability was measured by an optical analyzer Turbiscan® Lab Tower (Formulation, France). Monitoring of the vitamin A acetate concentration during shelf-life of emulsions was performed using HPLC.

3. Results and Discussion

3.1 Stability of Vitamin A during shelf-life study

The stability of vitamin A was monitored during six weeks at 40°C. (Table 1)

Table 1 Concentration of vitamin A (μM) in emulsions prepared applying the pressure of 100, 500, 1000 and 2000 bar with the addition of various concentrations of tocopherol (0, 2.5, 0.5 and 1 mg/g) during six weeks of storage. Average of $n=2$ is calculated.

AOX (mg/g of emulsion)	Pressure	Week					
		1	2	3	4	5	6
0	100	376 $\pm$ 19	389 $\pm$ 18	352 $\pm$ 21	355 $\pm$ 13	299 $\pm$ 0.1	157 $\pm$ 38
0	500	416 $\pm$ 3	399 $\pm$ 0.1	389 $\pm$ 8	379 $\pm$ 2	373 $\pm$ 13	282 $\pm$ 10
0	1000	440 $\pm$ 4	423 $\pm$ 1	411 $\pm$ 6	411 $\pm$ 6	399 $\pm$ 3	387 $\pm$ 1
0	2000	425 $\pm$ 4	410 $\pm$ 3	374 $\pm$ 12	376 $\pm$ 15	349 $\pm$ 32	290 $\pm$ 4
0.25	100	394 $\pm$ 3	392 $\pm$ 1	363 $\pm$ 13	340 $\pm$ 13	289 $\pm$ 15	226 $\pm$ 5
0.25	500	385 $\pm$ 6	378 $\pm$ 2	368 $\pm$ 8	333 $\pm$ 1	307 $\pm$ 4	288 $\pm$ 15
0.25	1000	4199 $\pm$ 6	389 $\pm$ 1	374 $\pm$ 1	365 $\pm$ 1	359 $\pm$ 1	337 $\pm$ 6
0.25	2000	4144 $\pm$ 3	388 $\pm$ 3	387 $\pm$ 5	375 $\pm$ 4	366 $\pm$ 5	349 $\pm$ 0.1
0.5	100	459 $\pm$ 10	422 $\pm$ 9	382 $\pm$ 3	383 $\pm$ 8	336 $\pm$ 12	290 $\pm$ 6
0.5	500	439 $\pm$ 19	415 $\pm$ 16	403 $\pm$ 1	381 $\pm$ 2	331 $\pm$ 2	312 $\pm$ 1
0.5	1000	490 $\pm$ 14	482 $\pm$ 1	475 $\pm$ 14	475 $\pm$ 26	445 $\pm$ 23	412 $\pm$ 16
0.5	2000	476 $\pm$ 5	454 $\pm$ 5	446 $\pm$ 14	434 $\pm$ 6	434 $\pm$ 2	411 $\pm$ 13
1	100	467 $\pm$ 1	451 $\pm$ 17	419 $\pm$ 13	420 $\pm$ 6	376 $\pm$ 4	336 $\pm$ 8
1	500	516 $\pm$ 1	496 $\pm$ 11	490 $\pm$ 5	484 $\pm$ 5	445 $\pm$ 25	420 $\pm$ 3
1	1000	516 $\pm$ 8	492 $\pm$ 7	468 $\pm$ 2	470 $\pm$ 13	433 $\pm$ 11	444 $\pm$ 3
1	2000	513 $\pm$ 1	497 $\pm$ 9	488 $\pm$ 11	444 $\pm$ 10	437 $\pm$ 13	410 $\pm$ 5

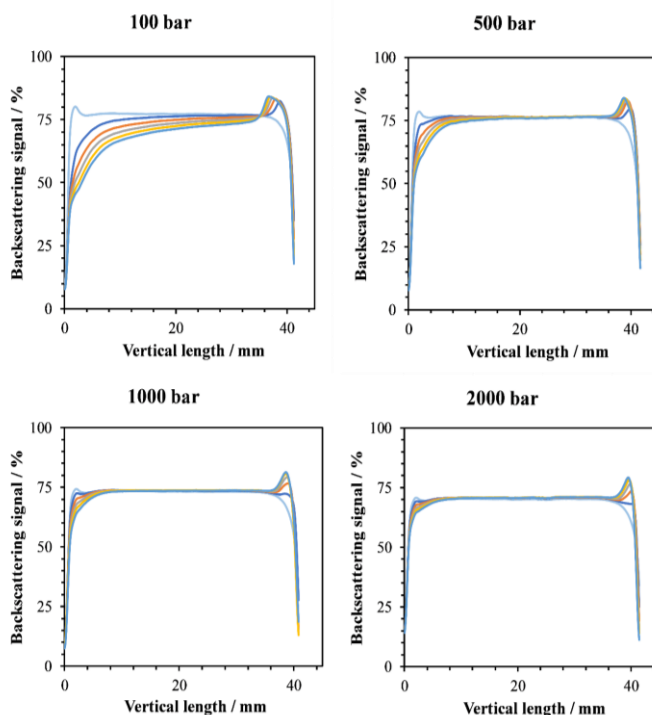
3.2 Statistical analysis

Three-way ANOVA analysis of the data showed that all three main factors made significant impact on the changes in the vitamin A concentration ($p < 0.001$). The concentration of tocopherol explained 48.7% of the total variance, followed by storage time (26.1%) and pressure (21.2%).

3.3 Turbiscan analysis

Fig. 1 shows the backscattering signals as a function of time acquired every 20 min during the first 120 hours. The resulting stability of the samples can be observed as a function of four different pressures (100, 500, 1000, 2000 bar). The results indicate that the most stable emulsions can be obtained by pressures comprised between 1000 and 2000 bar.

Figure 1 Backscattering signal (%) as a function of time in emulsions prepared at 100, 500, 1000 and 2000 bars during the first 120 h.



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Characterization of Novel Yeasts/Bacteria Probiotic Combination for Cereal-Based Fermented Beverage

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The principal activities of the PhD project are described. In the aim of developing a novel kind of beverage, oat and spelled flours were mixed with a combination of potentially probiotic strains: LG15 *Debaryomyces hansenii* strain, C9O4 *Lactobacillus plantarum* and *L. plantarum* LT52. Yeast and bacterial strains were preliminary characterized for some probiotic traits: *in vitro* resistance to gastrointestinal tract, adhesion to intestinal cells and mucin, and anti-genotoxic activity toward 4-Nitroquinoline-1-Oxide. Strains were then inoculated to a mix of flour (oat, spelled) and water which fermented for 48h at 30 °C. Beverages were then analysed for their volatile profile and physicochemical composition.

Caratterizzazione di una combinazione probiotica di lieviti e batteri da utilizzare in bevande fermentate a base cereali

Le principali attività di ricerca del progetto di dottorato sono descritte. Con l'obiettivo di mettere a punto una nuova tipologia di bevanda, farina di farro e d'avena sono state fatte fermentare con i seguenti ceppi: LG15 *Debaryomyces hansenii*, C9O4 *Lactobacillus plantarum* e LT52 *L. plantarum*. Prima dell'inoculo il lievito e i batteri sono stati testati per alcune proprietà probiotiche: resistenza *in vitro* al tratto gastrointestinale, adesione alle cellule intestinali e alla mucina e attività anti-genotossica nei confronti della tossina 4NQO. I ceppi così caratterizzati sono stati inoculati in una miscela di acqua e farina (farro o avena) lasciata fermentare per 48h a 30 °C. Le bevande ottenute sono state analizzate per la composizione fisico chimica e i composti volatili.

Key words: probiotic, cereal-based beverages, yeasts, bacteria

1. Introduction

This poster reports the main results of the activities of the PhD project concerning:

A1) the characterization of yeast and bacteria for their probiotic activities

A2) the development of a fermented beverage

2. Materials and Methods

The gastro-intestinal tract tolerance of all strains was determined by monitoring changes in total viable counts after exposing washed cell suspensions (yeast and bacteria mixed together) at 37 °C to a simulated gastric juice (SGF; pH 2.0), and to a simulated small intestinal juice (SIF; pH 8.0) (Prete et al., 2017). The cell-genotoxin interaction was evaluated using a co-incubation: bacteria and yeasts were cultured, in MRS and YPD broth at 37 °C and 30 °C for 24h, respectively. The 4-NQO was added to a final concentration of 0.1 mM and co-incubation was maintained at 37 °C for 150 min (under shaking) according to Caldini et al. (2002). Supernatants were successively analyzed for residual genotoxic and mutagenic activity. The microbial adhesion to intestinal cells was assessed according to Gonzalez et al., (2018) with some modifications for yeasts adhesion. NCM460 confluent monolayers was used and yeasts and bacteria were

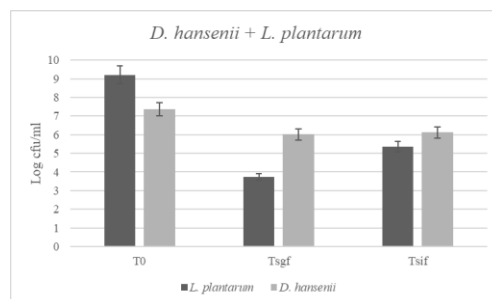
inoculated for 1.5h at 37 °C in 5% CO₂ atmosphere. The adhesion efficiency of strains was investigated on NCM460 cell line by enumeration by plating on MRS agar and YPD agar. Adhesion to NCM460 cells was calculated as percentage of adhered bacteria/yeasts correlated to the initial number of bacteria/yeasts added in each well. Bacterial ability to adhere to mucin layer was assessed too, according to Gonzalez et al., (2018). After strain characterizations, two kind of fermented beverages were produced: 5% oat and 5% spelled were inoculated separately with LG15 *D. hansenii* strain (10⁷ ucf/ml) and C9O4+LT52 *L. plantarum* strains (10⁹ ucf/ml) and fermented at 30 °C for 48h. Viable counts were also tested after 7 and 14 days. Volatile compounds were analysed using gas chromatography-mass spectrometry (GC-MS) combined with solid-phase microextraction (SPME), according to Lee et al. (2019). Organic acids (lactic, acetic, malic, succinic, and tartaric acid) and carbohydrates (glucose, fructose, and maltose) analysis was performed as described by Duarte et al. (2010).

3. Results and Discussion

3.1 Determination of the main probiotic characteristics

Both strains were able to survive in the solutions used to simulate the gastrointestinal tract: *L. plantarum* strains had a 40% recovery after SGF incubation and then they were able to recuperate after SIF incubation, reaching a survivor rate of 58%; *D. hansenii* strain exhibited a stronger tolerance with a percentage of survivor of 82% after SGF and 83% after SIF. In Fig. 1 microbial counts are shown for both strains. This result underlines the capability of the cells to recover from viable-but-non-cultivable condition caused by SGF exposure.

Figure 1 Microbial counts for *L. plantarum* and *D. hansenii* expressed as Log cfu/ml at time 0 (T0) and after the incubation with SGF (Tsgf) and SIF (Tsif).



Regarding anti-genotoxic activity, *L. plantarum* and *D. hansenii* strains resulted active, with an inhibition of 96,4% and 92,35% respectively. Due to the presence of mucus layer on the GI epithelium, the effect of mucin was tested to underline if it can affect the microbial adhesion property or it might have any impact on growth. Strains were tested alone and in combination. *D. hansenii* alone showed a percentage of adhesion of 85% and *L. plantarum* of 86%. In co-culture this percentage dropped to 78% for *L. plantarum* strains while *D. hansenii* strain confirmed a percentage of adhesion of 86%. All strains were able to adhere to intestinal cells.

3.2 Development of a fermented beverage

Mixed starter was used to develop a fermented beverage with 5% flour (oat or spelled). Fermentation was monitored checking pH, viable counts and volatile profile. Strains were tested singularly and showed an increasing of 1 Log after 48h. Initial pH was 6.3 and 5.3 for spelled and oat respectively. *L. plantarum* was able to acidify reaching a pH of 3.4 (spelled) and 4.5 (oat). Fermentation with *D. hansenii* resulted in a lower acidity of beverages with a final pH of 4.7 and 4.4 for spelled and oat respectively. Viable counts of mixed fermentation are shown in Figure 2. Spelled resulted a better substrate for yeast

and bacteria growth. Yeasts and bacteria were stable and were still found at concentrations of 6 and 8 Log cfu/ml respectively after 7 and 14 days. The volatile analysis of beverages resulted in the presence of fatty acids and their esters, alcohols, aldehydes, ketones and the mixed fermentation significantly changed the aroma profile of the four cereal broths, and each substrate showed a specific volatiles profile.

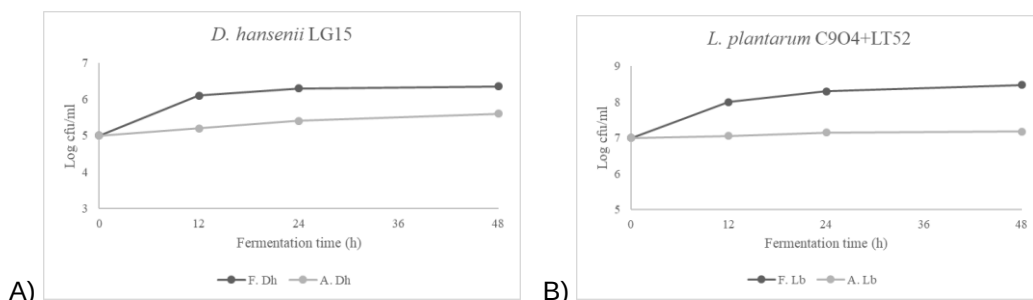


Figure 2 Microbial population during co-culture fermentation. A) Viable counts for *D. hansenii* in spelled (F. Dh) and oat (A. Dh). B) Viable counts for *L. plantarum* in spelled (F. Lb) and oat (A. Lb).

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Biotechnological Features of Lactic Acid Bacteria and their Application in Food Processing

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The doctoral thesis is focused on the application of lactic acid bacteria (LAB, CNR-ISPA Collection), selected for their peculiar biotechnological features (antimicrobial, probiotic, proteolytic, EPS production), to food preparation in order to improve their quality. The activities performed so far have concerned: A) use of LAB selected strains in food process to extend their shelf-life (processed fish), to provide probiotic properties (probiotic dehydrated apple snack), to improve nutritional/functional properties of bakery products (reduced addition of salt or fat); B) study of growth behaviour of selected LAB strains to optimize fermentation processes through predictive microbiology.

Studio delle caratteristiche biotecnologiche di ceppi lattici e loro applicazione in processi di trasformazione degli alimenti

La tesi di dottorato è focalizzata all'applicazione dei batteri lattici (LAB, Collezione CNR-ISPA) selezionati per peculiari caratteristiche biotecnologiche (antimicrobiche, probiotiche, proteolitiche, produzione di EPS) in processi di trasformazione di alimenti al fine di migliorarne la qualità. Le attività svolte finora hanno riguardato: A) l'applicazione di ceppi selezionati di batteri lattici in matrici alimentari per estendere la shelf-life (trasformati ittici), per sviluppare alimenti probiotici (snack di mela disidratata probiotica), per migliorare le proprietà nutrizionali/funzionali di prodotti panari (per ridurre l'aggiunta di sale o grasso); B) studio del comportamento di crescita di ceppi selezionati per ottimizzare i processi fermentativi mediante microbiologia predittiva.

Key words: Bio-preservation, probiotic foods, gastro-intestinal digestion, protein digestibility, fermentation products, predictive microbiology.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the completed (A1 - A2) and ongoing (A3 - B) activities concerning:

A1) the use of protective microbial strains (*Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum*), in combination with modified atmosphere packaging (MAP) and refrigerated storage, in tuna burgers to extend their shelf life;

A2) the suitability of an inclusion/coating/dehydration process to obtain dehydrated apple snack enriched with viable cells of the probiotic *L.paracasei* IMPC2.1;

A3) improvement of nutritional (protein digestibility) properties of bakery products using *L. plantarum* ITM21B fermentation products (Bio21B) and evaluation of exopolysaccharides (EPS) production by *Weissella cibaria* strain in fermentation products (FP_C43-11) containing wheat/pseudocereals flours.

(A4) determination of cardinal growth values of 8 selected LAB strains (*L. paracasei* and *L. plantarum*).

2. Materials and Methods

(A1) Three potentially biopreservative LAB strains (*L. casei* P1, *L. paracasei* 4.1 and *L. plantarum* 5BG) were screened for their protective performance in ready-to-cook tuna burgers stored at 4°C. The *L. paracasei* strain was selected as bio-preservative and tested under MAP conditions (5%O₂–95% CO₂) at 4°C. The shelf-life was determined by calculating the microbial acceptability limit (MAL: storage time needed to reach a mesophylic count of 7 log/g) and the sensory acceptability limit (SAL: storage time at which the overall quality reaches the acceptability threshold) using a reparameterized Gompertz equation (Danza et al., 2018).

(A2) To include the probiotic *L. paracasei* IMPC2.1 in apple slices, two procedures - vacuum impregnation (IP1) and soaking/stirring (IP2) - were evaluated. After probiotic inclusion, apple slices were coated or not with citrus pectin and dehydrated (4°C, 14 days). Probiotic coated (IP1, IP2) and uncoated (IP1U, IP2U) samples were subjected to simulated gastro-intestinal digestion to evaluate the strain survival (Valerio et al. *in press*).

(A3) *Protein digestibility*. Two *L. plantarum* ITM21B fermentation products, containing (Bio21B_H) or not gluten hydrolysate (Bio21B), were used in bread formulation in the presence or not of chickpea flour (CF) to prepare salt reduced (-50%) products. The protein content was analysed by Lab-on-a-Chip (LoaC) capillary electrophoresis and the *in vitro* protein digestibility (IVPD) was determined as reported Di Biase et al. (2019).

Fat reduction. The influence of dough yield (DY), flour type (amaranth, quinoa, wheat) and sucrose content on EPS production by *W. cibaria* C43-11, selected among 25 strains, was evaluated. The resulting FP were analysed for viscosity, EPS, pH, TTA, LAB count. A non-producer strain and FP not inoculated (Ctrl) were used as controls. Results were analysed by Principal Component Analysis (PCA).

(A4) Cardinal growth parameters (i.e. minimal, optimal and maximal) relevant to temperature, pH and a_w of 4 *L. plantarum* and 4 *L. paracasei* strains were determined using the turbidimetric method with the Bioscreen C. Data will be implemented in the decision making tool Sym'Previous to set the appropriate fermentation conditions for each food type.

3. Results and Discussion

3.1 Bio-preserving efficacy of LAB strains in tuna burger (A1)

L. paracasei 4.1 showed the best preserving performance during storage and significantly controlled aerobic mesophilic bacteria. The shelf-life of burgers was extended by 4 days respect to the control (Figure 1). The applied biotechnological procedure represents an effective approach for the mild preservation of fish products.

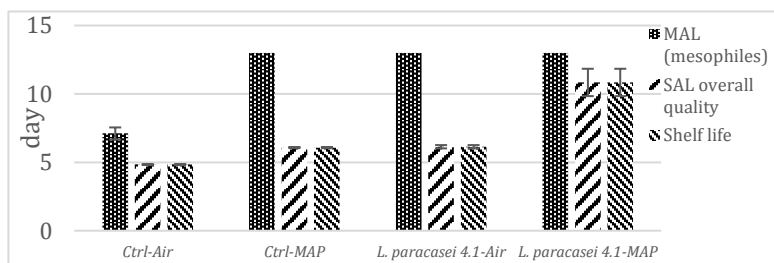


Figure 1
Shelf life (day;
mean±SD) of tuna
burger samples.

3.2 Development of a probiotic dehydrated apple snack and viability of the probiotic *L. paracasei* IMPC2.1 during simulated gastro-intestinal digestion (A2)

Both inclusion procedures (IP1 and IP2) allowed to obtain a dehydrated product harboring more than 9 log cfu of cells per 20 g portion. The IP2 procedure ensured the better bacterial survival during 30 days storage (4°C) and color parameters comparable to the fresh apple. The coating step did not influence the probiotic adhesion to the apple matrix. After simulated digestion a good survival rate, ranging from -2.3 to -2.7 logarithmic reduction was observed for the probiotic strain in apple slices, while a higher reduction occurred in skim milk (-3.9±0.1). Results confirmed the ability of the probiotic strain to adhere to the apple matrix and to survive during the digestive process.

3.3 Protein digestibility of salt reduced breads containing Bio21B (A3)

The addition of Bio21B and chickpea flour (CF_Bio21B) or wheat gluten hydrolyzate (Bio21B_H) to the bread formula significantly increased ($p < 0.05$) the protein digestibility respect to the reference product (Ctrl) and Bio21B bread samples (Table 1).

Table 1 Content and digestibility (IVPD) of proteins of breads.

Sample	total proteins (mg/gr bread)	IVPD (%)
Ctrl	18.63±1.05a	57.89±0.49a
Bio 21B	18.18±0.86a	56.20±0.25a
CF_Bio21B	22.38±0.22b	63.45±1.29b
Bio 21B_H	21.30±0.11b	62.68±1.03b

Evaluation of factors influencing EPS production by *W. cibaria* C43-11 for fat reduced bakery products (A3, ongoing)

The Principal Component Analysis allowed to highlight a positive correlation between EPS content and (FP_C43-11) viscosity. In particular, the FP formulated with amaranth flour and 6% sucrose (DY250) was selected for its application in fat reduced “focaccia” type bread.

3.4 Determination of cardinal growth parameters (A4, ongoing)

Cardinal growth parameters determinated for each strain showed a bacterial inter- and intra-species variability in growth abilities. Data will be completed in order to obtain a reliable prediction of growth in food conditions through predictive microbiology.

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Application of Different Commercial Lactase Preparations for the Production of Lactose-free Milk

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In this study, the impact of different lactase preparations on the profile of volatile organic compounds (VOC) as well as on the sensory properties of UHT lactose-free milk (LFM) during shelf-life were investigated by proton transfer reaction-mass spectrometry (PTR-MS) and sensory analysis, respectively. PTR-MS data were analyzed by multivariate statistics to spot temporal trends of the VOCs. Sensory analysis was performed with a trained panel.

Applicazione di diverse lattasi commerciali per la produzione di latte delattosato

In questo studio, l'impatto di diverse lattasi sul profilo dei composti volatili e sulle proprietà sensoriali del latte UHT delattosato nel corso della shelf-life sono stati investigati mediante proton transfer reaction-mass spectrometry (PTR-MS) e analisi sensoriale. I dati PTR-MS sono stati analizzati mediante statistica multivariata per evidenziare eventuali trend temporali nel profilo dei composti volatili. L'analisi sensoriale è stata svolta con un panel addestrato.

Key words: lactose-free milk, PTR-MS, shelf-life, VOCs, lactase, sensory analysis.

1. Introduction

High-quality milk should be characterized by a clean taste, without any odors or aftertaste (Francis et al., 2005). In UHT LFM, this is not trivial because of the higher sensitivity upon chemical changes compared to conventional UHT milk. The issue arises partially from the presence of unwanted proteolytic activity in the commercial lactases, which are sold with different degrees of impurities (Nielsen et al., 2018). In this frame, this study investigated the effect of the proteolytic side activity of the lactases on the milk quality during shelf-life through:

- A1) the assessment of the VOCs profiles by PTR-MS;
- A2) the sensory evaluation by Quantitative Descriptive Analysis (QDA).

2. Materials and Methods

Nine formulations of UHT LFM were produced industrially on three different weeks. Manufacturing was based on the addition of three different lactases (Lac1, Lac2, Lac3) before UHT treatment, in accordance with a procedure previously described (Dekker et al., 2019). Samples were then stored at 20°C under controlled conditions for 150 days and collected every 30 days (5 mL of each milk into 20 mL glass vials) for the PTR-MS analysis. A PTR-TOF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) in V mode was used and set up according to previous studies (Pedrotti et al., 2018). Headspace solid phase microextraction (HS-SPME) GC-MS was applied for better identification of the mass peaks detected by the PTR-MS. QDA was employed for the sensory analysis. Two points were taken for the sensory evaluation over shelf-life, namely after 30 and 120 days of storage. A trained panel assessed each UHT LFM sample based on the following descriptors: white color intensity, flavor intensity, mouthfeel, sweetness, cooked flavor, milky flavor, stale flavor, irregular flavor.

3. Results and Discussion

3.1 Analysis of the VOCs profiles by PTR-MS

PTR-MS data were analyzed by Principal Component Analysis (PCA). In Figure 1 a distinction of the UHT LFM samples due to storage time was demonstrated. Moreover, presence of biological variability in the milk emerged already at 0 days of storage, as the three different weeks of production were clearly distinguished, regardless the lactase preparation employed. However, PCA did not show any distinction of the samples based on the added lactase, suggesting a similar VOCs profile among the products.

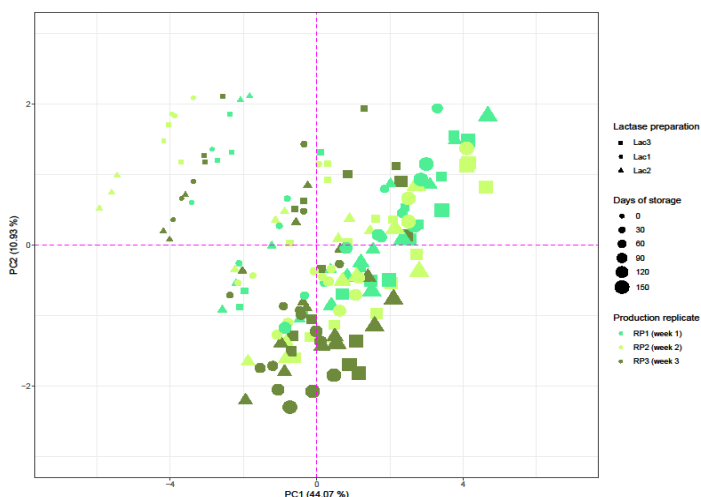


Figure 1 Principal Component Analysis (PCA) score plot of the 1st (PC1) and 2nd (PC2) component of the PTR-MS data.

Loadings considered relevant for the explanation of the spatial distribution illustrated in Fig. 1 were extrapolated and further considered via 2-ways ANOVA and Tukey post-hoc (when necessary) with Bonferroni correction. GC data were helpful to provide an identity to the selected mass peaks. The analysis confirmed storage time as significant factor for the changes in VOCs during shelf-life of UHT LFM. In particular, the effect was relevant for all the detected methyl-ketones, with 2-pentanone ($m/z = 87.0799$) and 2-heptanone ($m/z = 115.1117$) both registering the highest concentrations in the headspace and experiencing the sharpest increase during shelf-life. Besides storage time, the effect of the three tested lactase preparations (Lac1, Lac2, Lac3) on the VOCs profiling of UHT LFM was also investigated. Overall, only the mass peak $m/z = 107.048$ (identified as benzaldehyde) changed significantly over time based on the employed lactases (Figure 2). These results suggested that most of the unwanted proteolytic side activities originally contained in the tested lactase preparation were thermally inactivated by the UHT treatment before starting the shelf-life study. Nevertheless, the results indicated that benzaldehyde might be considered as a marker to monitor in UHT LFM as it can be formed as a result of the proteolytic activity from the lactase preparations (Jansson et al., 2017).

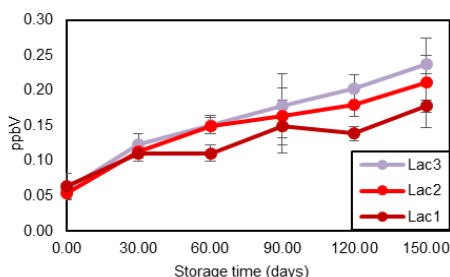


Figure 2 Temporal evolution of the mass peak $m/z = 107.048$, associated to benzaldehyde.

3.2 Sensory evaluation of the UHT LFM samples

Figure 3 reports the results of the sensory profile of the UHT LFM samples after 30 and 120 days of storage at 20°C. For each sensory session, data were analyzed by one-way ANOVA and Tukey post-hoc (when necessary). As expected, positive attributes like white color intensity and milky flavor decreased in intensity along shelf-life. Moreover, the UHT LFMs produced with different lactases did not change significantly in the sensory profile at each session. The results confirmed that the thermal inactivation of the proteolytic side activity of the tested lactases guaranteed positive performance in the products and did not end up in a differentiation during shelf-life.

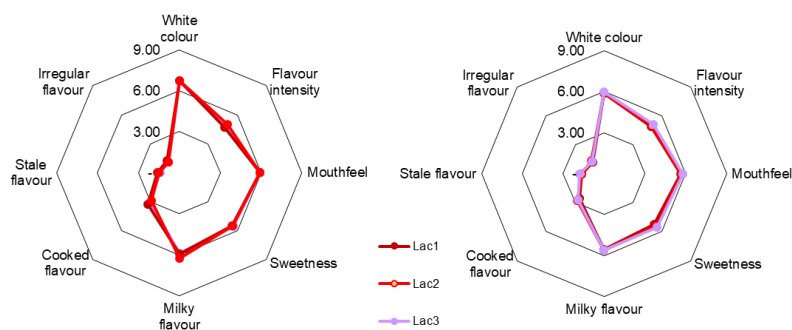


Figure 3 Sensory profiles of UHT LFM after 30 days (left) and 120 days (right) of shelf-life.

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Air Impingement Thawing in Food Service: the Study Case of Chicken Breast

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A test rig for air impingement thawing was set up. Subsequently, a thermal model of the thawing process of packaged chicken breast, selected for its wide diffusion in food service, was validated. An optimization model was used to identify the combinations of process variables based on selected performance indicators (thawing time, food temperature uniformity and maximum temperature). Identified thawing cycles were applied to frozen chicken, then analyzed for weight loss and water holding capacity. In next steps of the project, food quality effects of thawing in the developed test-rig will be compared to those of other thawing methodologies (refrigeration, microwave).

Scongelamento ad *Air Impingement* nella ristorazione: il caso studio del petto di pollo

È stato realizzato un prototipo per lo scongelamento con *air impingement*. Successivamente è stato validato un modello termofluido-dinamico dello scongelamento di petto di pollo, scelto per l'elevata diffusione nella ristorazione. È stato usato un modello di ottimizzazione per individuare le combinazioni delle variabili di processo basato su indicatori di *performance* (tempo di scongelamento, uniformità della temperatura dell'alimento, massima temperatura raggiunta). I cicli definiti sono stati applicati su pollo congelato, quindi analizzato per calo peso e *water holding capacity*. Nel prosieguo dell'attività si compareranno gli effetti qualitativi dei cicli effettuati nel prototipo con quelli di altre tecniche di scongelamento (refrigerazione, microonde).

Key words: Air impingement, thawing, chicken breast, food service.

1. Introduction

In accordance with the evolution of the PhD thesis project previously described (Bozzato, 2018), this poster reports the main results of the activities concerning:

A1) the development of an air impingement test rig and thermal modelling of the thawing process of chicken breast;

A2) the identification of thawing cycles through optimization techniques;

A3) the application of identified thawing cycles to chicken breast and evaluation of the thawing performance for the selection of optimal cycles.

2. Materials and Methods

An air impingement thawing test rig (Figure 1) was designed and assembled in Electrolux Professional's workshop. Variables of the thawing processes were air fan speed, temperature during up to five subsequent thawing steps, and distance of the samples from nozzles.

A thermal thawing model of chicken breast in PE bags was developed and validated (Tiberi et al., 2018). Thawing process variables were selected by Black-Box Optimization of performance indicators, using a multi-objective genetic algorithm (Muñoz

et al., 2015). Identified thawing cycles were applied on frozen chicken breast (8X3X1.5 cm), which was analysed for weight loss and water holding capacity (WHC). The latter was calculated as percentage ratio between area of pressed sample and area of water stain on filter paper (Grau & Hamm, 1953). Areas were measured by image analysis using an internally developed software (MatLab based).

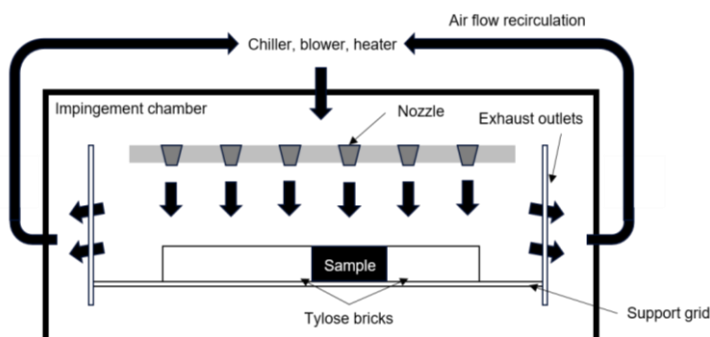


Figure 1 Schematic representation of the thawing test rig.

3. Results and Discussion

3.1 Development of the test rig and thermal modeling of the thawing process

Temperature values recorded during thawing of chicken breast in the test rig resulted in good agreement with those estimated by the selected model (Figure 2), demonstrating its accuracy during the whole process.

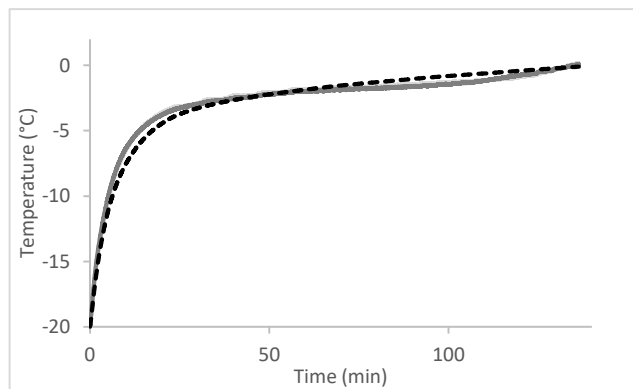


Figure 2 Model predicted (black dotted line) and experimental (grey continuous lines) temperatures of chicken breast core.

3.2 Identification of the air impingement thawing cycles

Process optimisation by multi-objective analysis allowed nine thawing cycles (Table 1) to be identified. Although five subsequent thawing steps were possible, all defined cycles had up to three phases. Modelled thawing times ranged from 85 to 270 min.

Table 1 Optimized air impingement thawing cycles parameters.

Cycle	Tray level	Fan speed	Step 1		Step 2		Step 3	
			Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
A	1	7	15	85				
B	2	4	8	23	5	113		
C	3	3	4	270				
D	1	7	15	68	10	24		
E	3	6	13	46	10	49	9	62
F	1	6	12	50	8	82		
G	3	6	8	188				
H	2	7	11	44	8	82		
I	1	3	7	14	4	241		

3.3 Thawing performances

Independently on thawing time, no significant differences in weight loss and WHC were observed among samples thawed by applying process parameters indicated in Table 1. Weight loss mean value was 2.5%, lower than previously reported values (about 4.8%) (Bedane et al., 2018) for thawing at refrigeration temperature. Mean WHC value resulted 21.7%, which was lower than data reported by Augustyńska-Prejsnar et al. (2018) for air, water and microwave thawing of chicken breast. These findings demonstrate the potential of air impingement to reduce thawing times in the food service, while minimizing exudates. The effect of air impingement thawing on chicken breast quality will be further analysed and compared to those associated to other thawing techniques, in conjunction with considerations on their economic and sustainability implications.

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Extravirgin Olive Oil Turbidity: Characterization and its Role During Shelf life

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PhD thesis project involve the study of positive characteristics related to turbidity (for example: high phenolic compounds content, antioxidant power, healthy and genuine image), combined with negative characteristics and problems that turbidity can bring to conservazione (for example: higher microorganism activity, unpleasure sensory characteristics development, formation of precipitare in bottle). This study could be usefull to giving suggestions and operative indication to the producers to obtain extravirgin olive oil with a desired and controlled turbidity grade.

Torbidità dell'Olio Extravergine di Oliva: caratterizzazione e ruolo in conservazione

Il progetto di tesi di dottorato prevede lo studio delle caratteristiche positive legate più o meno direttamente alla torbidità (alto contenuto fenolico, maggiore potere antiossidante, immagine salutistica e di genuinità), unite alle problematiche che questa può portare in conservazione (maggiore attività dei microorganismi, formazione di caratteristiche sensoriali indesiderate, formazione di precipitato in bottiglia). L'intento finale è quello di dare suggerimenti e indicazioni operative ai produttori per ottenere oli extravergine di oliva di voluta e controllata torbidità.

Key words: Veiled extravirgin olive oil, turbidity, filtration, water activity, water content, microorganism contamination

1. Introduction

Turbidity in extravirgin olive oil (EVOO) is a very controversial topic because is not clear if veiled oils are better than filtered ones during conservation for many reasons (Cayuela-Sánchez et al., 2019). In sight of this, first PhD activity allowed to select and develop a set of analyses to EVOO characterization. In accordance with methods described in literature, chemical, physic-chemical, biological and optical analyses have been chosen. This set of analyses has been applied to: (A) characterize olis with different turbidity grade; (B) shelf-life analysis of filtered and veiled EVOO subjected to HPP (High Pressure Processing) treatment.

2. Materials and Methods

2.1 Olive oil sample: For the first activity six different VEVOO were collected at the end of "decanter" and subjected to four different water and solid particles separation treatments to obtain: veiled oils (VO), filtered oils (FO), "only solid particles" oils (SO) obtained by liophilization, and "only water" oils (WO) obtained by filtration on glass wool.

For the second activity VEVOO sample have been collected at the end of decanter and half of it was immediatly filtered. After filtration, all samples were packaged into 250 ml PET bottles and half were processed in a HPP plant (608 MPa for 360 s at 15 °C). The full factorial design had included 4 theses: *CON* (i.e. not filtered and not treated with HPP), *FIL* (i.e. filtered and not treated with HPP), *HHP* (i.e. not filtered and treated with HPP), and *F-HHP* (i.e. filtered and treated with HPP). All the samples were

stored in a random position in an appropriate chamber at 20°C, while a lamp was left on for 8 h per day inside the box. All the samples were analyzed after 15 days, 1 month and 6 months of storage.

2.2 Chemical and sensory analyses: The acidity, peroxide value and UV spectroscopic indexes were measured according to EC Reg. 1989/2003. HPLC determination of the phenolic compound were performed in agreement with COI/T.15/NC No.3/Rev.4. The olive oil volatile organic compound content was determined using the HS-SPME-GC-MS technique (Fortini et al., 2017). The sensory evaluation followed the official procedure described in COI/T.20/NC No.15/Rev.10.

2.3 Suspended material determination, optical and microbiological analyses: Water content (%w/w) was analysed with 378598 HYDRANAL® - Moisture Test Kit. Water activity (A_w) was measured using a Rotronic Hygroskop DT hygrometer. The solid particle content was measure using the method described by Zullo et al. (2018). The microorganisms were enumerated according to the method reported by Zullo et al. (2010). The degree of turbidity (NTU) was measured using a Hach Model 2100 turbidimeter (Hach, USA). Confocal Laser Scanning Microscopy (CLSM) experiments were performer to observe the sospended material in oil.

3. Results and Discussion

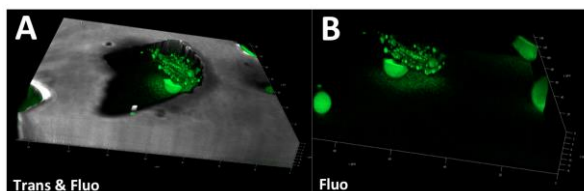
3.1 Characterization of extravirgin olive oil turbidity:

All the VO samples are characterized by high degree of turbidity grade that is related to a significant difference of water and solid particles contents, but not in proportional way. VO samples have water content >0.2% w/w, and A_w value >0.6, which are suitable for microbial activity. The VO samples were contaminated by microorganisms, but there is a non-proportional relationship between the microbial cell counts and the degree of turbidity grade, the water and solid particles contents. Comparing oils sample at the different separation treatments, is possible to say that the microbial cell count was mainly affected by the water content decrease. The FO samples were without microorganisms; the SO and WO samples had a surviving microbial population (i.e. 1.3 and 2.4 log UFC/g, respectively), but not significantly different from the FO samples. Consequently, the A_w of oil samples was significantly affected by the applied separation treatments (Tab.1). A_w values of the FO, SO and WO samples were <0.60, that is not suitable for microbial activity.

Oil samples at the different separation treatments	Degree of turbidity (NTU)	Water content (%w/w)	Solid particles content (%w/w)	A_w	Microbia I cell count (log UFC/g)	Total phenolic compounds
	***	***	***	***	***	***
VO	1296 a	0.24 a	0.23 a	0.68 a	3.8 a	708 ab
FO	15 c	0.05 c	0.00 b	0.43 c	n.d. b	559 c
SO	181 b	0.03 d	0.2 a	0.37 c	1.3 b	678 b
WO	59 c	0.11 b	0.00 b	0.53 b	2.4 b	736 a

Table 1 Mean value of the 6 oils for each treatment is reported. Different letters in the same column indicate significant differences ($p < 0.05$). In second row is reported the ANOVA results related to each analysis. * = $p < 0.05$; ** = $p < 0.01$; ***

Figure 1 Confocal scans of a VO sample with fluorescent probe. Panel A: overlay of the 3D scans obtained in fluorescent and transmission mode. Panel B: 3D scan in fluorescent mode.



The comparison of the oil samples in relation to the applied separation treatments show a significant effect on phenolic compounds content (Tab.1). Due to the affinity of phenolic compounds towards the water phase, the removal of water by a filtration treatment (FO) led to an approx. 20% decrease in the total phenolic content as compared to VO samples. In this study, the role of water is evidently important. For these reasons, CLSM was performed to observe the distribution of water inside the oil samples. Confocal images of a VO samples (Fig.1) show a water distribution in the form of (i) droplets dispersed in the oil phase, (ii) droplets and a water film adsorbed of the surface of solid particles. More investigations have to be done.

3.2 Shelf-life analysis of filtered and veiled EVOO subjected to HPP treatment

HPP was used as instrument to discriminate between the action of microorganisms and the effects of water and suspended solids. After 15 days of storage, only CON oils had measurable yeast content. Thus, both treatments were able to remove microorganisms: filtration through the removal of water and suspended solids, while HPP inactivating them using the high pressure. Furthermore, in the filtered samples water and solids were removed, and consequently a decrease of water activity was achieved with filtration. During the storage filtered samples (with and without HPP) showed the least phenolic fraction degradation and the lowest production of off-flavor compounds. In HPP samples the formation of fusty compounds was prevented, underling the role of microorganisms in the production of fusty defect during shelf life. On the other hand, HPP and CON samples showed a faster phenol degradation, highlighting the role of water in olive oil quality decrease.

4. Future activities

The future activities expected for my PhD include: (A) shelf-life analysis of oils with controlled turbidity grade obtained by mixing filtered oils with cloudy oils. This study could be usefull to undersand the behavior of oils with different content of water, solid particles, water activity, microorganism count and phenolic compounds content; (B) more extensive characterization of EVOO turbidity through the development of new methods for the determination of sugar, proteins and phospholipids.

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Application of Not Conventional Yeasts in Food Related Bioprocesses

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Tutor: Concetta Compagno

This report describes the first two activities of my PhD project. Using flow cytometry, we investigated the mechanisms involved in osmotic stress response. We saw that high salts concentration in elicits membrane depolarization and decreased membrane permeability in *Debaryomyces hansenii*. Successively we evaluated phytase activity in yeasts that can be employed to perform food related bioprocesses and we saw that, in all the analyzed strains, extracellular activity was always lower than the intracellular one.

Applicazione di lieviti non convenzionali per lo sviluppo di bioprocessi alimentari

Questo report descrive le prime due attività del mio progetto di dottorato. Grazie all'utilizzo della citometria a flusso, sono stati caratterizzati i meccanismi coinvolti nella risposta allo stress osmotico in *Debaryomyces hansenii*. Abbiamo visto che un'alta concentrazione di sali causa una depolarizzazione della membrana e ne diminuisce la permeabilità. Successivamente è stata valutata l'attività fitasica in lieviti che possono essere utilizzati in processi alimentari. È stato visto che in tutti i ceppi analizzati l'attività extracellulare risulta essere sempre più bassa di quella intracellulare.

Key words: not conventional yeast, osmotic stress, flow cytometry, membrane depolarization, enzymatic activity, phytic acid, antinutritional factors.

1. Introduction

In accordance with the PhD thesis project previously described (Capusoni, 2018), this poster reports the main results of the first two activities.

A1) The ability to grow in presence of high salt concentrations is very important in order to select good microorganisms for industrial application. To identify a good candidate to be developed into a "cell factory", a marine yeasts collection (Burgaud et al., 2010) has been characterized on the ability to grow in presence of high NaCl concentration. The mechanisms involved in osmotic stress resistance have been deeply investigated, in the most resistant strains, using flow cytometry (FC).

A2) Phytase are class of enzyme able to hydrolyze phytic acid (storage form of phosphorus in seeds). This molecule is classified as an antinutritional factor due on its ability to chelate metals. Monogastric animals are not able to metabolize this acid that accumulates in the environment leading to eutrophication. For this reasons phytase are commonly employed as feed additive. This enzymatic activity has been investigated on the strains selected during A1 and on other interesting yeasts species. Indeed, the latter, can be used in in food related bioprocess.

2. Materials and Methods

A1) The initial screening in presence of NaCl (ranging from 0.5 to 2 M) was performed on plates containing minimal medium Yeast Nitrogen Base (YNB glucose 2%, agar 2%, pH 6). Broth cultures were performed at 28 °C in flasks at 150 rpm. Cells were preinoculated in YNB and inoculated at OD_{600nm} 0.1 into the same medium supplemented or not with 4% (w/v) sea salts SS, or 2 M NaCl. To apply hyper-osmotic stress, cells growing in YNB were shifted in flasks containing 2 M NaCl, for 30 minutes

and 2 hours. For hypo-osmotic stress, cells growing in YNB 2 M NaCl were shifted in YNB for 30 minutes and 2 hours. The effect of salts on cell membrane permeability and on membrane potential was assessed by FC staining with SYBR Green I and DiBAC₄(3) respectively (Capusoni et al, 2019).

A2) The ability to degraded phytic acid was evaluated on *D. hansenii* strains selected in A1 and other yeasts species. Cells were precultured in YPD (1% Yeast extract, 2% Peptone, 2% D-glucose) and inoculated at 1 OD₆₀₀/mL in minimal media with 0.11g/L phytic acid as sole phosphate source and harvested at 10 OD₆₀₀/mL. Enzymatic activity was estimated on supernatant and using whole cells resuspended at 10 OD/mL in reaction buffer at 37°C by ammonium molybdate blue method (Klosowski et al 2018).

3. Results and Discussion

3.1 Osmotic stress response in *Debaryomyces hansenii*

A marine yeast (MYs) collection has been characterized on the ability to grow on solid medium containing NaCl. This analysis revealed that *D. hansenii* (BIO 2 and Mo40 strains) is among the most tolerant. This specie, is included in QPS EFSA (European Food Safety Authority) list and for this reason is exploitable in food bioprocess. Strains' halotolerance has been investigated carrying out broth culture in presence or in absence of SS or 2M NaCl. Table 1 shows that, in both strains, presence of SS did not affect growth in terms of rate, but slightly decrease biomass yield, while the presence of 2 M NaCl had not only a negative effect on both growth parameters but also on glucose consumption rate.

Table 1 Growth parameters of *D. hansenii* BIO2 and Mo40 cultivated in presence of sea salts (SS) and 2 M NaCl

	Bio 2				Mo40			
	μ_{max}	Biomass yield (Y)	q Glucose	Final biomass	μ_{max}	Biomass yield (Y)	q Glucose	Final biomass
	[h ⁻¹]	[g _{dw} /g _{glc}]	[mmol _{glc} /g _{dw} /h]	[g/L]	[h ⁻¹]	[g _{dw} /g _{glc}]	[mmol _{glc} /g _{dw} /h]	[g/L]
YNB	0.26 ± 0.01	0.42 ± 0.02	2.52 ± 0.03	8.88 ± 0.24	0.28 ± 0.01	0.39 ± 0.01	2.71 ± 0.13	7.71 ± 0.14
YNB SS	0.26 ± 0.01	0.36 ± 0.01	2.56 ± 0.04	7.45 ± 0.64	0.31 ± 0.01	0.37 ± 0.01	2.59 ± 0.13	7.90 ± 0.19
YNB 2M NaCl	0.15 ± 0.02	0.37 ± 0.01	1.95 ± 0.13	7.40 ± 0.14	0.16 ± 0.04	0.34 ± 0.02	2.05 ± 0.11	6.61 ± 0.16

Osmotic stress response has been further investigated by using FC. Looking at Figure 1 A is possible observe by staining cells with SYBR Green I, how the growth in presence of high salt concentration causes a decrease in membrane permeability. Figure 1B shows how the presence of salts causes membrane depolarization.

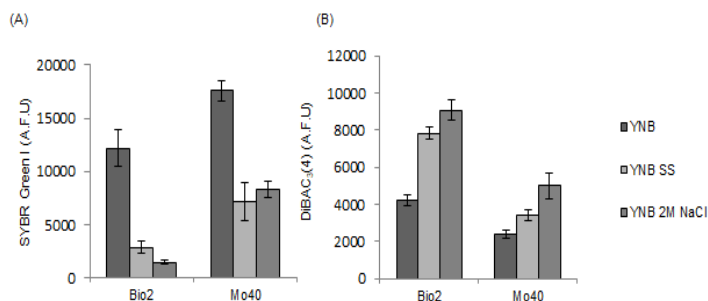


Figure 1 FC quantitative analysis of (A) SYBER Green I (B) DiBAC₄(3). Cells were collected in exponential phase in YNB, YNB SS and YNB 2M NaCl. (A.F.U. Arbitrary Fluorescence Units).

It is possible to hypothesize that a decrease of membrane permeability due to adaptation at high salt concentration is strictly associated to plasma membrane depolarization. This physiological response is very fast indeed it can be detected also after 30 min of salt exposition (hyper-osmotic stress). Successively, it was observed that the decreasing of membrane permeability and membrane depolarization are reversible phenomena. Indeed, the elimination of osmotic pressure during hypo-osmotic stress causes a rapid restoration of membrane polarization and restores cellular permeability, underlining how coordination of these mechanisms is fundamental in the control of cellular homeostasis.

3.2 Screening of phytase activity

Extracellular and intracellular phytase activities were investigated initially on 3 strains of *D. hansenii*: 2 identified from A1 and 1 isolated from gorgonzola cheese. The calculated activity was very low and for this reason a new screening was set up. We selected 20 strains belonging to several yeasts' genera (*Candida*, *Hanseniaspora*, *Dekkera*, *Torulaspora*, *Kluyveromyces* and *Zygosaccharomyces*) all exploitable in food process. Like reported in Table 2, in each strain, the measured extracellular activity was always lower than the intracellular one.

Table 2 Extracellular and intracellular phytase activity (BDL Below Detection Limit).

	<i>Z. kombutchensis</i> CBS8849 <i>K. lactis</i> CBS2395 <i>K. marxianus</i> Y1058 <i>H. uvarum</i> UMY 514 <i>H. uvarum</i> UMY 571 <i>D. bruxellensis</i> CBS 2499 <i>L. thermotolerans</i> NRRL Y8284 <i>T. delbrueckii</i> CBS 1466 <i>D. hansenii</i> BIO2 <i>C. utilis</i> M									
Extracellular (mU/mL)	BDL	BDL	9	BDL	BDL	BDL	BDL	BDL	6	164
Intracellular (mU/mg d.w.)	10	5	67	2	2	14	5	53	4	493

My PhD project will proceed characterizing the effect of pH and temperature on enzymatic activity and stability, the stereoselectivity and the substrate specificity. A good candidate is an enzyme able to work in animal's stomach (37°C and low pH) and stable during feed production process (high temperature). A preliminary screening shows us that MYs don't have nucleoside phosphorylase and denitrase activities. For this reason, we have decided to focus our attention on phytase leading a deep enzymatic characterization.

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Production of a Ready-to-Use Natural Antimicrobial with Application in the Wine Industry

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The main aim of this PhD thesis research project is the characterization, heterologous production and utilization of Kpkt. Naturally produced by the yeast *Tetrapisispora phaffii*, Kpkt is a killer toxin active against spoilage yeasts of interest for the wine industry. The research project is articulated as follows: modulation of Kpkt heterologous production in *Pichia pastoris* through the production of new expression vectors, optimization of Kpkt production in bioreactor, purification of Kpkt, evaluation of purified Kpkt effect on human cell lines, pilot scale production of Kpkt and utilization of Kpkt at winery scale. The second year was focused on Kpkt production in bioreactor and purification and on the evaluation of its effect on human cell lines.

Produzione di un antimicrobico naturale pronto all'uso con applicazione nell'industria enologica

Questo progetto di tesi di dottorato mira alla caratterizzazione, produzione e utilizzazione su scala pilota di Kpkt. Naturalmente prodotta dal lievito *Tetrapisispora phaffii*, Kpkt è una glicoproteina killer in grado di uccidere lieviti alterativi di interesse nell'industria enologica. Il progetto di ricerca è stato articolato come segue: modulazione della produzione eterologa di Kpkt in *Pichia pastoris* attraverso la costruzione di nuovi vettori di espressione; ottimizzazione della produzione di Kpkt in bioreattore, purificazione di Kpkt, studio del suo effetto su linee cellulari umane, produzione di Kpkt su scala pilota e sua utilizzazione in cantina. Il secondo anno è stato dedicato alla produzione di Kpkt in bioreattore, alla sua purificazione e alla valutazione del suo effetto su linee cellulari umane.

Key words: Kpkt, natural antimicrobials β -Glucanase, wine spoilage yeast, *Pichia pastoris*.

1. Introduction

In accordance with the PhD thesis project, the main results of the second year activities regard the following activities: **(A1)** Research of cultural parameters that maximize production of recombinant Kpkt in bioreactor; **(A2)** Recombinant Kpkt purification; **(A3)** Evaluation of the effect of purified Kpkt on human cell lines.

2. Materials and Methods

A1) For the production of recombinant Kpkt (rKpkt), *P. pastoris* clone #17 was pre-cultivated in 50 ml of BMGY culture medium (1% Yeast extract, 2% Peptone, 1.34% YNB,

4 × 10⁵ 5% biotin, 5% glycerol, phosphate citrate buffer pH 4.5), in 300 ml baffled flask. After 24 h at 30°C under agitation (180 rpm), the pre-culture was inoculated in 1.5 L of BMGY in bioreactor (Biostat Cplus, Sartorius). After 24 h at 25°C, 200 ml of 40% glycerol was added to reach an OD₆₀₀=400. After complete glycerol consumption (as indicated by GC analysis), rKpkt production was induced by adding methanol according to the fed batch technique. Methanol was added manually for 72 h so that its concentration never went below 0.5% (as confirmed by GC analysis). The cultivation took place at 20°C, stirring was regulated in cascade with the concentration of dissolved oxygen which was set at 25%. After 72 h the culture was centrifuged and microfiltered. For the production of native Kpkt, *T. phaffii* DBVPG 6076 was grown in 1.6 L of YPD (pH 4.5) at 25°C for up to 30 h. Stirring was regulated in cascade with the concentration of dissolved oxygen which was set at 20%. Cell free supernatant was utilized for well plate assay to assess the production of Kpkt and rKpkt as already reported (Chessa et al., 2017).

A2) For rKpkt purification a 10 ml Econo-Pac Disposable Chromatography Column (BIORAD) was used. The purification resin (TALON Metal Affinity Resin, Takara) was charged with cobalt ions and the purification protocol was based on the affinity between the His-Tag and the cobalt ions. Briefly, cell free supernatants were concentrated by ultrafiltration (cut-off 10 kDa) and 1 ml of 10X concentrated supernatant was loaded onto the purification column. The target protein was eluted with Imidazole elution buffer (Bio-Rad). Eluted fractions were loaded on SDS-PAGE (12% agarose) and tested on plate for killer activities.

A3) For the evaluation of the effect of native and recombinant toxins on human cell lines HaCat cells were cultured in liquid DMEM (Dulbecco's Modified Eagle Medium) containing phenol red, 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate for maintenance and propagation *in vitro*. The cells were grown at 37 °C, in the presence of 5% CO₂ at pH 7.4 and challenged with increasing concentrations of preparation of Kpkt and rKpkt. After 24h of incubation with the toxin the cells were treated with 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium (MTT) bromide and OD₅₁₂ was evaluated.

3. Results and Discussion

3.1 Research of cultural parameters that maximize production of rKpkt in a laboratory bioreactor.

The effects of different concentrations of protease inhibitors, carbon sources for biomass production and methanol feeding protocols were evaluated in order to maximize rKpkt production in bioreactor. While the concentration of protease inhibitors did not impact on rKpkt production and stability, the utilization of glucose, in place of glycerol, proved useful for the production of biomass prior to methanol induction. Methanol feeding was optimized in order to maintain its concentration $\geq 0.5\%$ (as confirmed by GC analysis) during cultivation.

3.2 Recombinant Kpkt purification

rKpkt coding gene harbours a His-Tag (consisting of six histidine residues) in C-terminus, thus affinity chromatography was utilized for the purification of the protein. The lack of killer activity in the fractions eluted by the column indicate the impossibility to purify

rKpkt with this technique, in spite of the several different attempts. Thus, immunoblotting techniques (western Blot, Dot-Blot) were applied to highlight rKpkt in the cell free supernatant of clone #17. For that two anti-His-Tag antibodies (rabbit monoclonal anti-His-Tag antibody and anti-rabbit secondary antibody or monoclonal Anti-polyHistidine–Peroxidase conjugated antibody, Sigma) and different techniques for the preparation of the samples were utilized (heat treatment at 90 or 95 °C for 5 minutes; no heat treatment). Also in these cases the two antibodies failed to highlight rKpkt in the supernatant, thus suggesting that either the tagged protein is expressed at low levels and require more than 100-fold enrichment, or the protein structure occludes the His-Tag, or His-Tag is degraded. Due to the need to obtain a ready-to-use natural antimicrobial preparation a different approach was utilized and applied to both Kpkt and rKpkt. Briefly, cell free supernatants containing native or recombinant proteins were concentrated by ultrafiltration (cut-off 10 kDa) and subject to freeze drying. The evaluation of both killer and β -glucanase activities of the lyophilized preparations indicated that this protocol leads to obtain stable and concentrated Kpkt preparations. In parallel Kpkt was produced in bioreactor according to the method described by Comitini et al. (2004).

3.3 Evaluation of the effect of Kpkt and rKpkt on human cell lines.

Seven different increasing Kpkt and rKpkt concentrations were used to evaluate their toxicity on HaCat cell lines. After 24h of incubation with the toxin the cells were treated with 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium (MTT) bromide. The MTT test is a standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan, giving the substance a blue/purple color. The blue-violet color is obtained when the cells are alive and process the MTT. The yellow color occurs when the cells have died and do not process MTT. Results are visible to the naked eye but live/dead cells were evaluated based on OD₅₁₂. Based on this test native and recombinant protein samples proved not toxic for human cell lines. This is an excellent result in view of Kpkt possible use as an antimicrobial agent in the oenological field.

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Functional Vegetable Ingredients and their Applications in Complex Food Matrices

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Functional vegetable ingredients are of high interest for the food industry for their technological effect and the increased consumer request of “natural” ingredients. Two new ingredients were assessed. The first was as physically-modified corn flour, which was firstly physico-chemical characterised and subsequently applied for fat reduction in a mayonnaise. The second was a highly viscous fiber-based syrup used as sugar replacer in an apricot filling for bakery products. Results highlighted the improved water-solids interaction functionality of the flour and its technological effect as fat replacer. Additionally, the “bulking agent” effect of the syrup in substitution of the sugar was confirmed.

Ingredienti vegetali funzionali e loro applicazione in matrici alimentari complesse

Gli ingredienti vegetali funzionali sono di grande interesse per l'industria alimentare per il loro effetto tecnologico e la tendenza dei consumatori verso prodotti più naturali. Tale progetto di dottorato ne ha valutato due: una farina di mais fisicamente modificata, inizialmente caratterizzata da un punto di vista chimico-fisico, successivamente usata per produrre maionesi con minor grassi e uno sciroppo semi-solido, a base di fibre vegetali, per la riduzione dello zucchero in un ripieno all'albicocca. Gli studi hanno evidenziato un'incrementata capacità di interazione con l'acqua della farina, la sua funzionalità tecnologica nelle maionesi e l'effetto strutturante in sostituzione dello zucchero dello sciroppo.

Key words: physically modified corn flour, reduced-fat mayonnaise, semi-solid fiber syrup, reduced-sugar.

1. Introduction

In accordance with the PhD thesis project previously described (Carcelli, 2018), this poster reports the main results of the first three activities concerning:

A1) physicochemical characterisation of physically modified corn flours (PMF) using a multidimensional and multianalytical approach;

A2) development and characterization of a fat reduced mayonnaises using PMF as fat replacer;

A3) development and characterization of a sugar reduced apricot filling for bakery application using a commercial ingredient semi-solid syrup, based on natural fibers from legumes and cereals

2. Materials and Methods

A1) Two physically modified corn flours [heated in a conventional cooker (M_1) and combination of heating and extrusion process (M_2)] and a native corn flour used as standard (C) were studied. C, M_1 and M_2 were initially characterized by amylose and resistant starch (RS) contents and flour hydration properties (WHC, WBC). Subsequently, flour-water interaction was examined at different water concentration (from 1:3 to 1:9) and

temperature (25°C and 100°C). Pastes such obtained were analysed using macroscopic [bostwick consistency, hardness (Texture Analyzer)] mesoscopic [G', G'' (rheometer)] and molecular [¹H molecular mobility (Low Resolution ¹H NMR)] level of investigation.

A2) A fat-replacer was prepared by heating (90°C) and mixing (25 min) M₂ in water (1:9 concentration). From a standard full-fat (FF, 80% oil) three mayonnaises with reduced fat amount (FA) were produced (FA60%, FA40%, FA25% oil) substituting oil with fat-replacer. Mayonnaises (as it, and treated at 65°C for 5 min) were characterized for physico-chemical [(hardness (Texture Analyzer); G' and G'' (rheometer), L*, a*, b*, ΔE (colourimeter), stability (centrifuge test and long term test)] and sensorial attributes (CATA and acceptability tests, 50 untrained judges).

A3), was used for its bulking agent effect to reduce sugar in apricot filling for bakery applications. Fillings were developed by partially replacing sugars ingredients (crystalline sucrose, glucose syrup) with MELTEC® in a 1:1 ratio, keeping pH and Brix constant, from a standard full sugar (FS) recipe achieving a 30, 50 and 70% sugar reduction (SR). Fillings were analysed for their stability (centrifuge test, bake stability), physico-chemical [hardness, adhesiveness (Texture Analyzer); apparent viscosity (rheometer); L*, a*, b*, ΔE (colourimeter); a_w (25°C, igrometer)] and sensorial attributes (CATA and acceptability tests, 50 untrained judges).

ANOVA with a Duncan post-hoc test was used to identify significant differences ($p \leq 0.05$) among samples. A Principal Component Analysis (PCA) was also performed (SPSS version 25.0, IBM SPSS Inc., Armonk, New York, USA) to analyse data of CATA test.

3. Results and Discussion

3.1 Physicochemical characterisation of physically modified corn flours (A1)

The amylose content in all flours (C, M₁, M₂) was about 27% and found comparable among samples, while a decrease in RS content was found when flour was physically treated (with no significant differences between M₁ and M₂ samples). WHC and WBC were found higher in treated samples than C with M₂ > M₁. Physical treatments had possibly altered starch granule integrity leading to a less ordered molecular structure with hydroxyl groups able to bind more water molecules (Camire et.al., 1990). Subsequently, flours ability to form homogenous pastes with water at different water concentration and temperature was assessed (Table 1). M₂ showed the higher ability to form pastes with water, with no syneresis in cold condition. In hot conditions, an increase in the ability of all flours to interact with water was attributed to a partially starch gelatinization, as expected. Higher Bostwick consistency, hardness value and G' was noticed for pastes obtained by M₂ in cold and hot condition and all samples had a solid like behavior (G' > G''). At molecular level M₂ pastes showed more rigid structure which can explain the higher texturing effect of the flour. Overall, results higlihted the ability of the multiscale method to have a global overview of the flour-water interactions and showed a higher water affinity of M₂, if compared with M₁ and C.

Table 1 *Flours ability to form homogenous paste with water at different temperature and concentration.*

Temperature (°C)	Flour	Concentration						
		1:3	1:4	1:5	1:6	1:7	1:8	1:9
25	C	X	X	X	X	X	X	X
	M1	V	V	X	X	X	X	X
	M2	V	V	V	X	X	X	X
100	C	V	V	V	X	X	X	X
	M1	V	V	V	V	V	V	V
	M2	V	V	V	V	V	V	V

V: homogenous pastes without syneresis X: water syneresis

3.2 Development and characterisation of a fat reduced mayonnaise using a physically modified corn flour as fat replacer (A2)

The technological functionality of M₂ was tested for the production of a fat replacer gel that has been used for the production of mayonnaise with reduced FA. Emulsion of all mayonnaises were well formed and had acceptable consistency, only exception FF which was unstable after heating. High and comparable emulsion stability was noticed among all samples. Sample with FA25% had hardness and G' comparable to FF, while FA40% and FA60% had higher hardness and G' than the other products. Rheological results overall corroborate the fat replacer ability to counterbalance absence of fat with the formation of a tridimensional structure created by the gel that surround the oil drops (Liu H. et al., 2006). Colour differences (ΔE) were observed between the full and reduced fat products due to lowering of a* and b* with reduced fat amount increase. Overall sensory acceptability was positive for all samples, with FA40% mayonnaise being the most preferred by a consumer panel (Figure 1-A). Reduced-fat mayonnaises were perceived of a paler coloured with a less persistent flavour than FF product. M₂ confirmed its technological functionality showing good results as fat replacer in reduced fat mayonnaise.

3.3 Development and characterization of a sugar reduced apricot filling for bakery application using a semi-solid syrup, MELTEC® (A3).

All fillings prepared were stable and well formed. SR70% had higher hardness, apparent viscosity, and adhesiveness compared to other fillings that did not differ among themselves. All SR products had a different color than full sugar (FS) counterpart due to lowering of a* and b* caused by the use of MELTEC®. Water activity increased in all SR samples as sugar was replaced with longer chain fibers which have lower water binding properties. Overall sensory acceptability was positive for all samples (Figure 1-B); in particular, the SR30% had acceptability comparable to the standard product for colour and taste and, a slightly lower for consistency. MELTEC® was found to be a valuable sugar replacer on a technological standpoint, as its use did not jeopardize workability and consistency of SR products. Additional tests in different food matrices may confirm its applicability.

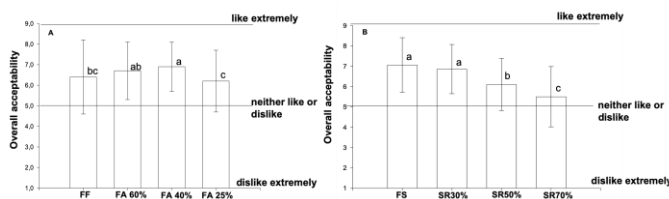


Figure 1 A - Overall acceptability of mayonnaises (A2); B - Overall acceptability of sugar reduced apricot fillings.

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Analytical Instrumental Approaches and Strategies to Support the Sensory Assessment of Virgin Olive Oils

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The first two activities of my PhD project are focused on the study of the relation between the main volatile compounds present in virgin olive oils and the related unique sensory attributes. Specifically, a protocol related to a SPME-GC-FID method for the identification and quantification of volatiles selected as markers of sensory attributes is presented as well as the development of a rapid screening method by HS-GC-IMS to support the Panel Test and aimed to discriminate samples based on the quality grades.

All the activities of this PhD project are developed in the context of the project OLEUM "Advanced solutions for assuring authenticity and quality of olive oil at global scale", funded by the European Commission within the Horizon 2020 Programme (2014–2020, GA no. 635690).

Approcci strumentali analitici e strategie per supportare la valutazione sensoriale degli oli vergini di oliva

Le prime due attività del mio progetto di dottorato sono incentrate sullo studio della relazione esistente tra i principali composti volatili presenti negli oli vergini di oliva e gli attributi sensoriali peculiari del prodotto. Nello specifico, lo studio è focalizzato sulla messa a punto e validazione in-house di un protocollo analitico SPME-GC-FID per l'identificazione e quantificazione di molecole volatili selezionate quali principali traccianti degli attributi sensoriali e sullo sviluppo di un metodo rapido di screening attraverso HS-GC-IMS a supporto del Panel Test e finalizzato alla discriminazione della qualità di oli vergini di oliva.

Key words: virgin olive oil, volatile compounds, sensory attributes, in-house validation.

1. Introduction

In accordance with my PhD thesis project, these are the main results of the first two activities concerning:

A1) analysis of the profile in volatile compounds of virgin olive oils by SPME-GC-FID: joint analytical protocol and in-house validation of the method.

A2) development of a rapid screening method (e.g. HS-GC-IMS) to support the Panel Test: building of a chemometric model to discriminate virgin olive oils, in particular "extra virgin" vs. "not-extra virgin" samples.

2. Materials and Methods

2.1 Analysis of the volatile compounds by SPME-GC-FID (A1)

During the first year of the OLEUM project, 180 commercial virgin olive oil samples were collected. A sub-set (60 samples) was considered for this preview investigation. Six panels evaluated all the oils, then, applying a decision tree developed in the context of the project, were classified in 12 EV (extra virgin), 30 V (virgin) and 18 L (lampante) and the volatile compounds were analyzed by SPME-GC-MS and SPME-GC-FID. Based on the statistical analysis (Cochran's C test, PCA), applied on sensory and instrumental data, it was possible to remove the outlier's samples as well as the less relevant volatile compounds (ND/< LOQ, Box & Whisker plots). The Analysis of Variance (ANOVA, $p <$

0.05) selecting a Browne-Forsythe test was also applied to select volatile compounds with marked effects on the three categorical variables (EV, V, L). Each involved OLEUM partner (SPME-GC-MS and SPME-GC-FID: 5 and 3, respectively) provided the obtained results of this selection process performed on its own dataset. For combining sensory and instrumental results a Multiple Factor Analysis (MFA) was performed with a dataset consisting of 54 sample and 32 volatile compounds, fruity and sensory defects (Fig. 1). This statistical approach was useful for the selection of 18 molecules mainly responsible for sensory attributes. During the second year of the OLEUM project, 152 commercial virgin olive oils sample were collected, a sub-set (60 samples) was considered and oils were classified in 27 EV, 20 V and 13 L by sensory analysis. Then, a joined protocol by SPME-GC-FID was developed and its in-house validation is now in progress. The main parameters of the method are summarized below: 1.900 ± 0.001 g of virgin olive oil weighted in a 20 mL glass vial plus 0.100 ± 0.001 g of 4-methyl-2-pentanol added as internal standard (IS approximate concentration = 2.5 mg/kg); the vial (hermetically closed with PTFE septum) was conditioned for 10 min at 40°C under agitation; fiber was exposed for 40 min then was inserted in the hot GC-injector port; volatiles were thermally desorbed 5 min at 260 °C with the purge valve off (splitless mode), eluted onto a capillary column (WAX: length: 60 m; I.D.: 0.25 mm; f.t. 0.5 μ m, Thermo Fisher Scientific) and detected by a flame ionization detector (FID). The carrier gas was helium at a flow rate of 1.5 mL/min. The oven temperature was held at 40°C for 10 min and then programmed to rise at 3°C/min to a final temperature of 200°C. A cleaning step was also added (20°C/min to 250°C for 5 min). To evaluate the performance of the method, several in-house validation parameters as repeatability, reproducibility, recovery, precision, limit of detection (LOD) and limit of quantification (LOQ), as well as the related linearity for each one of the 18 selected volatile compounds, will be determined.

2.2 Development of a rapid screening method by HS-GC-IMS (A2)

The same 2 sets of samples (60 + 60) described in paragraph 2.1 were analyzed by HS-GC-IMS with the aim to build a model able to discriminate different quality grades of virgin olive oils. Data processing involved sequential operations, starting from the export of raw data, obtaining a very large data matrix. For this reason, an important step concerned its reduction, going to select topographic zones of the chromatogram containing most of the information and then applying a subsequent periodic sampling of 10% obtaining a data matrix of 63000 variables. Subsequently, the selected spectra were arranged consecutively. Moreover, it was necessary to align the peaks, applying a smoothing procedure and a correction of the baseline. The standardized and centered data were then submitted to PCA (The Unscrambler 9.7 software) in order to further reduce the size of the matrix. Using the first 24 main components as independent variables, a PLS-DA (Partial Least Square–Discriminant Analysis) was performed. In particular, two PLS-DA elaborations were developed for two classes (EVOO vs NoEVOO and LOO vs NoLOO). Samples NoEVOO and NoLOO were subsequently classified in VOO vs LOO and EVOO vs VOO (Fig. 2). Finally, all models were validated by a full cross-validation.

3. Results and Discussion

3.1 Analysis of the volatile compounds by SPME-GC-FID (A1)

The correlation circle (Fig. 1) shows a projection of the variables in the factors plan and represent 32.11% of the total variability. When the projection of variables in the factors space is short, we can assume that these variables do not explain well the samples, so they are not relevant. The sensory defect of fusty-muddy sediment, found in 29 samples, was positively correlated with octane ($r = 0.568$), 3-methyl-1-butanol ($r =$

	Calibration	Full Cross Validation
EVOOs	100%	100%
NoEVOOs	100%	75%
LOOs	89%	78%
NoLOOs	91%	81%
NoEVOOs		
VOOs	96%	90%
LOOs	83%	72%
NoLOOs		
EVOOs	100%	83%
VOOs	77%	73%



Nutritional Characterization of Emerging Food Products: Microgreens

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The aim of this study was to evaluate the nutritional profile of six microgreens belonging to three species and two families: chicory (*Cichorium intybus* L., Puglia's local variety 'Molfetta', CM, and cultivar 'Italico a costa rossa', CR) and lettuce (*Lactuca sativa* L. Group *crispa*, cultivar 'Bionda da taglio', LB, and 'Trocadero', LT), from *Asteraceae*; and broccoli (*Brassica oleracea* L. Group *italica* Plenck, Puglia's local variety 'Mugnuli', BM, and cultivar 'Natalino', BN) from *Brassicaceae*.

Caratterizzazione Nutrizionale di Prodotti Alimentari Emergenti: Micro-ortaggi

Lo scopo di questo studio è stato valutare i diversi aspetti compositivi e il profilo nutrizionale di sei genotipi di micro-ortaggi, appartenenti a tre specie e due famiglie: cicoria (*Cichorium intybus* L., varietà locale pugliese 'Molfetta', CM, e cultivar 'Italico a costa rossa', CR) e lattuga (*Lactuca sativa* L. var. *crispa*, cultivar 'Bionda da taglio', LB, e 'Trocadero', LT), da *Asteraceae*; e broccoli (*Brassica oleracea* L. var. *italica* Plenck, varietà locale pugliese "Mugnuli", BM e cultivar "Natalino", BN) da *Brassicaceae*.

Key words: Microgreens, proximate composition, mineral, bioactive compounds.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the following activity:

A1) Evaluate the proximate composition, mineral content and bioactive compound content of six microgreens, belonging to three species (*Cichorium intybus* L., *Brassica oleracea* L. Group *italica* Plenck, and *Lactuca sativa* L. Group *crispa*), including two Italian local varieties.

2. Materials and Methods

Harvested microgreens were freeze-dried and then used for the analyses. The dry matter (DM) concentration was measured with an automatic moisture analyzer at 105 °C. Proximate analysis of samples was carried out as follows: ashes were determined by using a muffle furnace according to AOAC method 923.03; protein content ($N \times 6.25$) was determined by the Kjeldahl nitrogen method according to AOAC method 955.04; fat content was determined after Soxhlet extraction according to AOAC method 920.39; dietary fiber content was determined by an enzymatic–gravimetric procedure according to AOAC method 991.43; sucrose, fructose and glucose were determined by an enzymatic assay (K-SUFRG, Megazyme, USA); total carbohydrates were calculated by the difference of protein, lipid and ash on a dry matter basis. Mineral content was determined by TXRF and ICP-AES, according to Allegretta *et al.* (2019). Total chlorophyll (TCH) content was determined spectrophotometrically using the method of Lichtenthaler and Buschmann (2015) with minor modifications. Antiradical activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical scavenging capacity test, according to Difonzo *et al.* (2015). Total phenolic compounds (TPC) were determined on the same methanolic extract by the Folin–Ciocalteu assay. Total anthocyanins (TA) were extracted with 70:29.5:0.5 (v/v) methanol/water/HCl (37%). The absorbance of the

solution was determined at 535 nm. Alpha-tocopherol (α -T) extraction was performed according to Santos *et al.* (2012). Total carotenoids (TC) were determined spectrophotometrically using Lichtenthaler's formula.

3. Results and Discussion

3.1 Proximate composition of microgreens

The proximate composition of the microgreens is reported in Table 1. The DM content shows no significant differences among the microgreens ($6.0 \text{ g } 100 \text{ g}^{-1}$, as an average). As expected, the lipid content was negligible and comparable to the typical values of common leafy vegetables. Proteins were higher in *Brassicaceae* than in *Asteraceae* microgreens, reaching a maximum amount of $3.0 \text{ g } 100 \text{ g}^{-1}$. Low levels of dietary fibre were also found: in particular, the chicory genotypes exhibited the highest levels, not exceeding $0.70 \text{ g } 100 \text{ g}^{-1}$. Sugars reached the maximum amount in CR ($0.80 \text{ g } 100 \text{ g}^{-1}$) and the minimum level in BN ($0.19 \text{ g } 100 \text{ g}^{-1}$). Finally, the ashes reach values around $1 \text{ g } 100 \text{ g}^{-1}$, in agreement with the data reported in the literature.

Table 1 Proximate composition of microgreens ($\text{g } 100 \text{ g}^{-1} \text{ FW}$) and results of the statistical analysis. Different letters in the same row indicate a significant difference according to Tukey's test ($P < 0.05$). LOD, limit of detection.

	CM		CR		BM		BN		LB		LT	
Dry matter	6.4±0.7	a	6.3±0.7	a	6.8±0.6	a	6.0±0.9	a	5.2±0.8	a	5.5±0.6	a
Lipids	0.3±0.0	ab	0.3±0.0	b	0.4±0.1	a	0.3±0.0	b	0.3±0.0	b	0.3±0.1	ab
Proteins	1.9±0.0	c	2.4±0.0	b	3.0±0.1	a	2.8±0.1	a	2.6±0.1	b	2.4±0.0	b
Fibre	0.62±0.07	ab	0.70±0.06	a	0.36±0.03	c	0.26±0.0	c	0.43±0.06	c	0.44±0.08	bc
Glucose	0.11±0.01	bc	0.26±0.01	a	0.17±0.05	b	0.04±0.0	c	0.03±0.0	c	0.17±0.0	b
Fructose	0.14±0.07	ab	0.22±0.01	a	0.03±0.01	bc	<LOD	c	0.05±0.01	bc	0.12±0.02	abc
Sucrose	0.19±0.07	bc	0.32±0.01	ab	0.38±0.01	a	0.14±0.04	c	0.12±0.04	c	0.26±0.05	abc
Ashes	0.9±0.1	b	1.1±0.02	ab	1.2±0.1	a	1.1±0.2	a	1.0±0.1	ab	1.0±0.1	ab

3.2 Mineral content

Table 2 shows the results of elemental characterization of *Asteraceae* and *Brassicaceae* microgreens using both TXRF and ICP-AES. Despite differences between the samples, K was the most abundant element in all the microgreens analysed, with concentrations ranging between 2495 (BM) and $4496 \mu\text{g g}^{-1}$ (CR), depending on the genotype. The others most abundant elements in all the samples were, in order, Ca and P (in chicory and lettuce microgreens) followed by S (in the two Brassica varieties). Fe and Mn were the most prevalent micronutrients, in agreement with the outcomes reported by Pinto *et al.* (2015). The concentrations of Cd and Pb in all the samples complied with the safe limits imposed by the EU regulation for leafy vegetables, which amount to 200 and $300 \mu\text{g}$ per kg FW for Cd and Pb, respectively.

Table 2 Mineral content ($\mu\text{g g}^{-1}$ FW) of microgreens and results of the statistical analysis. Different letters in the same row indicate a significant difference according to Tukey's test ($P < 0.05$)

	CM		CR		BM		BN		LB		LT	
P	944±45	ab	1039±44	a	568±17	d	793±17	c	536±29	d	872±53	bc
K	3736±261	b	4496±142	a	2495±103	c	2921±53	c	2865±126	c	4735±372	a
Ca	1272±84	c	1546±31	bc	1878±129	a	2027±23	a	1008±50	d	1466±6	bc
Mg	252±26	a	237±17	ab	207±2	b	220±5	ab	200±16	b	248±8	a
S	588±71	d	708±27	c	1387±19	b	1575±18	a	248±11	e	670±3	cd
Na	105±30	c	80±10	c	227±5	b	256±46	b	474±15	a	73±9	c
Fe	14.1±2.8	ab	12.1±0.4	bc	6.5±0.3	c	5.9±0.2	c	4.3±0.2	c	16.6±0.5	a
Zn	4.4±0.3	b	4.5±0.1	bc	3±0.1	c	4.6±0.1	b	4.7±0.1	b	5.2±0.1	a
Mn	7.5±1	b	6.8±0.9	c	5.3±0.9	c	8±0.1	b	13.2±0.6	a	6.6±0.1	bc
Cu	0.83±0.05	b	1.18±0.01	a	0.29±0.01	d	0.39±0.01	c	0.45±0.02	c	0.86±0.06	b
Ni	0.26±0.01	a	0.29±0.02	a	0.09±0.2	c	0.09±0.01	c	0.1±0.01	c	0.15±0.03	b
Cd	44±3	b	35±3	bc	19±1	c	29±2	bc	61±4	a	74±2	a
Pb	<14		<14		<14		<14		<14		<14	

3.3 Bioactive compound content

Table 3 Bioactive compounds of microgreens. Different letters mean a significant difference at $p < 0.05$ according to Tukey's HSD test for multiple comparisons. 05).

	CM	CR	BM	BN	LB	LT
TCH $\mu\text{g g}^{-1}$	601±50 b	703±65 a	365±42 c	608±31 b	335±11 c	317±21 c
DPPH $\mu\text{mol TE g}^{-1}$	15.2±3.5 cd	12±1.5 d	29.8±1.9 ab	37.1±1.5 a	20.1±0.3 c	22.5±1.7 bc
TPC $\mu\text{g g}^{-1}$	359±8.6 c	252±7.3 d	654.2±11.3 b	791.4±46.1 a	307.7±3.6 c	313.9±0.2 c
TA $\mu\text{g g}^{-1}$	5.31±0.25 e	5.34±0.11 e	17.36±0 b	126.6±0.39 a	8±0.24 d	15.75±0.17 c
α -T $\mu\text{g g}^{-1}$	11.2±1.09 d	27.39±2.33 cd	75.68±7.75 a	48.56±10.5 b	44.76±4.7 c	20.68±1.42 cd
TC $\mu\text{g g}^{-1}$	143±2.25 b	155.2±1.15 a	77.5±2.61 f	92.57±3.05 e	128.8±5.94 c	115.3±8.14 d

As far as bioactive compounds (Table 3) are concerned, chicory (CM, CR) and BN were very rich in chlorophyll. Regarding the hydrophilic antioxidant activity, the 2 *Brassicaceae* (BM, BN) showed high values of both antiradical activity and total phenols. Among the phenolic antioxidants, the anthocyanins produced a significant fraction only in broccoli microgreens (BM, BN). As for lipophilic antioxidants, α -tocopherol was detected in a wide range of concentrations, from 11 to 76 $\mu\text{g g}^{-1}$ FW. Mugnoli microgreens (BM) were the richest in this important bioactive compound. While for total carotenoids there were higher values in the 2 *Asteraceae* (CM, CR, LB, LT).

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Novel Biotechnologies in Dairy Products

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This PhD thesis project aimed to investigate the dairy biotechnology in order to set up different strategies to obtain low-fat traditional cheese with similar sensory characteristic to full fat products, in order to preserve the regional culture. Moreover, the consumption of typical foods increases their future development. Over consumption of dietary fat is associated with various chronic illnesses and it has increased the consumer awareness and demand for low-fat foods, including cheeses.

Biotechnologie innovative nel settore lattiero

Il seguente progetto di dottorato ha riguardato l'investigazione di biotecnologie innovative nel settore lattiero caseario in modo da promuovere differenti strategie per produrre formaggi a ridotto tenore in grasso, mantenendo inalterate le qualità sensoriali ed i tradizionali processi produttivi. Questa necessità di produrre alimenti a ridotto tenore in grasso nasce dalla maggiore consapevolezza dei consumatori riguardo all'impatto che una dieta ricca in grassi possa comportare sulla propria salute.

Key words: Microbiology, fat-replacers, burrata cheese, typical dairy products

1. Introduction

The following poster describes the main outcomes obtained of two main activities concerning:

A1) Polyphasic approach to study physico-chemical, microbiological and volatile organic compounds of a traditional Burrata cheese

A2) Setup of different biotechnological strategies in order to reach a low fat burrata cheese product by changes to the manufacturing process including the use of skim milk, addition of EPS-producing strains and fat replacers.

2. Materials and Methods

A1) Samples of traditional full-fat burrata, a creamy cheese included on the list of traditional agri-food products (PAT) (Minervini et al., 2017), were recovered from a local dairy plant (Igualat, Noci, Bari, Italy), kept at 4°C and analyzed each 5 days during 15 days of storage. Microbiological analyses were carried out as described previously (Minervini et al. 2017). The pH 4.6-insoluble and -soluble nitrogen fractions of the samples were analyzed by urea polyacrylamide gel electrophoresis (Urea-PAGE) and reverse phase high pressure liquid chromatography (RP-HPLC), as described by Di Cagno et al. (2003) and Gobbetti et al. (2002), respectively. The bacterial diversity was estimated by 16S metagenetics analysis carried out at RTLGenomics (Lubbock, TX) using the Illumina MiSeq platform (Minervini et al. 2017)

A2) An integrated approach based on biochemical, microbiological, and sensorial analyses of cheeses was used to obtain burrata cheese with a significative reduction (ca 50%) of fat content compared to full fat cheese. Commercially available freeze-dried *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* were purchased from Clerici-Sacco S.r.l. (Cadorago, Italy). Those strains were

selected based on their capacity to produce exopolysaccharides in milk and cream milk in order to improve product functionality. Powdered xanthan and carrageenan were purchased from Farmalabor S.r.l. (Canosa di Puglia, Italy). Eight types of burrata cheese were manufactured at the industrial plant Ignalat (in a vat of ~200 liters), located in Noci (Bari, Italy) namely, (i) Cheese made with low fat cream milk and governing liquid supplemented with *Lc. lactis* strain (BP); (ii) Cheese made with low fat cream milk added of xanthan and standard governing liquid (BPX); (iii) Cheese made with low fat cream milk added of carrageenan and standard governing liquid (BPC); (iv) Cheese made with low fat cream milk added of xanthan; EPS producing strain and standard governing liquid (BPXE); (v) Cheese made with low fat cream milk added of carrageenan; EPS producing strain and standard governing liquid (BPCE); (vi) Cheese made with low fat cream milk; EPS producing strains added in milk and cream, respectively, and standard governing liquid (BPEL); (vii) Cheese made with low fat cream milk; EPS producing strain added in milk, and standard governing liquid (BPEL2); (viii) Cheese made with low fat cream milk; EPS producing strain added in cream, and standard governing liquid (BPEL3). Traditional burrata cheese was used as control (CC). Experimental burrata cheeses were characterized by compositional and physicochemical analyses; cultivable microbiota and microbiome analysis; proteolysis and concentration of free amino acids; and sensory analysis as reported by Minervini et al. (2017).

3. Results and Discussion

3.1 Compositional, microbial and biochemical characterization of traditional full fat burrata cheese

This study provides more in-depth knowledge of the biochemical and microbiological characteristics of traditional burrata cheese. The biotechnology proposed in this study should be considered a useful tool to improve the functional value of traditional burrata cheese. During 16 days of storage, traditional burrata cheeses showed different microbiomes and biochemical and sensory features. Based on culture-dependent methods, Burrata cheeses are considerably sensitive to the growth of spoilage bacteria, as presumptive *Pseudomonas*, while lactobacilli and lactococci seem to decrease during time (Figure 1)

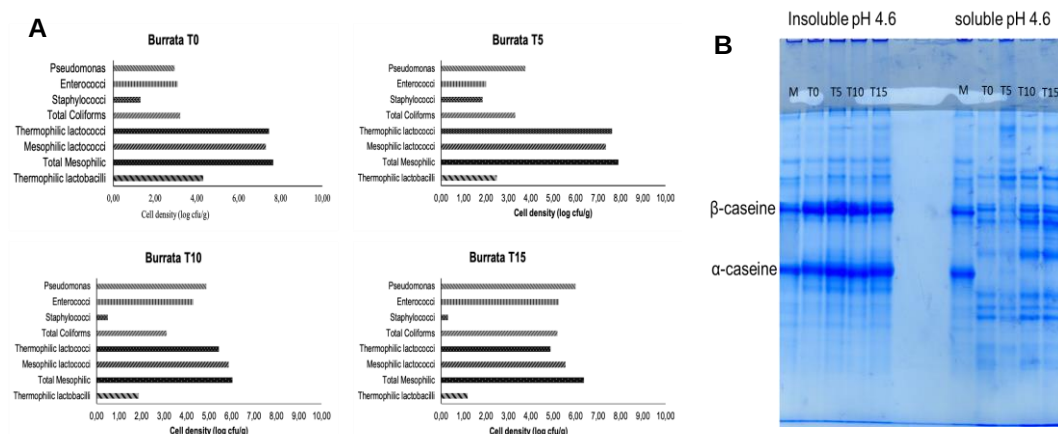


Figure 1 Cell density of principal microbial groups (A) and UREA-Page (B) of pH 4.6 insoluble and soluble nitrogen fractions in full fat traditional burrata cheese at different time of storage (T0, T5, T10 and T15 days).

Urea-PAGE profiles of pH 4.6-insoluble N fractions showed very reduced hydrolysis of caseins throughout storage with some statistical ($P < 0.05$) differences among the storage times (see Figures 1A).

3.2 Experimental low-fat burrata cheese using xanthan and carrageen as fat replacers and EPS producing strains Figure 2 shows the cell densities of principal microbial groups found in low-fat experimental burrata cheeses. Compared to the control, cheeses containing fat replacers and EPS producing strains showed an increase in cell viability of mesophilic lactococci and thermophilic streptococci during storage. Respect the traditional burrata cheese the use of fat replacers and EPS producing strains may have reduced the cell densities related to alterative bacteria such as presumptive *Pseudomonas* spp., staphylococci and total coliforms. In particular, staphylococci were not detectable after 13 days of storage. Although further analysis are in progress, these preliminary results may suggest that the addition of EPS-producing strains and fat-replacers to burrata cheese is a promising method to obtain a significative reduction of fat content without negative affect on flavour and textural features of the cheese.

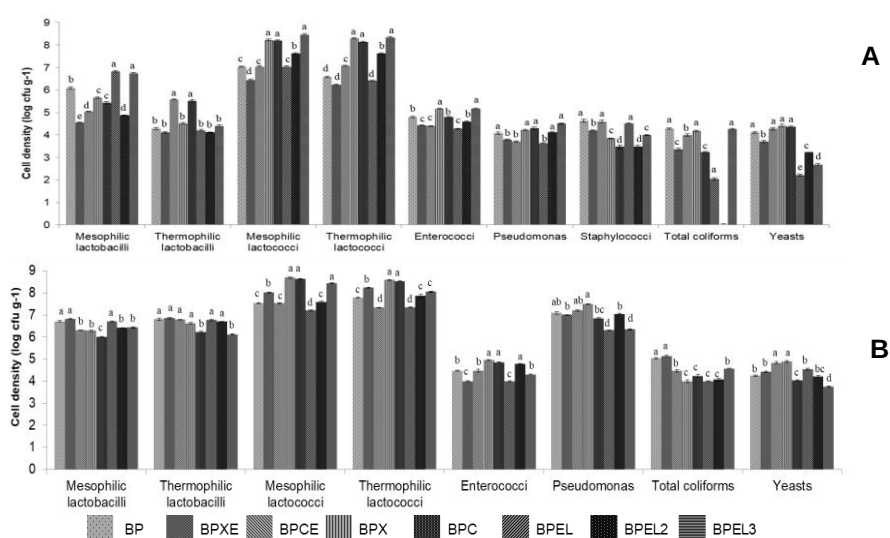


Figure 2 Cell densities of principal microbial groups in experimental burrata cheeses after 2 (A) and 13(B) days of storage at 4 °C

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Development and Optimization of Analytical Methods for the Extractions of Food Bioactive Compounds Using Conventional and non-Conventional Solvents

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The first activities of the PhD thesis project are subsequently described. An analytical method for the analysis of phenolic compounds in extra virgin olive oils (EVOO) by high-performance liquid chromatography (HPLC) coupled with photodiode array (PDA) and mass spectrometry detectors (MS), was optimized and validated. Extracts obtained by conventional liquid-liquid extraction with organic solvents were analysed and the chromatographic profile of thirty samples from four Italian regions highlighted the presence of secoiridoids, phenolic alcohols, flavonoids, and phenolic acid classes. The chromatographic method, after optimization, was validated studying some parameters, e.g., intra-day and inter-day retention time and peak area precision, limit of detection, limit of quantification, and linearity. Subsequently, a green method of extraction based on the use of deep eutectic solvents (DESS) for EVOO phenolic compounds was optimised, using the previously optimized and validated analytical method for extract analysis.

Sviluppo ed ottimizzazione di metodiche analitiche per l'estrazione di composti bioattivi da alimenti, utilizzando solventi convenzionali e non-convenzionali

In seguito sono riportate le prima attività svolte del progetto di dottorato. In primo luogo è stato ottimizzato e validato un metodo analitico per l'analisi dei composti fenolici dell'olio extra vergine di oliva mediante cromatografia liquida ad alte prestazioni accoppiata a rivelatore a serie di fotodiodi e spettrometria di massa. Gli estratti ottenuti mediante estrazione convenzionale, utilizzando solventi organici, sono stati analizzati e il profilo cromatografico di trenta campioni di olio provenienti da quattro regioni italiane, ha mostrato la presenza delle classi dei seicoridoidi, alcoli fenolici, flavonoidi e acidi fenolici. Il metodo cromatografico, in seguito all'ottimizzazione, è stato validato studiando alcuni parametri come la precisione intra-day ed inter-day dei tempi di ritenzione e delle aree dei picchi, limite di rilevazione, limite di quantificazione e linearità. Successivamente, utilizzando per le analisi degli estratti il metodo precedentemente ottimizzato e validato, è stato ottimizzato un metodo di estrazione "green" per i composti fenolici dell'olio extra vergine di oliva, basato sull'utilizzo dei solventi eutettici profondi.

Key words: extra-virgin olive oil, green extraction, HPLC-PDA/MS, phenolic compounds, deep eutectic solvents.

1. Introduction

This poster reports the main results of the first two activities concerning:

A1) Optimization and validation of an analytical method for phenolic compounds analysis in EVOO by HPLC-PDA/MS;

A2) Optimization of a green method for the extraction of phenolic compounds in EVOO based on DESS.

2. Materials and Methods

For the first activity EVOO phenolic compounds were extracted by liquid-liquid extraction (LLE) using a mixture of methanol/water (70:30 v/v). Phenolic compounds separation was carried out using a Pentafluorophenyl Column (150 × 2.1 mm I.D., 2.7 µm d.p.). Elution was performed at 40 °C with water/0.1% HCOOH (solvent A) and methanol/acetonitrile at 1:1 (v/v)/0.1% HCOOH (solvent B) at a constant flow-rate of 0.2 mL/min. The gradient elution profile was as follows: 0–5 min 5% B, 5–15 min 5–30% B, 15–40 min 30–50% B, 40–50 min 50–100% B. The injection volume was 2 µL. Data were acquired using a PDA in the range 210–400 nm and the chromatograms were extracted at 280 nm. Chromatograms were acquired in the MS instrument using ESI as interface in negative ionization mode using the following parameters: nebulizing gas flow (N₂): 1.5 mL min⁻¹; Event time: 1 s; mass spectral range: 100–800 m/z; scan speed: 1000 amu/sec; detector voltage: 1.5 kV; Interface temperature: 250 °C; CDL temperature: 300 °C; heat block: 300 °C; interface voltage: –3.50 kV; Q-array: 0.0 V; Q-array RF: 150.0 V. The optimized method, validated using a mixture of representative phenolic compounds that could be present in EVOOs, was applied to the analysis of phenolic profile of thirty samples of monovarietal and multivarietal EVOOs samples including PDO oils from different areas in Italy (Sicily, Lazio, Tuscany, and Puglia).

With regard to the second activity, four DESs being ChCl-glycerol (DES1), ChCl-lactic acid (DES2), ChCl-triethylen glycole (DES3) and ChCl-urea (DES4) were considered for the study. In order to optimize the DES extraction method, different parameters were studied: water content in DES (10, 20, 30 and 70 %) and ratio of sample amount (mg) to DES volume (mL) (1:1, 2:1, 3:1, w/v). In order to evaluate the extractive capacity of each tested DES, phenolic compounds were separated and detected by HPLC-PDA/MS method described above. Extraction with DES was also compared with conventional extraction, based on the use of a mixture of methanol/water (70:30 v/v).

3. Results and Discussion

3.1 Optimization and validation of an analytical method for phenolic compounds analysis in EVOO by HPLC-PDA/MS

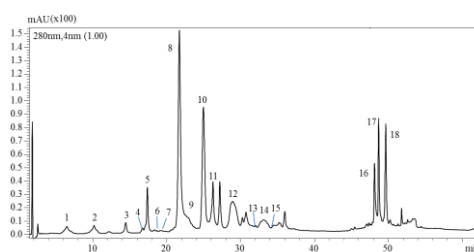


Figure 1 HPLC-PDA chromatogram of phenolic compounds present in one of the analyzed EVOO sample. 1: Hydroxytyrosol, 2: Tyrosol, 3: Vanillic acid, 4: Ligstroside derivative, 5: p-cumaric acid, 6: Hydroxydecarboxymethyl elenolic acid, 7: Elenolic acid, 8: Dialdehydic form of oleuropein aglycon, 9: Oleuropein aglycon, 10: Oleuropein aglycon. 11: Lygstroside aglycon, 12: Oleuropein aglycon, 13: Luteolin, 14: lygstroside aglycon, 15: Apigenin, 16: Unknown, 17: Unknown, and 18: Unknown.

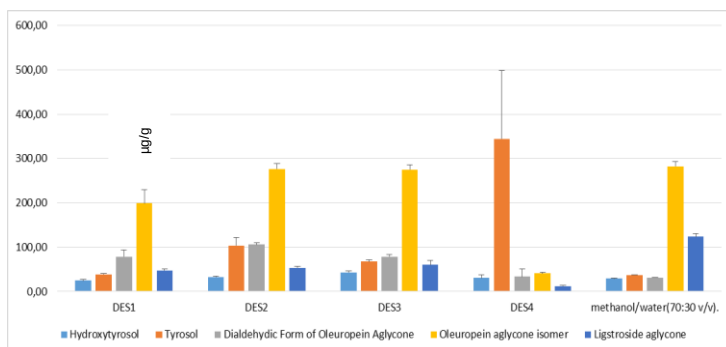
In all samples secoiridoids were the main compounds ranging from 85 to more than 99% (w/w) of the total concentration of detected phenolic compounds. Flavonoids and

phenolic alcohols percentage ranged from 0.3 to 1.5% (w/w) and from 0.1 to 12% (w/w) respectively. The content of vanillic and p-coumaric acids, belonging to the class of phenolic acids was in the range 0.1–0.6% (w/w) of total concentration of analyzed compounds. These results are in accordance with literature data (Bakhouché et al., 2013). Comparing the different Italian geographical areas the highest total phenolic compounds concentration, calculated as the sum of concentration of analyzed compounds, was found in the Puglia area. An ANOVA test showed a significant statistical difference between Puglia and Sicily total concentration of phenolic compounds, in accordance with results reported in literature (Antonini et al., 2015).

3.2 Optimization of a green method for the extraction of phenolic compounds in EVOO based on DESs

A total of five phenolic compounds, previously identified, were selected (1,2,8,10,11), for the evaluation of DESs extraction efficiency. Results showed that extraction repeatability was quite good for all the selected DESs except for DES4, corresponding to the urea-based one which gave RSD% values of compounds concentrations till 50%. Taking into consideration all the solvents, the order of efficiency for the extraction of phenolic compounds from olive oil was DES2 > DES3 > methanol/water (70:30 v/v) > DES1 (Figure 2).

Figure 2 Concentration ($\mu\text{g/g}$) of phenolic compounds extracted with DES1, DES2, DES3, DES4 and mixture methanol/water (70:30 v/v).



As low pH of the choline chloride-lactic acid DES could cause the degradation of secoiridoids derivative containing tyrosol and hydroxytyrosol in their structure, we selected DES 3 as the best extractive solvent. In order to optimize extraction conditions, the selected alcohol-based DES (DES3) was prepared adding 10, 20, 30, and 70% (v/v) of water. The highest and repeatable extraction efficiency was obtained with DES added of 20% water. We then evaluated the sample to solvent ratio optimal condition considering 1:1, 1:2 and 1:3 (w/v) ratio. For the final method validation we selected ChCl-triethylen glycole 1:2 (molar ratio) added of 20% water using a sample to solvent ratio of 1:1 (w/v) and room temperature as extraction conditions.

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Nutritional, Physicochemical, and Sensory Evaluation of Gluten Free Bread Supplemented with *Digitaria Exilis* Stapf. Flour

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The first activities of the PhD thesis project are described. Different percentages of Fonio (*Digitaria exilis* Stapf.) flour (25-50-75-100%) were substituted in a gluten free (GF) bread formulation and compared to the conventional GF breads formulations. Physicochemical properties have been assessed on flours, doughs and GF breads produced.

Valutazione nutrizionale, fisicochimica e sensoriale di pane senza glutine fortificato con farina di *Digitaria Exilis* Stapf.

Nel contributo presentato vengono descritte le prime attività del progetto di tesi di dottorato. È stato studiato l'effetto della sostituzione di farine convenzionali usate per la produzione di pane senza glutine, con diverse percentuali di *Digitaria exilis* Stapf. (25, 50, 75, 100%).

Sono state le proprietà fisico-chimiche delle farine, impasti e pani senza glutine prodotti studiate.

Keywords: *Digitaria exilis* Stapf., wholegrain, gluten free bread, dough, rheology

1. Introduction

Fonio (F) is a gluten free grain suitable for use in the diet of celiac patients (Jideani & Jideani, 2011). GF diet is often characterized by an excessive caloric intake and a reduced intake of fibre, minerals and complex carbohydrates. The total or partial substitution of rice flour and corn starch in GF bread with F flour could be a strategy to increase fibre content as well as phenolic and antioxidant activity in the final product. The aim of this study is to find the optimal percentage of substitution of F in GF bread to improve its sensory and nutritional quality. In accordance with PhD project this poster reports the main results of the first activities concerning:

A1) the determination of the viscometric and rheological properties of flours and doughs at different percentages of F flour.

A2) Textural properties of the breads produced substituting different percentages of F to the conventional GF formulations.

2. Materials and Methods

GF control (C) was prepared using a conventional GF formula based on 50% corn starch, 50% rice flour (R), 90% water, 1,8% NaCl, 1,8% yeast, 5% sunflower oil, 1,5% guar gum, and 1,5% psyllium fiber.

F-enriched GF breads were prepared by replacing the rice flour and the corn starch with increasing percentages of F, as shown in Table 1. In addition, to compare the behavior of F-enriched GF breads with other widespread GF formulations, two other R based formulations were prepared. The Water Level (WL) of the doughs was adjusted in order to obtain the same consistency of the control (C) at 90% WL, using a rotational rheometer. A Power Law model was used to predict the new WL based on single frequency measurements of G^* for each sample (Nunes, M. H. B., et al. 2009).

Table 1 *PhD thesis experimental design*

CODE	RICE (%)	CORN STARCH (%)	FONIO (%)	WATER LEVEL (%)
C	50	50	0	90
F25	25	25	50	88,3
F50	0	50	50	88,5
F75	0	25	75	97
F100	0	0	100	110
R75	75	25	0	98
R100	100	0	0	110

Abbreviations: C, control; F100, fonio 100%; F75, fonio 75%; F50, fonio 50%; F25, fonio 25%; R75, rice 75%; R100, rice 100%.

2.1 Viscometric properties of flours and dough rheological measurements

The pasting properties and the Stirring Number (SN) of flours were evaluated using a Rapid Visco Analyzer (RVA-4, Newport Scientific, Warriewood, Australia), whereas the rheological properties of the doughs were studied with a frequency sweep test using a rotational rheometer (Anton Paar, MCR92, GmbH, Inc., Graz, Austria).

2.2 Bread analysis

12 breads were prepared for each formulation. Two loaves from each formulation were analysed after 3 h from processing. Bread mechanical properties (hardness, cohesiveness, springiness and resilience) were evaluated with a TPA test, by using a TA-XTplus Texture Analyzer (Stable Micro Systems) equipped with a P36R.

3. Results and Discussion

3.1 Flour and dough characterization

Data on SN and pasting properties of the samples with different levels of F were reported in Table 2. SN values, which represent the α -amylase activity, resulted lower when the percentage of F added increased. Regarding the viscometric properties, the peak V with higher F addition levels worsened the viscosity profile with respect to the C. On the contrary, the samples with the highest percentages of F substitution showed the lowest BD value. Doughs with 75 and 100% of F, had higher thermostability and lower shear thinning and disintegration of swollen systems than from other samples. FV, that shows the ability of the material to form a viscous paste, decreased as the amount of F increased. The highest values of SB, which are related to amylose retrogradation were recorded in R100 and R75. F flours retrograded to a less extent than the other samples. The sample R75 showed the highest Peak time (6.17 min). The peak time of F samples were lower than samples without F.

The rheological properties of the doughs were reported in Table 2. Frequency sweep showed that the elastic (or storage) modulus, G' , for all GF doughs was greater than the viscous (or loss) modulus, G'' , at a frequency of 10 Hz, suggesting a solid elastic-like behaviour of all doughs. However, the addition of F affected the rheological behavior of the doughs to a different extent, where F100 showed higher G' e G'' values, showing a more elastic and viscous behaviour. In general elastic and viscous moduli increased with increasing percentages of F used (from 50% upwards). The highest value of G' and G'' moduli were obtained for R100.

Table 2 *Pasting properties of F flour and reological properties of the doughs at 90% WL*

Parameters	C	F25	F50	F75	F100	R75	R100
Peak V (Cp)	3702 c	3243 d	2307 e	1959f	1981 f	4099 b	4475 a
BD (Cp)	786 b	790 c	691 c	509 d	520 d	709 b	919 a
FV (Cp)	5210 c	4160 d	2941 ef	2856 f	3142 e	6112 b	7493 a
SB (Cp)	2293 c	1706 d	1325 e	1406 e	1681 d	2722 b	3937 a
Peak time (min)	5,73 b	5,47 c	5,14 d	5,20 d	5,14 d	6,17 a	5,73 b
PT (°C)	79,80 c	79,05 cd	79,05 cd	81,88 b	77,45 d	83,13 ab	83,88 a
SN (Cp)	2974 c	2521 d	2222 e	2248 e	2022 f	3389 a	3240 b
G'	6774 e	6909 e	6071 e	9918 e	20879 b	12034 c	23689 a
G''	1857 d	1820 d	1706 d	2614 c	5045 b	2930 c	5414 a

Abbreviations: Peak V, Peak viscosity; BD, Breakdown; FV, Final viscosity; SB setback; PT, pasting temperature; SN, stirring number. Different letters in the same row show significant differences according to LSD test ($p < 0,05$).

3.2 Textural properties

As reported in Table 3, the F samples (F50, F75, F100) showed values of hardness significantly higher than the other samples. Only F25 did not exhibit significant differences when compared to the R100, R75 and C samples. Regarding cohesiveness, that describes the extent to which the food structure can be deformed before it ruptures, all F-enriched GF breads showed values significantly lower with respect to the control, except for the sample F25. The tendency of these breads to be less cohesive than the other samples is, undoubtedly, a defect that occurs in many GF breads, which usually show a high susceptibility to fracture or crumble. As evidenced by the lowest values of resilience observed in all the F-enriched GF breads, the incorporation of F significantly decreased the capacity of the crumb to return to its original state after compression, except for sample F25 which did not show significant differences ($p < 0,05$) with respect to C and R100 breads. At last, F75 and F100 showed springiness values significantly lower than the other samples.

Table 3 *GF breads texture parameters*

	Hardness (N)	Springiness	Cohesiveness	Resilience
C	10,95 d	0,98 a	0,56 b	0,29 b
F25	11,99 d	0,99 a	0,56 b	0,29 b
F50	18,29 c	0,98 a	0,44 c	0,21 c
F75	51,31 b	0,95 b	0,40 d	0,16 d
F100	61,10 a	0,95 b	0,41 cd	0,16 d
R75	9,21 d	0,97 a	0,59 a	0,32 a
R100	8,83 d	0,97 a	0,57 ab	0,29 b

Different letters in the same row show significant differences according to LSD test ($p < 0,05$).

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Gluten Free Biscuits Formulations with Unconventional Flours and Bioactive Ingredients

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The present poster communication reports and compares the results of the characterization of gluten free cereal (CF), pseudo cereal (PCF) and legume (LF) flours. In particular, data related to the composition of flours starch are presented: damaged starch, resistant starch, non resistant starch, and in vitro starch digestion rate which allows to estimate the glycemic index. Pasting and functional properties and total phenolic and flavonoid contents have been also evaluated. The aim of the project is to produce gluten free (GF) biscuits with a reduced glycemic index. Hence, this characterization is helpful in order to select the flours, understand their composition and eventually to evaluate how they affect biscuit quality.

Formulazioni di biscotti senza glutine attraverso l'impiego di farine non convenzionali ed ingredienti bioattivi

Questa comunicazione riporta e confronta i risultati inerenti la caratterizzazione di farine senza glutine ottenute da cereali, pseudocereali e legumi. In particolare, vengono presentati i dati relativi alla composizione dell'amido delle farine: amido danneggiato, amido resistente, amido non resistente e digeribilità in vitro dell'amido, che consente di stimare l'indice glicemico. Inoltre, sono state valutate anche le proprietà viscoamilografiche e funzionali ed il contenuto di fenoli e flavonoidi totali. Lo scopo del progetto è di produrre biscotti senza glutine a ridotto indice glicemico. Questa caratterizzazione è quindi utile al fine di selezionare le farine, comprendere la loro composizione ed eventualmente per valutarne l'influenza sulla qualità dei biscotti.

Key words: biscuits, flours, gluten free, glycemic index, pasting properties.

1. Introduction

In accordance with the PhD thesis project previously described (Di Cairano, 2018) this poster reports the main results of the first activity concerning:

A1) the physico-chemical characteristics and pasting properties of GF flours.

2. Materials and Methods

CF, PCF and LF were characterized. Millet, oat, rice and sorghum flours were analysed for CF group; amaranth, buckwheat, quinoa and teff flours for PCF and lentil, pea, chickpea and red lentil flours for LF. Water absorption capacity (WAC), oil absorption capacity (OAC), water solubility index (WSI) and bulk density (BD) were evaluated according to Chauhan et al. (2016) and Kaur et al. (2015). Total phenolic content (TPC) and total flavonoid (TFC) content were determined according to Folin-Ciocalteu method and AlCl_3 colorimetric assay. Pasting properties were evaluated using a Micro-

ViscoAmilograph (Brabender OHg, Duisburg, DE). The temperature profile employed was 30-90-30°C, with a 20 min holding time at 90°C and a heating/cooling rate of 3.0 °C/min. Total starch (TS), damaged starch (SDAM), resistant starch (RS) and non resistant starch (NRS) were determined using Megazyme assay kit, K-SDAM, K-RSTAR (Megazyme Int., Wicklow, IR) following manufacturer instruction. Hydrolysis index (HI) was determined according to Molinari et al. (2018) with some modification. The starch was hydrolysed with α -amylase and amyloglucosidase. Samples from the reaction solution were taken each 30 min for 3 h. The glucose freed from the reaction was measured spectrophotometrically. HI was obtained by dividing the area under the hydrolysis curve of each flour by the corresponding area of a reference sample. Predicted glycemic index (pGI) was calculated according to the equation $pGI = 39.71 + 0.549 \times HI$ (Goñi, Garcia-Alonso, & Saura-Calixto, 1997). All the assays for starch characterization were made on raw flours. Statistical analysis was performed using R 3.6.0. ANOVA and Tukey HSD test were executed on raw data or properly transformed data. When it was not possible to use ANOVA, Kruskal-Wallis non parametric test was employed followed by Dunn post hoc test.

3. Results and Discussion

3.1 Functional proprieties and phenolic content

For what concerns functional properties no significant differences (data not shown) were found for WAC and BD, whereas the three groups of flours resulted to have different WSI ($L > PC > C$) and OAC ($PC > L > C$).

In recent year, the enhancement of GF products with ingredients richer in phytochemicals is gaining interest (Rocchetti et al., 2017); hence the inclusion of flours more nutrient dense may improve nutritional profile of GF biscuits. In our samples, PCF had the highest TPC (1.38 ± 1.46 mg GAE/g) whereas no significant differences have been found between CF and LF. TFC was higher for LF (1.85 ± 1.02 mg QE/g).

3.2 Pasting properties

LF were different from C and PC flours. In particular, they have a lower viscosity (181 ± 29.23 BU), and a lower setback which means a reduced tendency to retrograde (101 ± 26.34 BU). Pasting properties are usually affected by several factors, such as starch composition, amylose/amilopectin ratio, and the presence of other soluble and insoluble components (Lii, Lai, & Shen, 2004).

3.3 Total starch, resistant starch, damaged starch content and predicted glycemic index

The three groups of samples did not show any differences for TS and SDAM content, whereas LF had the lowest SDAM and highest RS content (Table 1). pGI is affected mostly by starch composition. A significant negative correlation has been recorded between RS and pGI of maize flour (Camelo-Mendez, Agama-Acevedo, Tovar, & Bello-Pérez, 2017). However, TS, NRS and RS of analyzed samples showed no correlations with pGI, except for a slight positive correlation between SDAM

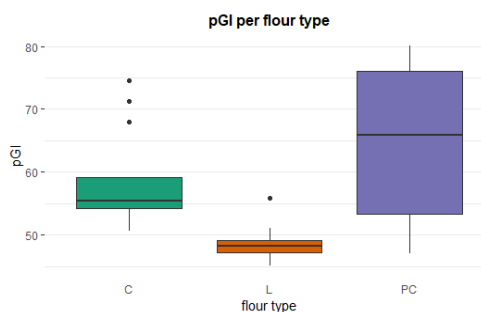


Figure 1 Predicted glycemic index of cereal (C), pseudocereal (PC) and legume (L) flours.

content and pGI ($r=0.44$, $p\text{-value}=0.007$). LF presented the lowest pGI ($p<0.05$), with a small variability inside the group (Figure 1).

Table 1 Total starch, damaged starch, non resistant starch and resistant starch content, hydrolisis index and predicted glycemic index of gluten free cereal, pseudocereal and legume flours.

	TS (% db) ^o	SDAM (% w/w)	NRS (% db) ^o	RS/TS (% db) ^{*a}	HI [*]	pGI [*]
CF	54.29±19.67 ^{ns}	3.85±1.99 ^a	50.22±17.69 ^{ns}	4.51±4.58 ^a	34.18±14.56 ^a	58.48±8.00 ^a
PCF	43.98±10.99 ^{ns}	2.67±2.33 ^a	47.45±7.00 ^{ns}	0.94±0.57 ^c	44.74±24.97 ^b	64.27±13.71 ^a
LF	49.26±7.99 ^{ns}	1.57±0.53 ^{ab}	40.88±10.26 ^{ns}	7.11±2.93 ^b	16.00±5.32 ^a	48.49±2.92 ^b

CF: cereal flours, PCF: pseudocereal flours, LF: legume flours.

Data are expressed as mean ± standard deviation. Different superscript letters within a column indicate statistically significant differences at $p < 0.05$. ^oOne way ANOVA and Tukey HSD test were carried on arcsin square root transformed data. ^oANOVA was carried on log 10 transformed data. ^{*}Kruskal-Wallis test and Dunn test.

PCF presented a large variability compared to the other groups. Considering the flours inside each group, no differences have been found between LF, whereas for what concern PCF, buckwheat and teff flour had the lowest pGI, and sorghum flour had the lowest pGI for CF group (data not shown). The next step of this work is to prepare formulations able to lead to GF biscuits with a low glycemic index. This activity of characterisation and, in particular the pGI, are going to be the starting point for the formulation of the flour blends to employ in the recipes.

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Development of Active Multilayer Bio-Films for Food Packaging Application

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The first activities of the PhD thesis project are described. Antioxidant active film based on chitosan, sodium caseinate and rosemary essential oil has been realized and characterized. Furthermore, antimicrobial film obtained by including *Lactobacillus curvatus* into CH/SC were studied in term of cell vitality. Finally, monolayer and multilayer packaging system were realized for shelf extension of food samples.

Sviluppo di film multistrato attivi per il food packaging

In questo lavoro sono riportate le attività del progetto di dottorato realizzate durante il primo anno. Film attivi ad attività antiossidante a base di chitosano, caseinato di sodio e olio essenziale di rosmarino sono stati realizzati e caratterizzati in termini di proprietà chimico-fisiche, proprietà barriera e proprietà antiossidanti. Film attivi ad azione antimicrobica sono stati realizzati mediante l'aggiunta di ceppi di *Lactobacillus curvatus* produttori di batteriocine. I film sono stati studiati in termini di vitalità cellulare. Infine, le soluzioni a base di biopolimeri sono state utilizzate per realizzare un film monostrato e multistrato a base di PLA.

Key words: Active packaging, DPPH test, biopolymers, lactic bacteria, rosemary essential oil

1. Introduction

In accordance with the PhD thesis project previously described (Di Giuseppe et al., 2018), this work reports the main results of the activities concerning:

A1) Development of active materials: using biopolymers like chitosan, sodium caseinate and whey protein as biopolymer matrix to develop antioxidant and antimicrobial (Volpe, et al., 2017) active films. As active compounds, rosemary essential oil and lactic bacteria strain producing bacteriocine has been used. The objective of the work was the characterization of antioxidant, antimicrobial and physical properties of the film (A1.1); Design an active monolayer and multilayer by coating commercial biodegradable film with the active coating (A1.2)

2. Materials and Methods

Films of CH 2% (W/v), SC 4% (W/v), were obtained by casting, as reported in a previous work (Volpe et al., 2017). Film thickness was measured using a micrometer model H062 with sensity of $\pm 2\mu\text{m}$ (Metrocontrol Srl, Casoria, NA, Italy). Five replicas were conducted for each sample treatment. Five measurements were taken at random position and the mean values were calculated. Hydrophilic antioxidant activity (HAA) was evaluated using 2, 2'-diphenyl-1-picrylhydrazyl free radical scavenging assay (DPPH) according to (Hromiš et al. 2014). IC50 of active film were evaluated after 24 h of incubation in DPPH solution. A standard calibration curve was construct measuring the antioxidant activity of known quantities of active compounds. The color of the samples was

evaluated using a colorimeter (Minolta CHROMA METER CR-300). The color values of L (black/white), a^* (redness/greenness) and b^* (yellowness/blueness) were measured and averaged from three random positions for each sample, and the total color difference (E) was calculated. The film opacity was determined according to the method described by (Liu et al., 2017) by measuring the absorbance at 600 nm using the UV-VIS spectrophotometer (Jasco V-550 UV / VIS Spectrophotometer). The film surface density was calculated as: $\rho = C \times V$, where C is the blend solution concentration (mg ml^{-1}), V is the volume of the solution poured into the petri dish (ml) and A is the surface area of the petri dish (cm^2). The humidity of the films was evaluated by gravimetric method, maintaining the film at 105°C for a time sufficient to reach a constant weight. The results are expressed as relative humidity percentage (RH%). The water vapor permeability (WVP) and water vapor transmission rate (WVTR) of the films was evaluated using a gravimetric test according to ASTM Standard Method E 96 (ASTM, 1993) using a Payne permeability cup (Carlo Erba, Milan, Italy). The solubility of the films at different pH was tested with the procedure reported by Lanciotti et al. (2003). The mechanical properties of the films were evaluated with an Instron dynamometer (Universal Testing Machine, Instron 5900R-4467, Massachusetts, USA), following the method D882-00 (ASTM, 2001) equipped with a load cell of 1 kN.

3. Results and Discussion

3.1 Determination of the chemico-physical properties of biodegradable films

Generally, the addition of Rosemary Essential Oil (REO) into biopolymer matrix didn't modify the analyzed chemico-physical properties of the film (table 1-2). Nevertheless, the presents of the REO decreases slightly the WVP, WVTR and solubility at pH 8 of the films. On the contrary b^* value and the surface density increase in samples with REO. The Elongation at break increase when CH was added to SC.

Table 1 RU, permeability and color properties of active biopolymer films.

Film	RU (%)	WVP	WVTR
		($\text{g m}^{-1} \text{s}^{-1} \text{Pa}^{-1}$)	($\text{g m}^{-2} \text{h}^{-1}$)
SC	11.18 $\pm$ 0.59a	9.08 $\times 10^{-11}$ $\pm$ 7.70 $\times 10^{-12}$	3.90 $\times 10^{-3}$ $\pm$ 10 $\times 10^{-11}$ b
SC+REO	11.39 $\pm$ 1.09a	8.16 $\times 10^{-11}$ $\pm$ 8.05 $\times 10^{-12}$	3.89 $\times 10^{-3}$ $\pm$ 10 $\times 10^{-11}$ b
SC/CH	12.13 $\pm$ 0.33a	9.49 $\times 10^{-11}$ $\pm$ 8.89 $\times 10^{-12}$	3.67 $\times 10^{-3}$ $\pm$ 10 $\times 10^{-10}$ a
SC/CH +REO	16.74 $\pm$ 1.96b	8.97 $\times 10^{-11}$ $\pm$ 9.30 $\times 10^{-12}$	3.48 $\times 10^{-3}$ $\pm$ 10 $\times 10^{-10}$ a
S2-C	12.60 $\pm$ 2.50	2.25 $\times 10^{-10}$ $\pm$ 1.80 $\times 10^{-11}$	3.25 $\times 10^{-4}$ $\pm$ 1,5 $\times 10^{-11}$
S2-M	14.60 $\pm$ 2.44	3.70 $\times 10^{-10}$ $\pm$ 2.32 $\times 10^{-11}$	5.10 $\times 10^{-3}$ $\pm$ 2,3 $\times 10^{-11}$

IC₅₀ is reflected in the level of bleaching action and antioxidant activity (Hromiš et al., 2014). Generally, REO showed a low IC₅₀ value as single active compounds. The IC₅₀ of CH/SC film and film realized with CH/SC /1.5% REO shows not significant differences (table 3).

Table 2 *Mecchanical and physical properties of active biopolymer films.*

Film	Young modulus (MPa)	Tensile strenght (MPa)	Elongation at break (%)	ps (mg/m ²)	Thickness (μm)
SC	634±512	9.77±7.16	4.38±1.55 ^a	6.99±0.47 ^a	57.19±12.45 ^a
SC+Reo	788±277	10.00±3.70	3.02±0.85 ^a	8.48±0.36 ^c	52.76±13.14 ^a
SC/CH	294±128	12.25±2.63	15.20±5.57 ^b	7.73±0.22 ^b	67.35±15.97 ^b
SC/CH +Reo	450±260	9.23±6.08	13.52±6.99 ^b	8.85±0.25 ^c	69.81±15.87 ^b

Film realized with chitosan and with chitosan/1.5%Reo shows a particular situation where the IC₅₀ are 45.66 mg/g and 54.93mg/g respectively; these results suggest that in both cases the essential oil is linked to the biopolymers and can not be donor a hydrogen to the free radical (DPPH).

Table 3 *IC₅₀ of active compound and biopolymer films incorporate with rosemary essential oil*

Biopolymer/active molecules	IC ₅₀ (mg/g)	Biopolymer/active molecules	IC ₅₀ (mg/g)
CH	45.96	Rosemary essential oil (Reo)	0.01
SC	18.96	CH+1.5%Reo	54.60
CH/SC	51.63	SC+1,5%Reo	0.04
		CH/SC+1.5%Reo	48.47

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Determination of Different Oxidative Stress Markers in Biological Matrices by Means UHPLC-MS/MS

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In this PhD thesis project, the objective was the development of analytical methods for the evaluation of oxidative stress through the monitoring of specific biomarkers; in this way it is possible to evaluate the change in the physiological state by different point of view. The goal is also the evaluation of changes in the oxidative state of the organism resulting from the exposure of xenobiotics or due to incorrect dietary regimes, and the potential to rebalance the antioxidant defenses of our body through the use of specific dietary regimes

Determinazione di differenti markers dello stress ossidativo in matrici biologiche tramite UHPLC-MS/MS

In questo progetto di dottorato si è preso come obiettivo lo sviluppo di metodi analitici per la valutazione dello stress ossidativo tramite il monitoraggio di specifici biomarkers allo scopo di valutare il cambiamento dello stato fisiologico. Il fine ultimo è inoltre la valutazione di cambiamenti nello stato ossidativo dell'organismo conseguenti all'esposizione di eventuali xenobiotici o dovuti a regimi alimentari scorretti, e la potenzialità di riequilibrare le difese antiossidanti del nostro corpo tramite l'utilizzo di regimi alimentari mirati.

Key words: OX markers, Isoprostanes, Oxisterols, Extraction, UHPLC-MS/MS.

1. Introduction

The aim of this PhD thesis report is to present the development of different analytical methods for the monitoring of different OX markers in biological matrices:

A1) Determination of the levels of isoprostanes in the urine by means of dispersive Liquid-Liquid Micro Extraction (dLLME) and subsequent micro Solid Phase Extraction (μ SPE) cleanup: a reliable analytical method was developed in order to have a high sensitivity, and at the same time succeed in cleaning up the sample, in particular 8-isoprotoglandine F2 α (8-iso-PGF2 α) and 5 isoprostane F2 α (5-IPF2 α);

A2) Identification and quantification of membrane oxysterols in both swine and kelp spermatozoa through a Liquid-Liquid Extraction (LLE) and subsequent clean up μ SPE. The presented method is evaluated on the following oxidized forms: 20d-hydroxycholesterol, 25-hydroxycholesterol, 25-hydroxycholesterol, 22-Hydroxycholesterol, 7 β -hydroxycholesterol and desmosterol;

2. Materials and Methods

Different matrices show different difficulties to face as reported below. In the case of isoprostanes we must deal with compounds present in very small concentration in the urine [1]; furthermore, they are a challenge for UHPLC-MS/MS analysis due to the relevant matrix effect [2]. Given this problem, we opted for a dLLME extraction which

allows a significant enrichment factor of the analytes and a preliminary clean-up: on 1 mL of urine 250 μ L of buffer pH 5 are added together with 0.6 mg of NaCl, to finish are added 900 μ L of isopropanol and 100 μ L of chloroform. everything is vortexed for 1 min and in an ultrasonic bath for 10 minutes, to finish the sample is centrifuged at 4000 rpm for 20 minutes at a temperature of 4 ° C, then proceed to μ SPE clean up, in fact, after having taken the chloroform portion and having brought the sample to dryness, it is resuspended in a 90:10 pH5 buffered H₂O:MeOH solution and loaded into a C18 cartridge previously activated with MeOH and conditioned, washing is carried out with a solution 80:20 pH5 buffered H₂O:MeOH and finally eluted with 50 μ L of a 50:50 pH5 buffered H₂O:MeOH solution. The eluate is then sent for UHPLC-MS / MS analysis, which were performed by negative ESI mode, using a Kinetex XB-C18 2.6 μ m column for the chromatographic run

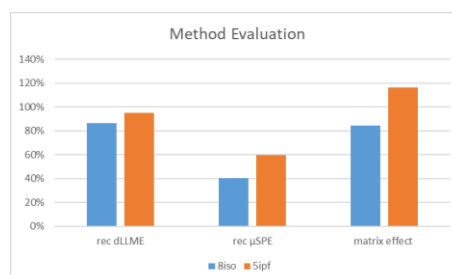
With regard to oxysterols, the difficulty was the rupture of the plasma membrane where they are found physiologically [3], to do this we chose to use a sonication process consisting of an immersion probe to break the bonds between the membrane phospholipids followed by an LLE extraction and then a μ SPE clean up to concentrate the sample, after resuspending the chloroform portion dry-put with 112 μ L of a precipitating solution, centrifuged and removed, 88 μ L of H₂O are added and loaded in a cartridge previously conditioned with acetonitrile, washed on H₂O and subsequently the analytes are eluted with 50 μ L of an ACN solution: MeOH 20mM HCOOH, in this case the eluate is then sent for UHPLC-MS / MS analysis, which were performed by positive APCI mode, using a Kinetex XB-C18 2.6 μ m column for the chromatographic run

3. Results and Discussion

3.1 Determination of Isoprostanes in urines

The results obtained for the different classes of compounds were satisfactory, in fact it was possible to obtain a good extraction with the dLLME technique, succeeded in concentrating the sample and then performing a clean up μ SPE which led to a lower matrix effect in the sample, managing to further concentrate the same starting from an initial sample volume of 1 mL and a final elution volume of 0.05 mL, leading to having a LOQ in model conditions of 12 ppt in the standards. In the real samples the values of LOQ because was below the physiological values, even having used composts coming from individuals who conduct a healthy diet combined with a regular sporting activity. In the figure 1 are reported the values from the validation carried out in the matrix going to evaluate the individual recoveries strengthen different steps of the extraction and comparing them to each other, for the fortification downstream of the process for the evaluation of the matrix effect and the recoveries of the standards were placed in the elution solvent and evaluated by comparing it with an extraction performed on a model solution. We can see how the step of dLLME results to have a good extractive power, while μ SPE does not have the same extractive power also due to the minimal desired amount of organic solvent in the elution solution, so as to obtain that most of the interferents remain in cartridge, resulting in a reduced matrix effect, that was one of the main goals of the project.

Figure 2 The graph shows the results obtained from the comparison on the various fortifications on matrices for the evaluation of the method



3.2 Determination of Oxysterols in cellular membranes

For oxysterols, a different approach was applied, in fact they are not prone to matrix effect as Isoprostanes and the requested LOQs are significantly higher. They, on the other hand, still face difficulties that have represented a challenge: in fact they are present in the cell membranes and it was therefore difficult to completely and rapidly break the membrane phospholipids; in this case in the spermatozoa of pig and of kelp is the chosen matrix but some results is also obtained on Zebrafish embryos. It was then decided for the use of a sonicator equipped with probe immersion which allows the membrane to be broken in a few seconds. Subsequently our analytes were extracted with a method appropriately miniaturized for the occasion. The subsequent μ SPE step allows us to work with small volumes of sample, so as to maintain an adequate enrichment factor for analysis; in fact another difficulty of these compounds is their low sensitivity in mass spectrometry without a derivatization process [4]. This step was avoided by working on the extraction method and using an APCI source. The table shows the values obtained from the validation of the method where they have been reported.

Table 1 *Enrichment factor with relative RSD (%) for each analytes*

	20D	25	24	22	7b
Enrichment	249%	277%	127%	243%	164%
STD	16%	19%	3%	20%	18%

Table 2. *Validation data for the analytical method.*

	LOQ (NG/ML)	ACC (%)	PREC (RSD%)	EQ	R ²
20D-HYDROXYCHOLESTEROL	1	97	16	$y = 0.0007x - 0.00006$	0.9925
25-HYDROXYCHOLESTEROL	2.4	95	18	$y = 0.0011x - 0.01$	0.993
24-HYDROXYCHOLESTEROL	1.3	94	3	$y = 0.0018x - 0.0163$	0.9948
22-HYDROXYCHOLESTEROL	2	101	20	$y = 0.0007x - 0.0066$	0.9867
7B-HYDROXYCHOLESTEROL	0.8	109	18	$y = 0.0119x + 0.0127$	0.9955
7B-HYDROXYCHOLESTEROL-D7	0.2	103	18	$y = 0.0079x - 0.007$	0.9944
DESMOSTEROL	12	92	21	$y = 0.0034x - 0.018$	0.9879

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Malting of Hemp Seeds (*Cannabis sativa* L.) to Improve their Antioxidant and Nutritional Values

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Part of the first three activities of the PhD project is described. Firstly, the nutritional and antioxidant profile of hemp seeds of *Futura75* and *Secuieni jubileu* cultivars (cvs) was evaluated. Secondly, the first two steps of the malting process (soaking and germination) were performed and their effect on the seeds' antioxidant profile was analysed. Nutritionally, the seeds of both cultivars showed similar features although *Secuieni jubileu* seeds had a higher phytate content; moreover, 66h and 90h of germination significantly improved the antioxidant profile of the seeds.

Maltazione dei semi di canapa (*Cannabis sativa* L.) finalizzata al miglioramento del loro profilo antiossidante e nutrizionale

Parte delle prime 3 attività del progetto di dottorato è descritta. È stato valutato il profilo nutrizionale e antiossidante dei semi di canapa delle *cultivars* (cvs) *Futura75* e *Secuieni jubileu*. Sono state condotte le prime due fasi della maltazione (ammollo e germinazione) ed è stato analizzato il loro effetto sul profilo antiossidante dei semi. Nutrizionalmente, i semi di entrambe le cvs hanno caratteristiche simili, tuttavia i semi di *Secuieni jubileu* hanno un maggior contenuto di fitati; inoltre, 66h e 90h di germinazione hanno migliorato significativamente il profilo antiossidante dei semi.

Key words: Germination, malting, hemp seeds, antioxidant profile, total phenolic content, nutritional profile.

1. Introduction

In accordance with the PhD thesis project previously described (Farinon, 2018), this poster reports the main results of a part of the first three activities concerning:

A1) Nutritional and functional characterization of *Secuieni jubileu* and *Futura75* hemp seeds;

A2) Experimental malting trials performed in a laboratory scale;

A3) Evaluation of the malting effect on the nutritional and functional features of the *Secuieni jubileu* and *Futura75* seeds.

2. Materials and Methods

Seeds of *Futura75* and *Secuieni jubileu* cvs were used. Grounded seeds were analyzed for moisture, protein, fat and ash content in accordance with AOAC methods. Carbohydrates were calculated by difference. The phytic acid content was analyzed on defatted, milled seeds by the Phytic acid assay kit (Megazyme®, Ireland). Steeping was carried out on cleaned seeds in 2.5 L of deionized water (20±1°C) for 5h with a 5 min air-rest-period each 30 min. Soaked seeds were germinated for 18h; 42h; 66h; 90h at an average temperature and relative humidity of 24°C and 75% respectively. After each step, an aliquot of each cv was freeze-dried and milled. Samples were extracted with 80% ethanol for 4h, in the dark, at 4°C. The following assays were performed: TPC (Total

Phenolics Content), FRAP (Ferric Reducing Antioxidant Power) and TEAC (Trolox Equivalent Antioxidant Capacity). All analyses were carried out at least in triplicate. One-way analysis of variance (ANOVA) with Tuckey's post-hoc-test was performed to establish significant differences ($P \leq 0.05$).

3. Results and Discussion

3.1 Hemp seeds proximate composition

The proximate composition and phytate content of the two hemp seeds cvs are shown in Table 1.

Table 1 Proximate composition and phytate content (g/100g dw), of Futura75 and Secuieni jubileu hemp seeds cultivars.

Hemp seeds cultivar	Moisture	Protein ¹	Fat	Carbo-hydrate ²	Ash	Phytate
Futura75	7.04±0.58 ^b	24.31±0.16 ^a	32.31±1.23 ^a	31.98	4.36±0.01 ^b	2.74±0.20 ^b
Secuieni jubileu	8.15±0.97 ^a	21.85±0.14 ^b	32.88±0.66 ^a	31.39	5.73±0.01 ^a	3.74±0.15 ^a

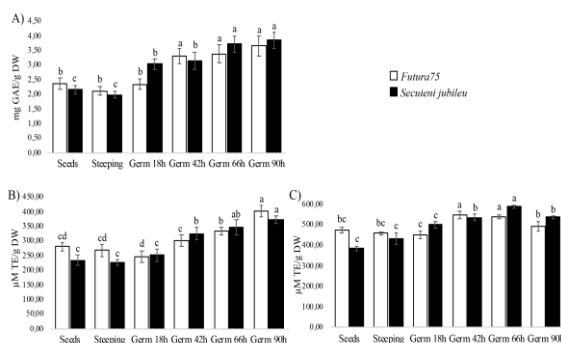
¹Conversion factor: 6.25. ²As difference. Means ± standard deviations with different letter by column indicate statistically significant differences ($p \leq 0.05$).

The proximate composition of both cvs are consistent with those obtained in other works for different industrial hemp cvs (Vonapartis et al., 2015). As expected, the most representative nutrient is the fat. Both *Futura75* and *Secuieni jubileu* seeds had the same fat content (about 32% of dry weight), but they were significantly different for the protein ash and pytate content. In fact, the seeds of *Secuieni jubileu* contained a slightly lower amount of protein but higher ash and phytate content in comparison to *Futura75* ones. Since phytate is an antinutrient, strategy to reduce its content seems to be particularly important to improve the nutritional value of *Secuieni jubileu* seeds and the first two steps of the malting process could be useful to achieve this goal (Gupta, et al., 2013).

3.2 Antioxidant profile of untreated steeped and germinated hemp seeds

The antioxidant profile of *Futura75* and *Secuieni jubileu* untreated, steeped and germinated seeds extracts was analysed and the obtained results are shown in Figure 1.

Figure 1 A) TPC; B) FRAP; C) TEAC of Futura75 and Secuieni jubileu hemp seeds untreated (seeds), steeped (steeping) and germinated (Germ) at different germination time (18h, 42h, 66h, 90h). Data are means ± standard deviation of three replicates of three independent experiments; different letters within the same cv indicate significant differences ($P < 0.05$). GAE: Gallic Acid Equivalent; TE: Trolox Equivalent



As shown, TPC (1a), FRAP (1b) and TEAC (1c) values of untreated seeds was slightly lower in *Secuieni jubileu* than in *Futura75* but in both varieties they increased

during germination with a similar trend and reached the highest value after 66h or 90h of germination. The increase was more remarkable in *Secuieni jubileu* seeds (78% for TPC, 71% for FRAP; 50% for TEAC) than in *Futura75* (55% for TPC, 40% for FRAP; 13% for TEAC). These results are in accordance with the fact that the antioxidant activity is closely related to polyphenols, compounds with strong antioxidant power. In fact, it is found a high positive correlation between TPC and FRAP ($R = 0.72$) and TPC and TEAC ($R = 0.75$). The increase of TPC during the germination was reported for several species (Cevallos-Casals et al., 2010). Also, several literature data had showed a higher antioxidant capacity in sprouts in comparison to seeds for many plant species (Frassinetti et al., 2018; Gan et al., 2017) and this is probably due to the rise in the content of antioxidant compounds such as vitamins and polyphenols during germination, other than in their type and chemical nature. Interestingly, the FRAP trend obtained in this work was closely like to the TPC once with the highest value at 90h of germination, otherwise TEAC assay showed a gradual increase of the antioxidant capacity until 66h where it reached the highest value, then there was a slightly but significant, decrease. This is could be due to a degradation of tocopherols or bioactive peptides. Tocopherols are chain-breaking compounds with high antioxidant power, abundant in hemp seeds. Their antioxidant function can be detected by TEAC but not by FRAP assay and some literature data had shown a tocopherols' increase during early germination, followed by a decrease during long germination time (Gan et al., 2017). Moreover it has been shown that the hydrolysis of hemp seeds' proteins produce small peptides with antioxidant activity and the degree of peptides' bioactivity depend on the hydrolysis time (Teh et al., 2016). Hence, it is possible that a shorter germination time could produce bioactive and antioxidant peptides which are further hydrolysed to smaller and no more bioactive after longer germination time. Overall, studies concerning the changing in TPC and antioxidant power upon germination are manly focused on cereals and pseudo-cereals and only few works had paid attention on hemp seeds (Frassinetti et al., 2018), but no one focused on *Secuieni jubileu* cv. According to our results, the first two step of the malting process can effectively improve the antioxidant profile of both *Futura75* and *Secuieni jubileu* hemp seeds and 66h of germination seems to be the best time for both cultivars.

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Metagenomics Approach to Investigate the Microbiota Composition of Salame Piemonte PGI

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During these two years of the PhD thesis project, the first three activities are described. Salame Piemonte PGI is a fermented sausage typical of North of Italy. The maturation of this product is characterized by the lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) bacteria activity. In order to select a starter culture is essential to understand the composition of microbial communities. Therefore, we used a shotgun sequencing approach simultaneously to culture dependent-method.

Approccio metagenomico per investigare la componente microbiologica del Salame Piemonte IGP

Sono descritte le principali tre attività svolte durante questi primi due anni del progetto di tesi di dottorato. Il Salame Piemonte IGP è un alimento fermentato tipico del Nord d'Italia. La maturazione di questo prodotto è caratterizzata dall'attività dei batteri lattici e staphylococchi coagulasi-negativi. Nell'ottica di selezionare colture starter è essenziale comprendere al meglio la composizione della comunità microbica. Abbiamo, quindi, adottato un approccio di sequenziamento metagenomico in parallelo a tecniche coltura dipendenti.

Keywords: metagenome analyses, starter culture selection, Salame Piemonte, fermentation, bacteria.

1. Introduction

In accordance with the PhD thesis project previously described, this poster reports the main results of the first three activities concerning:

- A1) Bacteria isolation and identification from Salame Piemonte samples;
- A2) Physiological characterization of the isolates;
- A3) Shotgun sequencing approach to investigate the microbial communities.

2. Materials and Methods

Three different batches of Salame Piemonte production without the use of starter culture were analysed. During the maturation process (50 days) six-time of sampling were analysed due to the culture-dependent method. Fifteen strains from LAB and for CNS were isolated at every time. The first screening for the characterization of the bacteria populations more prevalent in the product studied was carried out by REP-PCR with the primer (GTG)₅ (Laulund *et al.*, 2017; Rantsiou *et al.*, 2005) and successively by sequencing. After the identification of the strain, a physiological characterization of the isolates was performed (Englezos *et al.* 2018). This test was done to select the best strains to use in a starter culture. Compressively 270 strains of LAB and 270 strains of CNC were testing for different growth condition and technological performed. Total DNA was extracted from salami matrices for each time of sampling. The DNA of three biological

replicates was pooled before further processing and time zero for each batch was eliminated because the samples weren't enough good. The samples were sent to the America company for a shotgun sequencing. The obtained data were analyzed due to bioinformatic and static tools to obtain a complete frame of the microbial community (Ferrocino *et al.* 2018).

3. Results and Discussion

3.1 LAB and CNS isolates

The results of bacteria identification should be pointed out that, *Lactobacillus sakei* for LAB (Fig. 1a) and *Staphylococcus xylosus* for CNS (Fig. 1b), were present in all the productions never low than 50% of the relative abundance. In particular, the March's batch was composed of different lactic acid bacteria species other than *L. sakei*. In particular, in March production we can find *Lactobacillus plantarum*, *Lactobacillus coryniformis*, *Lactobacillus curvatus* and a big per cent of *Pediococcus acidilactis* that are no present in the other two batches.

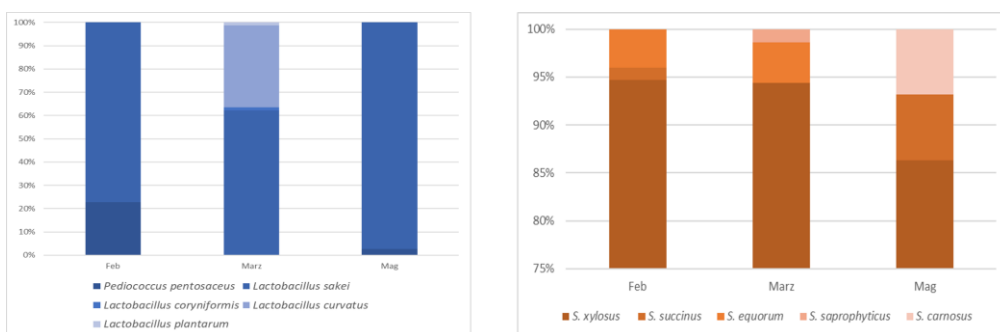


Figure 1 LAB (a) and CNS (b) species present in the three batches.

3.2 Growth curve at different temperature

Each test was performed in MRS broth for LAB and BHI broth for CNS, in microplates with an inoculum of 2% from overnight culture. The strains were tested for growth rate at 30°C, 20°C, 15°C and 10 °C. The growth was evaluated with a spectrophotometric method at 630 nm to obtain a growth curve. The same procedure was used for the growth rate in broth with 2-3 and 4% salt concentrations at 30°C. The optical density measures obtained were analyzed with R software (Growthcurver package).

The acidification capability of LAB strains was evaluated measuring the pH value after 8 hours from the inoculum at 30°C and 20°C and after 23 hours from the inoculum at 15°C and 10°C. Nitrate reductase activity was evaluated for CNC strains. An aliquot of overnight culture in YT-broth added of KNO₃ (250 ppm) was added of Grieff-Ilosvay's reagent and incubated at room temperature for 15 min. The nitrite production from nitrate was determined by a change in the broth colour.

All the data obtained were elaborated, a heatmap for each microplate was used to easily select the best strains. In the heatmap, the strains were chosen for good growth at all the temperature and a not strong acidity for the pH. For LAB group the selected strains were: two *P. pentosaceus* and two *L. sakei*; for CNS three strains of *S. xylosus* were selected.

3.3 Metagenomics analyses

The metagenomics approach evidence the *L. sakei* dominance in all the three batches followed by *P. pentosaceus* and *L. curvatus* (Fig.2). The presence of *S. xylosus* was ever very low. From the comparison of the three batches, we can observe a separation of their microbiota.

Strain-level biodiversity will have to be considered, in particular for *L. sakei*, to understand the relationship between the microbiome and final food characteristics.

Another added advantage is that these data generated by WGS can also be used for secondary analyses, such as the detection of the virulence gene, antibiotic resistance gene profiling, etc.

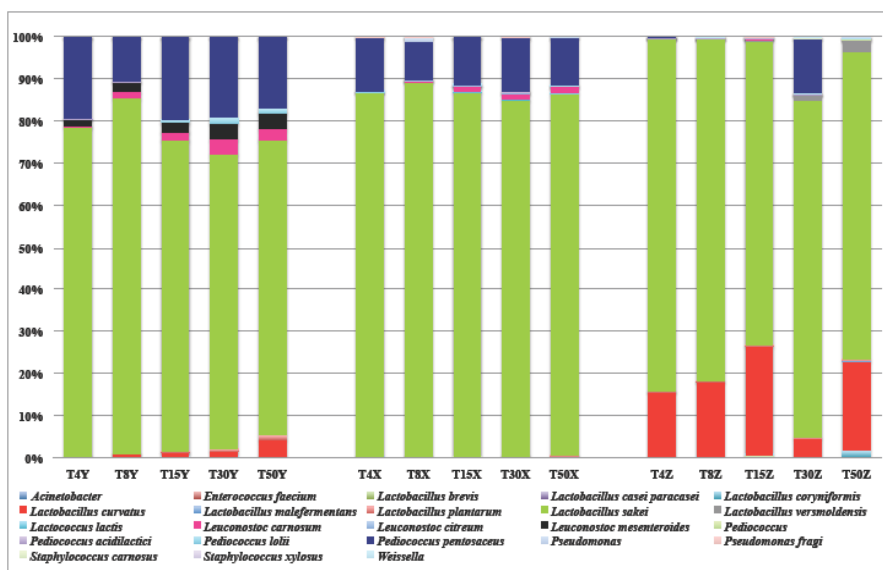


Figure 2 Dominance of *Lactobacillus sakei* never lower than 70% of the relative abundance in the three batches.

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Production of Functional Ingredients Using Bioactive Compounds from *Pleurotus Ostreatus*

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The first activities of the PhD thesis project are described. Firstly, the major bioactive compounds of *Pleurotus ostreatus*, namely: β -glucans, ergosterol, vitamin D₂ and phenolics were evaluated. Secondly, a food grade extraction procedure for the recovery of β -glucans was developed by measuring the yield and antioxidant activity. β -glucan-rich extract was used as emulsifying agent for the encapsulation of an oxidisable compound. The stability of oil-in-water (O/W) emulsions was determined checking the variation of oil droplets size distribution over time. The left-over insoluble solids could be exploited as a source of vitamin D₂ and ergosterol.

Produzione di ingredienti funzionali usando composti bioattivi da *Pleurotus ostreatus*

Le prime attività del progetto di tesi di dottorato sono descritte. In primo luogo, sono stati misurati i principali composti bioattivi di *P. ostreatus*, ovvero: β -glucani, ergosterolo, vitamina D₂ e fenoli. Successivamente, è stata messa a punto una procedura di estrazione dei β -glucani misurando la resa e l'attività antiossidante. I β -glucani sono stati utilizzati come emulsionanti per l'incapsulamento di un composto ossidabile. La stabilità delle emulsioni olio-in-acqua (O/W) è stata determinata attraverso la variazione della distribuzione dimensionale delle gocce d'olio nel tempo. Il residuo di estrazione può essere utilizzato come fonte di ergosterolo e vitamina D₂.

Key words: *Pleurotus ostreatus*, β -glucans, emulsion stability, encapsulation, vitamin D₂, sustainability.

1. Introduction

This PhD project aims at the development of applications of *P. ostreatus* biomass grown on a “sustainable” substrate as an ingredient in added-value foods. In accordance with the PhD thesis project previously described (Gallotti, 2018), this poster reports the main results of the first two activities concerning:

A1) Evaluation of the major bioactive compounds, namely: β -glucans, ergosterol, vitamin D₂ and phenolics;

A2) Development of a “food grade” extraction procedure of β -glucans and application of the extract for the stabilization of O/W emulsions.

2. Materials and Methods

For the study, a selected commercial strain of *P. ostreatus* rich in β -glucans was dried at 40 °C and grinded to a fine powder before use. Vitamin D₂ and its precursor ergosterol were extracted according to the procedure of Sławinska et al. (2016) and identified by the HPLC procedure by Huang et al. (2015). Hot-water soluble β -glucans fraction was extracted from *P. ostreatus* powder following a method adapted from Synytsya et al. (2009) and then modified as described by Gallotti et al. (2019). The

resulting extracts are, namely: extract from Washed solids, W; extract from Unwashed solids, UW, obtained removing a washing step with distilled water before the extraction. The amount of β -glucans in the mushroom powder and in the extracts was measured with an enzymatic kit purchased from Megazyme (Bray, Co. Wicklow, Ireland). The phenolic content was determined spectrophotometrically by the Folin-Ciocalteu assay, while the antioxidant activity was determined by the ferric ion reducing antioxidant power (FRAP) method. The O/W emulsions were prepared using maltodextrin with dextrose equivalents of 12 (MD, DE12) as wall material, β -glucans extract as emulsifier and commercial sunflower oil with 500 mg/kg of α -tocopherol as a model for lipophilic active compounds. To prepare the emulsions, a two-step protocol with first the production of a pre-emulsion without wall material and then the addition of an aqueous solution of MD and emulsifying agent was used. Emulsions were homogenized at lab scale (100 mL of volume) by a Polytron homogenizer and at pilot scale (1L of volume) by a rotor-stator homogenizer. The emulsion stability was estimated from the evolution of the oil droplet size distribution measured by laser light diffraction with a Mastersizer at the time of preparation and after 2 hours. From the volume size distribution obtained, the median diameter d_{50} and d_{10} and d_{90} , corresponding to the diameters for which respectively 50, 10 and 90% of the particles have a smaller size, were determined and the span was calculated as $\text{span} = (d_{90} - d_{10})/d_{50}$. Experimental data were analyzed by one-way ANOVA using LSD (Least Significant Difference) as a multiple range test.

3. Results and Discussion

3.1 Characterization of the biomass of *P. ostreatus* and its extracts

The major bioactive compounds of *P. ostreatus* biomass are reported in Table 1. The levels of these compounds in the selected strain were relatively high compared to those reported in the literature (Lavelli et al., 2018)

Table 1 Characterization of *P. ostreatus* biomass.

Total glucans g/100g d.w.	β -glucans g/100g d.w.	Vitamin D ₂ mg/100g d.w.	Ergosterol mg/100g d.w.	Total phenolics mg gallic acid equivalents/100g d.w.
36.0 $\pm$ 0.9	35.0 $\pm$ 0.9	0.26 $\pm$ 0.09	154 $\pm$ 23	272 $\pm$ 19

Data are average $\pm$ SD.

3.2 Encapsulation of an oxidisable target using *Pleurotus* β -glucans as an emulsifying agent

The total glucans and β -glucans contents (Tab. 1) were used to calculate the yield of extraction reported in Table 2. The extraction method for β -glucans previously proposed by Synytsya et al. (2009) was modified by removing a washing step in order to increase β -glucan yield from 7.7 to 17.2 % (Tab. 2). Different formulations of emulsions were then tested (Tab. 3) at lab and pilot scale, using both the W and UW extracts, maintaining the same percentage of dry matter (around 40%w/w). The results about stability (Table 3) show that no significant changes have occurred in the microstructure of the emulsions, confirming that they were physically stable when the percentages of β -glucans was more than 0.08% for the emulsions with 4%w/w of oil. The amount of emulsifier required depends on the oil quantity, thus for emulsions with 8%w/w of oil was necessary to increase the percentage of β -glucans up to 0.13%w/w to maintain the stability; less percentages of β -glucans resulted in not stable emulsions (data not shown).

In conclusion, the water-soluble extract of *P. ostreatus* can act as an efficient emulsifying agent, while the insoluble solids could be exploited as a source of vitamin D₂ and ergosterol.

Table 2 Characterization of the β -glucans-rich extracts obtained from *P. ostreatus*.

Extract	Dry matter %	Yield total glucans %	Total glucans g/100g d.w.	Yield β -glucans %	β -glucans g/100g d.w.	FRAP $\mu\text{mol Fell eq./100g d.w.}$
W	1.0 ^a $\pm$ 0.0	11.2 ^a $\pm$ 0.6	20.1 ^a $\pm$ 1.1	7.7 ^a $\pm$ 0.6	13.4 ^a $\pm$ 1.1	0.07 ^a $\pm$ 0.01
UW	1.4 ^b $\pm$ 0.1	20.4 ^b $\pm$ 1.1	26.2 ^b $\pm$ 1.3	17.2 ^b $\pm$ 0.8	21.6 ^b $\pm$ 1.1	0.13 ^b $\pm$ 0.00

Data are average $\pm$ SD. Values in the same column with differing superscripts are significantly different (LSD, $p < 0.01$).

Table 3 Formulations of emulsions and size distributions at the time of preparation ($t = 0$) and after two hours ($t = 2\text{h}$)

Emulsion	Formulation			Size distribution			
	Oil %	MD %	β -glucans %	d50 ($t = 0$) (μm)	d50 ($t = 2\text{h}$) (μm)	Span ($t = 0$)	Span ($t = 2\text{h}$)
E4_lab_W	4.0	35.4	0.08	2.02 ^a	2.00 ^a	1.4 ^b	1.4 ^b
E4_pilot_W	4.0	35.4	0.09	2.49 ^a	2.56 ^a	1.4 ^b	1.2 ^b
E4_pilot_UW	4.0	35.6	0.09	2.50 ^a	2.59 ^a	1.1 ^b	1.2 ^b
E6_lab_UW	8.0	31.4	0.13	2.84 ^a	2.96 ^a	1.4 ^b	1.4 ^b
E6_pilot_UW	8.0	31.5	0.13	2.76 ^a	2.83 ^a	1.1 ^b	1.1 ^b

D50 and span values in the same row with differing superscripts are significantly different (LSD, $p < 0.01$).

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Rapid Oxidative Stress Test for Lipid Soluble Antioxidants

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This work aims to develop fast, simple and direct methods to monitor oxidation reactions in lipid food systems, based on electrochemical sensors. Briefly, in the first part of the work, different stressing agents, such as radical initiators, hydrogen peroxide and metal catalyzers will be evaluated in terms of their capacity to induce oxidation in different food lipid systems. In the second part, rapid and fast monitoring methods will be implemented thanks to the use of electrochemical sensors. Overall, the work has the objectives to (1) develop novel sensors for the online monitoring of the storage stability of lipid foods (2) apply these methods for the screening of novel powerful lipophilic natural antioxidants.

Test di stress ossidativo rapido per antiossidanti solubili in lipidi

Questo lavoro propone metodi rapidi, semplici e diretti per monitorare la reazione di ossidazione lipidica, sulla base di sensori elettrochimici. La prima parte del lavoro consiste nello studio di diverse fonti di stress ossidativo, quali iniziatori radicalici, acqua ossigenata e catalizzatori metallici. Nella seconda parte del lavoro, questi elementi di stress ossidativo verranno implementati in metodi rapidi e semplici per il monitoraggio delle reazioni di ossidazione lipidica. In generale, questo lavoro permetterà di (1) implementare nuovi sistemi di monitoraggio dello stato ossidativo di alimenti contenenti lipidi e (2) utilizzare questi metodi rapidi come screening per la selezione di nuovi antiossidanti naturali che possano controllare o prevenire la fase di induzione ossidativa.

Keywords: cyclic voltammetry, lipophilic antioxidants, oxidative stress.

1. Introduction

In accordance with the PhD thesis project previously described, this poster reports the main results of the first three activities concerning:

A1) the development of novel fast and simple methods for the evaluation of the oxidative stress test of lipid foods by using different oxidative stress systems and electrochemical sensors;

A2) the application of the method for the direct analysis of the storage stability lipid foods, such as extra virgin, virgin and sansa oil samples;

A3) the application of the methods for the screening of new natural lipophilic antioxidants and their capacity to retard the induction step of the lipid autoxidation reaction.

2. Material and Method

Electrochemical measurements were performed by using the Autolab PGSTAT 128N potentiostat (Metrohm, Germany). A three-electrode system was used throughout the study. The efficacy of the cleaning and pretreatment procedure was verified by analyzing a standard solution of ferrocene methanol (10 mM) in 1-propanol containing LiCl (20 mM). The antioxidant activity of lipid soluble antioxidants, α -tocopherol, catechin,

retinyl acetate, caffeic acid and 3-hydroxytyrosol were measured with a single protocol, in homogeneous conditions by CV. The procedure consisted in a glass cell containing 10 mL of 1-propanol with LiCl (20 mM) as supporting electrolyte. All the signals were recorded and analyzed by software NOVA (version 1.11).

3. Results and discussion

3.1 Oxidative stress evaluation of lipid soluble antioxidants

The cyclic voltammetric profile of 100 μM of α -tocopherol is shown in Fig. 1. Upon the introduction of 150 μL of 200 mM of azo-bis-iso butyronitrile (AIBN) solution into the voltammetric cell maintained at 60 $^{\circ}\text{C}$, the magnitude of the oxidation peak is reduced over time. The approach was finally applied on catechin, caffeic acid, retinyl acetate and hydroxytyrosol. The shape of the anodic and cathodic scan reveals the irreversible nature of the system.

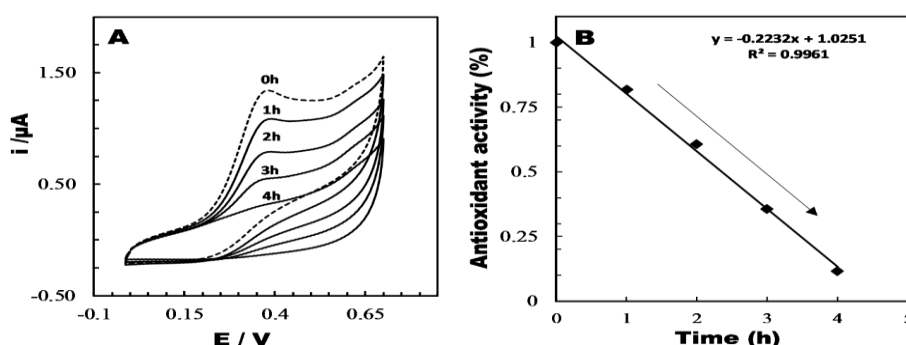


Figure 1 Cyclic voltammograms of 100 μM α -tocopherol antioxidant solution before (dashed line) and after (solid lines) addition of 150 μL of AIBN solution (200 mM) using a glassy carbon electrode in 1-propanol solvent system with LiCl (20mM) as supporting electrolyte at 60 $^{\circ}\text{C}$ (A) and the antioxidant activity of the tocopherol solution as a function of time (B). Scan rate is 0.1Vs $^{-1}$.

3.2 Hydrogen Atom Transfer (HAT) capacity and Electron Transfer (ET) capacity of olive oils

Finally, the method was applied to evaluate HAT and ET capacity of commercial extra virgin olive oils. Fig. 2 (A) shows the cyclic voltammograms of increasing concentrations of EVOO (A), without addition of AIBN. Peaks were increasing linearly with the increment of oil concentration. Similar CV profile was analyzed for virgin and sansa olive oil. EVOO showed lowest peak potential (0.35 V) than VOO and SOO. In general, lower the peak potentials correspond to greater antioxidant power because low energy required to abstract an electron (Lemma et al., 2014, Haman et al., 2017). Fig 2 (B) shows the cyclic voltammograms of after addition of 150 μL of the AIBN solution (200 mM). The oxidative stress prompted by AIBN in EVOO resulted in a decrease of the peak current (Shahidi et al., 2010; Morozova et al., 2016).

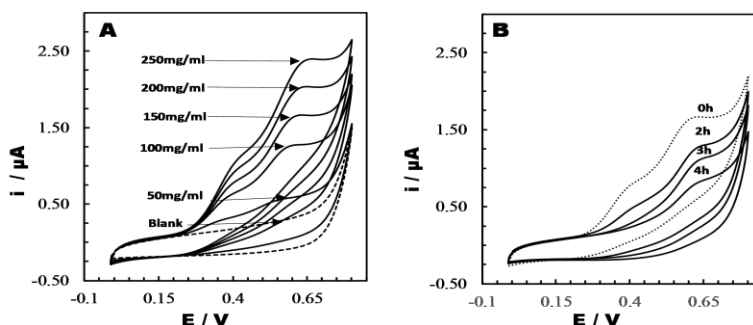


Figure 2 Cyclic voltammograms of EVOO by glassy carbon electrode in 1-propanol solvent system with LiCl (20mM) as supporting electrolyte: **(A)** increasing concentrations of EVOO **(B)** EVOO: HAT capacity

In this study, a simple, sensitive and direct electrochemical method was proposed to characterize the antioxidant capacity of lipid soluble antioxidants based on the reaction between the antioxidants with a radical initiator AIBN. According to the obtained results, it was possible to rank the hydrogen atom transfer capacity of the studied. Finally, the proposed method allows the direct measurement of the oxidative stability of the lipophilic antioxidants in complex matrixes, such as edible oils, without any pretreatment of the samples (commercial edible olive oils).

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Development of New Functional Dairy Products

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The PhD thesis project aims to develop functional dairy products. The first innovative functional product, is a phytosterols-enriched ricotta cheese. This functional product will aim to lower the LDL-cholesterol in consumers with risk of hypercholesterolemia and cardiovascular diseases. During the first 2 years of the PhD project, different studies have been carried out for the development of this product. Firstly, the ricotta composition has been studied; then, a new analytical method for phytosterols quantification have been developed; Thirdly, studies of the composition and the stability of functional ingredients (phytosterols) during ricotta processing and storage were assessed. Finally, the chemical composition of the developed products was studied and compared with normal ricotta cheese. The next year will be dedicated to assess the bioaccessibility of the functional compounds (phytosterols) in ricotta cheese during in vitro digestion models.

Sviluppo di nuovi alimenti funzionali nel comparto lattiero caseario

Il progetto di tesi di dottorato si propone di sviluppare prodotti caseari funzionali. Il primo prodotto è una ricotta arricchita da fitosteroli. Durante i primi 2 anni del progetto di dottorato, sono stati effettuati diversi studi per lo sviluppo di questo prodotto. In primo luogo, la composizione della ricotta è stata studiata; dopodiché sono stati sviluppati nuovi metodi estrattivi ed analitici per quantificare i fitosteroli. In terzo luogo, è stata valutata la composizione e la stabilità degli ingredienti funzionali (fitosteroli) durante la produzione e la conservazione della ricotta. Infine, la composizione chimica dei prodotti sviluppati è stata studiata e confrontata con la ricotta normale. Il prossimo anno sarà dedicato alla valutazione della bioaccessibilità dei composti funzionali (fitosteroli) nella ricotta durante i modelli di gestione in vitro.

Key words: Functional foods, phytosterols, ricotta cheese.

1. Introduction

Among the chronic diseases related to the human diet, cardiovascular disease, which is associated to high levels of total cholesterol, is one of the most leading cause of death in the world (Han et al., 2016). Nowadays, the growing consumer awareness of their blood cholesterol level, has guided the food industry to respond by proposing new food products, which could satisfy consumer expectations. Therefore, during the recent years, the development of low-fat food products has considerably increased especially in the dairy sector. Another strategy has consisted to develop functional dairy products (F.D.P) containing phytosterols, which are steroid compounds occurring in plants and having a lowering effect on blood LDL-cholesterol. This development of new F.F is an interconnected process involving food industry companies, European commission regulations, marketing and scientists (Küster-Boluda & Vidal-Capilla, 2018). In this regard, researchers from the University of Camerino and a cheese producing company named SABELLI have come together to produce a new phytosterols-enriched ricotta cheese. To our knowledge, it is the first time that a ricotta cheese enriched in phytosterols is developed. This poster reports the main results of the studies made to realize this F.D.P.

2. Materials and Methods

Ricotta samples were supplied by SABELLI Spa (Italy). Phytosterols and ingredients were supplied by ACEF Spa (Italy). The development of the phytosterol-enriched ricotta cheese followed a 7 steps-study:

- Studies on the ricotta composition;
- Development and validation of new analytical methods for phytosterols quantification;
- Study on the composition of the functional ingredient used for ricotta enrichment;
- Development of an extraction method for phytosterols extraction in ricotta cheese;
- Study of the stability of phytosterols ingredients during ricotta processing (heat-stability);
- Study of the stability of phytosterols during storage;
- Chemical composition of the obtained enriched product.

3. Results and Discussion

The developed method showed a good linearity ($R^2 \geq 0.995$) and high reproducibility ($RSD \% \leq 10\%$). It was an analytical innovation allowing the detection of both sterols and stanols by HPLC-DAD through a pre-column derivatization method.

This analytical method has been applied on functional ingredient and allowed to determine the phytosterols present in the ingredient. The extraction method showed high recoveries ($\geq 80\%$).

Phytosterols demonstrated a good stability to heat-treatment and during ricotta processing ($\geq 85\%$ of stability). Moreover, phytosterols remained stable during ricotta shelf-life (4-weeks). The developed functional cheese presented a similar nutritional profile compared to the original ricotta cheese.

These results confirmed that the production of a phytosterol-enriched cheese is technically and chemically feasible. Future analyses will be performed in order to assess the bioaccessibility of phytosterols in ricotta cheese. Moreover, sensorial analyses and consumer acceptance will be also assessed.

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Modelling of synergistic enzyme-assisted extraction of pigments from vegetable matrices

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The first two activities of the PhD thesis project are described: i) chemical characterization (moisture and cell wall polysaccharides) of vegetable products (tomatoes); ii) characterization of the enzyme preparations, evaluating the electrophoretic profile, kinetic parameters and optimum of temperature and pH by using the Response Surface Methodology (RSM). Considering the polysaccharide composition of tomato cell wall, it was possible to identify the suitable extraction conditions (T and pH) for the tailored enzymatic mix, thus enhancing the recovery of red pigments from tomatoes.

Ottimizzazione dell'estrazione sinergica assistita mediante enzimi di pigmenti da matrici vegetali

Le prime 2 attività del progetto di tesi di dottorato hanno riguardato: i) caratterizzazione chimica dei vegetali (pomodori; umidità e principali carboidrati costituenti); ii) caratterizzazione dei preparati enzimatici (profilo elettroforetico, parametri cinetici e optimum di temperatura e pH usando la tecnica statistica RSM). Considerando la composizione polisaccaridica della parete cellulare, è stato possibile identificare le condizioni di estrazione più adeguate (T e pH) per la miscela enzimatica selezionata, al fine di facilitare il recupero dei pigmenti rossi.

Keywords: Tomato red pigments, Cell wall polysaccharides, Enzyme preparations, Kinetic parameters, RSM analysis

1. Introduction

In accordance with the PhD thesis project previously described, this poster reports the results of the following activities:

A2) Chemical-physical characterization of vegetables to be enzymatically processed (i.e. tomatoes);

A3) Characterization of enzyme preparations to be employed: electrophoretic profile, kinetic parameters, optimal process conditions (T and pH) for their synergistic application.

2. Materials and Methods

Fully ripened red colored tomatoes of 'Ciliegino' variety were analysed for moisture, cellulose, hemicellulose (xylans) and pectin content (Ververis et al., 2007), and for glucose, xylose and galacturonic acid, using the specific assay kits. The selected enzyme preparations were characterized in terms of: i) protein content by Bradford's method; ii) electrophoretic profile; iii) kinetic parameters. The following activity assays were performed: polygalacturonase (PG), cellulase (CL) and xylanase (XL) (Ramalingam et al., 2013), amylase (AM, Ul-Haq et al., 2010) and pectin lyase (PL, Cerreti et al., 2017). For each enzyme preparation, the effect of temperature (20-70 °C, in acetate buffer pH 4.5) and pH (2.5-8, in McIlvaine buffer) on the enzymatic activity was investigated. RMS was applied and the experiments were carried out with a central composite face-centered

design with quadratic model, which was used to study the combined effect of the two independent variables (T and pH). The experimental design and statistical analysis were performed using Minitab 17.1.

3. Results and Discussion

The moisture content of fully ripened tomato was $94 \pm 1\%$ (wet weight basis) and the cell wall polysaccharides composition in dry weight basis (dm) was as follows: cellulose 4.7 ± 0.3 g/100gdm; pectin 2.1 ± 0.2 g/100gdm and hemicellulose 1.4 ± 0.1 g/100gdm. The 4 commercial enzyme used in this study were characterized by total proteins amount (Table 1), which ranged from 0.022 mgBSAeq/mL (PG) to 0.082 mgBSAeq/mL (AM).

Table 1 Protein content, kinetic parameters (V_{max} , K_M , k_{cat} , K_a), and molecular weight (MW) of tested enzyme preparations

Enzyme	Protein content (mg _{BSA} /m)	V_{max} (I.U./mg _{BSAeq})	K_M (mg/mL)	k_{cat} (min ⁻¹)	K_a (min ⁻¹ /mg mL ⁻¹)	R^2	MW (kD)
PG	0.022±0.001	3805±274	0.70±0.11	1.51x10 ⁸ ±274	2.15x10 ⁸ ±5.41x10 ⁶	0.97	69
PL	0.022±0.001	6±0.4	6.44±0.55	2942±0.45	457±3.36	0.98	40
CL	0.029±0.007	626±37	6.53±0.81	1.12x10 ⁷ ±37	1.85x10 ⁶ ±2.68x10 ⁴	0.98	55
XL	0.033±0.003	84±2	0.0071 ±0.0007	3.04x10 ⁵ ±2.2	4.25x10 ⁷ ±4.20x10 ⁵	0.97	24
AM	0.082±0.002	17±1	0.17±0.01	50488 ±0.41	2.93x10 ⁵ ±2.65x10 ³	0.98	49

Observing the SDS-PAGE profile (Fig. 1), the protein fingerprint was characterized by a dominant band, thus indicating the high purity of all the commercial enzymes which appeared monocomponent preparations. Concerning PG, the band at 40 kDa corresponded to a PL, as proved by the enzymatic assay.

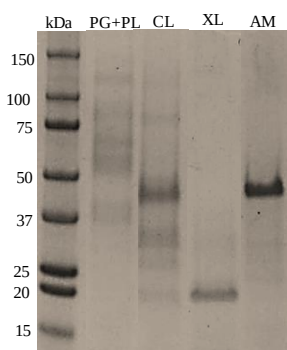


Figure 1 Protein profiles, obtained by SDS-PAGE electrophoresis, of each commercial enzyme preparation. Relative molecular mass of protein standards (kDa) are indicated.

The kinetic curves of the 4 tested enzymes followed the hyperbolic behaviour described by the Michaelis-Menten equation and the relative parameters (K_M , V_{max} , k_{cat} and K_a) are reported in Table 1. The effect of T and pH on the activity of each enzyme was evaluated by the RSM analysis. The variance for each factor evaluated was partitioned into linear, quadratic and interactive components and was represented using the second order polynomial function as follows (1):

$$y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_{12} + b_{22}X_{22} + b_{12}X_1X_2 \quad (1)$$

For all response variables, the R^2 values (higher than 0.90) suggested that the regression models well explained the enzyme activity behaviour when T and pH changed (Table 2), with no lack of fit. The squared effects of T and pH were all highly significant

(p -value < 0.001). The effect of T was always slightly higher than the effect of pH , except for XL . All of the interaction effects were statistically significant (p -value > 0.05), proving the synergistic effect exerted by the two variables on all the enzyme activities, with the only exception of XL .

Table 2 Regression coefficients, model p -value, lack of fit, R^2 and adjusted R^2 for the different polynomial models (Note: Subscripts: 1 = temperature; 2 = pH ; *Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level.).

Regression coefficient	PG	CL	XL	AM
b_0	+96.40	+85.30	+91.78	+84.69
b_1	-6.05***	+6.02	+1.18	-3.01
b_2	-2.84*	-0.90	+9.68**	+2.21
b_{11}	-37.46***	-32.19***	-37.60***	-21.71***
b_{22}	-33.48***	-29.02***	-33.97***	-50.08***
b_{12}	-9.30*	+10.18*	-	+4.52*
Model p -value	0.000	0.000	0.000	0.000
Lack of fit p -value	0.209	0.119	0.073	0.186
R^2	0.991	0.929	0.948	0.981
R^2_{adj}	0.987	0.879	0.926	0.967

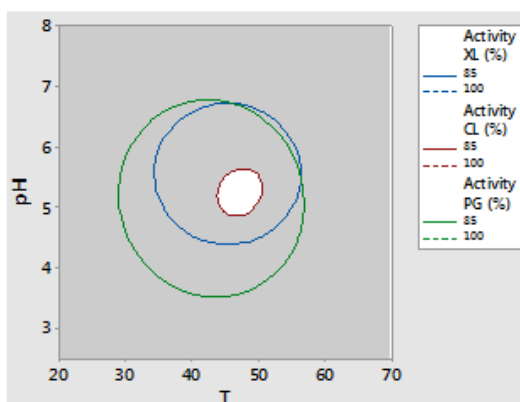


Figure 3 The optimum region by overlaying contour plots of the three enzyme activities evaluated as a function of Temperature and pH

This statistical approach allowed us to identify the optimal range of T and pH for maximizing the enzyme activities when applied in mix, thus enhancing process flexibility. In order to optimize the process conditions (T and pH) for the extraction of lycopene from tomatoes

(considering their chemical composition) the application of a tailored enzymatic mix (based on CL , PG and XL) was enhanced by RSM. The contour plots of CL , PG and XL were superimposed in an overlay plot (Fig. 3). Graphical optimization displays that the optimal conditions for the simultaneous application of CL , PG and XL (activity higher than 85%) were: 45-55 °C and pH 5-6.

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Improvement of the Use of Food Resources and Reduction of Post Harvest-Losses in African Countries

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The first PhD thesis project activities are described. Firstly, hyperenergetic and sustainable meals “Pappa di Parma” were developed against infant malnutrition in Tanzania. After safety and quality assessment, the Pappa feasibility and the families’ acceptability were tested in the Rukwa region (Tanzania). Data collected indicated Pappa formulas as possible sustainable long-term solutions to decrease infant malnutrition in Tanzania. Secondly, the effect of sprouting and drying conditions of sorghum on flour technological properties was studied considering different flour blends (sorghum, wheat and cassava in different proportion). Sprouting affected the technological quality of flour while drying conditions did not affect same properties.

Miglioramento dell’utilizzo di risorse alimentari e diminuzione delle perdite post raccolta in prodotti locali di paesi africani

La prima attività ha riguardato lo sviluppo di formulati iperenergetici e sostenibili contro la malnutrizione infantile in Tanzania (“Pappa di Parma”), la loro caratterizzazione e implementazione nella regione di Rukwa. Lo studio ha mostrato che la Pappa di Parma può rappresentare uno strumento accessibile e sostenibile per la lotta alla malnutrizione. La seconda attività ha riguardato lo studio delle proprietà tecnologiche di blends di farine di sorgo (germinato ed essiccato in due condizioni) – frumento – tapioca, in diverse proporzioni. La germinazione, e non le condizioni di essiccamento della granella germinata, ha influenzato le proprietà tecnologiche delle farine studiate.

Key words: Pappa di Parma, Tanzania, sustainability, infant malnutrition, sorghum, sprouting, technological properties, thermal properties, pasting properties.

1. Introduction

In accordance with the PhD thesis project previously described (Marchini, 2018), this poster shows the main results of the first two activities (A1 and A2):

A1) Development, characterization and local implementation of “Pappa di Parma” formulas against infant malnutrition in Tanzania.

A2) Effect of different drying conditions after germination on the technological properties of sprouted sorghum flour and its blends with common wheat and tapioca flour. The nutritional characterization analysis are ongoing.

2. Materials and Methods

A1) After the definition of a Tanzanian food composition database and the nutritional needs for malnourished children (Nutrition Unit, University of Parma), the meals were developed using basic technologies and raw ingredients accessible to Tanzanian families. The ingredients were processed (i.e. root boiling, flour rehydration, seed toasting, pulse soaking), reduced in size using a pestle and mortar, hand stirred and mixed with fat phase creating a homogeneous and sweet creamy meal, acceptable to children. Moisture content (MC%, 105°C in oven to constant weight), water activity (a_w ,

25°C, igrometer), pH and colour (L^* , a^* , b^* , colorimeter Minolta CM-2600d) were measured at day 0 and 90. To verify the product quality during storage, peroxide value (PV, AOAC Method 965.33) after lipid extraction (AACCI Method 30-25.01) was analyzed at day 0, 2, 7, 15, 50 and 90 after production, at 25 and 40°C, with and without being vacuum sealed and airtight, and in light and dark conditions. To verify the Pappa feasibility and acceptability, thanks to the partnership with the St. Joseph University (Tanzania) and Golfini Rossi ONLUS, the meals were prepared and tested at Mvimwa Abbey (Rukwa Region, Tanzania) on 287 children and their mothers by the compilation of a simple questionnaire.

A2) White sorghum seeds were soaked for 16h at 25°C and 90% UR, germinated in an industrial sprouting plant (Bühler AG, Uzwil, Switzerland at Molino Quaglia, Vighizzolo D'Este, Italy) for 72h at 25°C and dried for 12h at 40°C (SgA) or 6h at 50°C (SgB). Ungerminated sorghum grain (S) was used as control. All samples were grounded in a labscale mill (Bona S.r.l, Monza, Italy), and after ricostitution to whole grain sorghum flour, mixed with whole grain common wheat flour (W) and tapioca flour (T) to obtain the following blends: 100:0 (S; SgA; SgB; W; T), 50:50 (S:SgA; S:SgB; T:S; T:SgA; T:SgB; W:S; W:SgA; W:SgB; SgA:SgB) and 33:33:33 (W:S:T; W:SgA:T; W:SgB:T). Proximate analysis, water and oil holding capacity (WHC and OHC), swelling power and solubility (Sp and S0 at 60, 70, 80 and 90°C), starch pasting properties [(Pasting Temperature (PastingT), Peak Temperature and Viscosity (PeakT and PeakV), Final Viscosity (FinalV), Breakdown (BD) and Setback (ED) with MVAG; Marti et al, 2017], thermal properties [starch gelatinization enthalpy (ΔH_g) and amylose–lipid complexes melting enthalpy (ΔH_{al}) with DSC heating from 30°C to 120°C at 5°C/min (DSC Q100, TA Instruments, New Castle, DE, USA)] were measured. Significant differences ($p \leq 0.05$) among samples were assessed by one-way ANOVA with a Duncan post-hoc test. A Principal component analysis (PCA) was also performed (SPSS version 25.0, IBM SPSS Inc., Armonk, New York, USA).

3. Results and Discussion

Table 1 *Pappa di Parma formulas.*

PAPPA 1	PAPPA 2	PAPPA 3	PAPPA 4
Palm oil 9%	Palm oil 8,5%	Palm oil 6,6%	Palm oil 12,8%
Peanuts 9%	Peanuts 12,8%	Peanuts 8,9%	Sunflower seed oil 10,3%
Sunflower seeds 16%	Bean flour 25,5%	Sesame 11,1%	Sesame butter 17,9%
Cassava flour 16%	Corn flour 10,6%	Bean flour 24,5%	Soya flour 25,6%
Milk powder 22,7%	Cassava flour 8,5%	Cassava flour 11,1%	Sugar 23,1%
Sugar 16%	Sugar 12,8%	Sugar 17,8%	Baobab 10,3%
Avocado 11,3%	Avocado 21,3%	Avocado 20%	

Table 2 *Pappa di Parma physico-chemical characteristics.*

	PAPPA 1	PAPPA 2	PAPPA 3	PAPPA 4		
				25°C, vacuum, dark stored	25°C, no vacuum, light stored	40°C, no vacuum, light stored
<i>Day</i>						
a_w						
0	0.90±0.00 ^b	0.99±0.00 ^a	0.97±0.00 ^a		0.45±0.00 ^{CA}	
90	n.a.	n.a.	n.a.	0.39±0.00 ^B	0.36±0.00 ^C	0.32±0.00 ^D
MC (%)						
0	29.37±0.95 ^c	58.56±0.3 ^a	43.49±0.14 ^b	2.72±0.00 ^B	2.96±0.07 ^{DA}	1.58±0.02 ^C
90	n.a.	n.a.	n.a.	2.60±0.06 ^B	4.14±0.02 ^{BA}	
pH						
0	5.78±0.08 ^c	6.29±0.1 ^a	6.13±0.10 ^b	3.83±0.05 ^B	3.76±0.03 ^B	3.78±0.03 ^B
90	n.a.	n.a.	n.a.	3.76±0.03 ^B	1.26±0.49 ^{AB}	
PV (mEq/kg)						
0	n.a.	n.a.	n.a.	1.84±0.02 ^A	0.85±0.01 ^B	1.69±0.00 ^A
90	n.a.	n.a.	n.a.			

Values are expressed as means±SD. Values followed by different lowercase letters in each row are significantly different according to Duncan's test ($P \leq 0.05$). Values followed by different capital letters are significantly different according to Duncan's test ($P \leq 0.05$).

A1) Four formulas of Pappa were developed (Table 1) and characterized (Table 2). Pappa n.1, 2 and 3 needed to include water in their formulas and a_w and pH values resulted not able to ensure the long-term product safety, as expected. Instead, Pappa 4 didn't include water in its formula and a_w and pH values guaranteed a long-term stability. Moreover, the low PVs quantified (maximum $1,84 \pm 0,02$ mEQ/kg for 25°C vacuum sealed airtight sample) at day 90 confirmed the stability of the product in all storage conditions tested, as reported by Manary, 2005. The meals acceptance in terms of taste (> 93%), the mothers' availability to get involved in the preparation, ingredient and production costs accessibility, showed Pappa di Parma as possible concrete solution against child malnutrition.

A2) Principal Components Analysis (PCA) was performed to understand the effect of sorghum grain germination and drying conditions on technological quality of flours (Fig. 1). Based on a first PCA on the whole dataset, T and T: SgB samples were excluded as outliers, due to their high thermal and pasting properties. We obtained a PCA model where the first two components explained 67.2% of variance. PC1 (47.2%) clusters SgA, SgB and their blends, grouped in the negative side (Fig 1b) and is mainly represented by Δ Hal, WHC, OHC, Sp60, PastingT, PeakT parameters (Fig 1a). PC2 groups T blends with SgA and SgB, in its positive side (Fig 1b) with a major role played by variable S0, while samples in negative side (S blends) depended on Sp80, Sp90, ED and FinalV (Fig. 1a). PCA output discriminated samples depending by sprouting treatment, but not by drying condition (SgA, SgB and their blends). According to Marchini, 2018, following steps are the investigation of sprouting and drying conditions effects on nutritional quality of sorghum, to optimize formulas and processes for sorghum-pulses flat bread development.

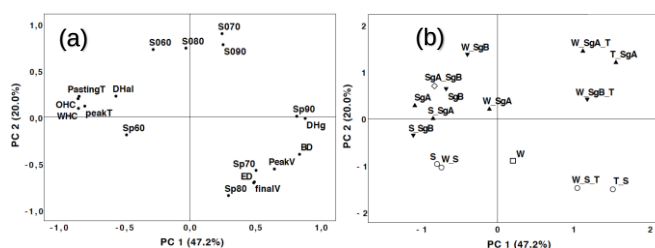


Figure 1 Principal Component Analysis (PCA) results obtained for the two principal components, showing (a) the projection of the variables on the factor plane, and (b) the projection of the cases on the factor plane. $\square$ W; $\circ$ S; $\blacktriangledown$ SgB; $\blacktriangle$ SgA; $\diamond$ SgA:SgB.

4. Acknowledgements

We would like to thank Prof. A. Pagani, A. Marti (DeFENS, University of Milan, Italy), Lucio Quaglia (Molino Quaglia SpA) for kindly providing the sorghum grain sample, supporting us in the germination process and in MVAG analysis, and Prof. G. Costantino for supporting us in the PCA analysis.

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Seaweed Biomass Fermentation: a New Approach for Food Production

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During the second PhD year the ability of microorganisms to growth on algal matrix was investigated. The fermentability of macroalgae was studied in order to obtain fermented food but also antimicrobials and compounds of interest for food industry. It was also evaluated the ability of food borne pathogens to develop on commercial seaweed and their growth kinetics.

Fermentazione di biomassa algale: un nuovo approccio per le produzioni alimentari

Durante il secondo anno di dottorato si è indagata la capacità di microrganismi di crescere su matrice algale. Si è studiata la fermentescibilità di macroalghe al fine di ricavare alimenti fermentati ma anche antimicrobici ed altri composti di interesse per l'industria alimentare. Si è valutato anche come i microrganismi patogeni di origine alimentare siano in grado di svilupparsi su alghe di interesse commerciale e la loro cinetica di crescita.

Key words: Seaweed, Lactic acid bacteria, Fermentation, Food borne pathogens

1. Introduction

Seaweeds are multicellular eukaryotic organisms usually classified into three groups on the basis of their pigmentation: red algae (Rhodophyta), green algae (Chlorophyta) and brown algae (Phaeophyceae). Seaweeds are of great interest for their content in natural compounds and metabolites with medical and technological properties, but represent also a viable and economical source as staple foods or functional ingredients for food productions. Lactic acid fermentation has been applied by centuries to preserve food and to obtain aromatic compounds. In accordance with the PhD thesis project previously described (Martelli, 2018), this poster reports results concerning:

A4) Development of functional foods with fermented seaweeds: The development of seaweed-based functional foods and ingredients is a great opportunity for the unused seaweeds that live the Mediterranean Sea. The main goal is the valorization of seaweeds by lactic acid fermentation to produce functional foods or ingredients (A4.1). Fermentation processes, functional and technological characterization, food safety (A4.2) and food design will open new perspectives to consider seaweeds as an innovative food matrix to obtain innovative and functional food products.

2. Materials and Methods

2.1 Bacterial strains used for fermentation

Different bacterial strains isolated from both vegetable and dairy matrices and belonging to different species were used to evaluate seaweeds fermentation: *Lactobacillus casei* (2240), *Lactobacillus paracasei* (4186), *Lactobacillus rhamnosus* (1473), *Lactobacillus rhamnosus* (GG), *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum* (285), *Bacillus subtilis* (5002) were used to test the fermentability of seaweeds and three strain of *Bacillus cereus* (Isolated from seaweed

samples) were used for the microbiological challenge test. All the strains, except for the commercial strain *L. rhamnosus* GG, belong to the collection of the Department of Food and Drug of the University of Parma.

2.2 Seaweeds fermentation conditions and monitoring

The fermentability was tested on three different species of seaweeds: *Himanthalia elongata*, *Undaria pinnatifida* and *Palmaria palmata*. The matrix was rehydrated with 75% of water and then inoculated individually with each bacterial suspension in order to obtain a final concentration of 7 Log CFU/ml. The fermentation was monitored by means of counts in bacterial culture media.

3. Results and Discussion

3.1 Raw *Himanthalia elongata* fermentation

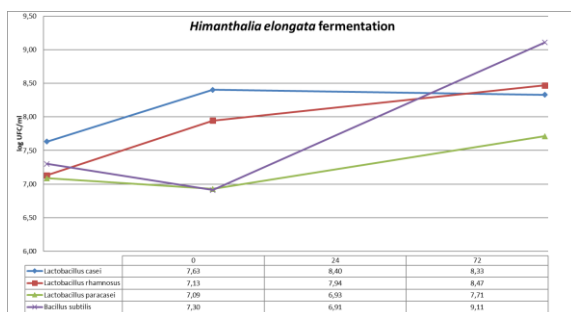


Figure 1 Growth curve of LAB and *B. subtilis* on *Himanthalia elongata*.

Himanthalia elongata is a species of brown seaweed belonging to the order of Fucales that grows copiously on the coast of the atlantic ocean. The aim of the study was the evaluation of *Lactobacillus casei* group bacteria and *Bacillus subtilis* capacity to grow on raw seaweed as the sole source of nutrients. The fermentation of *H. elongata* was carried on for 24 and 72 hours in sterilized glass cans. The four strain tested showed a different attitude to grow on the matrix. *L. casei* group bacteria (*L. casei* 2240, *L. paracasei* 4186, *L. rhamnosus* 1473) growth was determined, by means of counts in MRS agar medium, after 24 and 72 hours of incubation at the optimal temperatures of the tested strains, 37 °C. *Bacillus subtilis* 5002 growth was measured after 24 and 72 hours at 30° C on nutrient agar (NA). Contrary to the results obtained in the study by (Gupta & Abu-Ghannam, 2011), in which it was observed that neither the raw form of *H. elongata* nor that thermally processed were able to support the growth, in the present work we can see how the strains were able to grow in the substrate.

3.2 *Undaria pinnatifida* and *Palmaria palmata* fermentation

Considering the results obtained with the fermentation of *H. elongata*, the same fermentation process was carried out with other two different species of algae *Undaria pinnatifida*, an asiatic brown algae, and *Palmaria palmata*, a red algae typical of the atlantic ocean. With this experiment was tested how the fermentation process changed with a raw or a sterilized seaweed. *L. rhamnosus* 1473 was used to test the fermentability

of the samples. Fermentation of these seaweed species was not possible. Seaweeds have a lot of bioactive compounds such as protein (phycoerythrin, phycocyanin), small peptides, polyphenols (phlorotannins) and sulfated polysaccharades (fucans and fucoindans) with antimicrobial activity (Pérez, Falqué, & Domínguez, 2016).

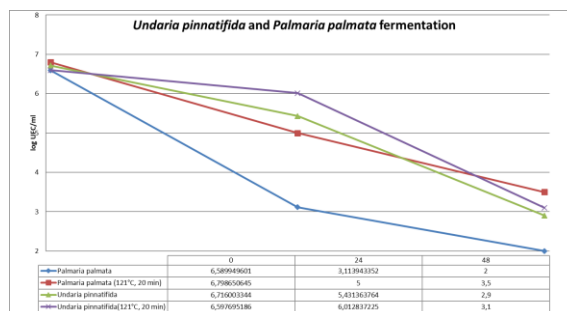


Figure 2 Growth curve of *L. rhamnosus* on *Undaria pinnatifida* and *Palmaria palmata*.

3.3 Behaviour of *Bacillus cereus* on seaweed: microbiological challenge test

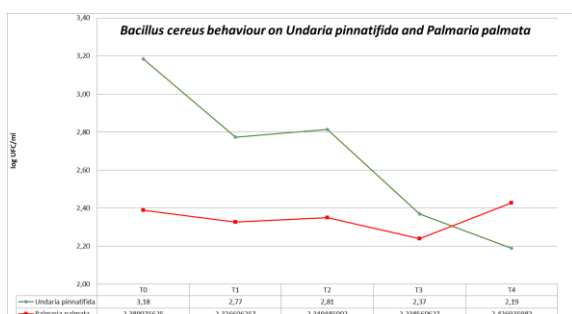


Figure 3 Growth curve of *B. cereus* on *Undaria pinnatifida* and *Palmaria palmata*.

Based on the results obtained with the lactic acid fermentation, two microbiological challenge test were carried out considering two products made of different species of seaweeds (*Undaria pinnatifida* and *Palmaria palmata*) to evaluate the behavior of *Bacillus cereus* in a rehydrated seaweed product during the shelf life. The algae were contaminated (3 Log cfu /g) with a mixture of three strains of *B. cereus* previously isolated from seaweed samples.

The contaminated seaweed has been divided into aliquots and subsequently kept at 4°C to simulate the storage conditions. *B. cereus* growth was monitored by plate count just after contamination (T0) and after 1 (T1), 2 (T2), 3 (T3) and 4 (T4) days of storage. *U. pinnatifida* and *P. palmata* did not sustain the growth of inoculated *B. cereus*. *P. palmata* showed a bacteriostatic effect, while *U. pinnatifida* had a bactericidal effect against *B. cereus*.

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Enzyme-Assisted Aqueous Extraction of Oil from Oilseeds By-Product

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The aim of this industrial PhD project, founded by MIUR through the Research and Innovation National Operative Program (PON) 2014-2020 and developed in collaboration with the Besana S.p.A. Company, is to study enzymatic processes able to degrade oilseeds structure in order to improve the technological high quality oil extraction. In order to obtain more information about oil extractability, the preliminary activities of this project are described. Firstly, the different enzymes, such as protease, cellulase and pectinase, which degrade the principal components of the oilseed structures were tested. Moreover, the optimum seeds flour: buffer ratio was investigated.

Estrazione di olio da scarti di oleaginose mediata da enzimi in fase acquosa

Lo scopo del progetto di dottorato industriale, finanziato dal MIUR attraverso il PON di Ricerca e Innovazione 2014-2024 e sviluppato in collaborazione con Besana S.p.A., è lo studio di processi enzimatici in grado di degradare la struttura di oleaginose per il miglioramento tecnologico del processo di produzione di olio di alta qualità. Per ottenere maggiori informazioni sull'estrazione dell'olio, sono descritte le attività preliminari di questo progetto. Inizialmente, sono stati testati i diversi enzimi, quali proteasi, cellulasi e pectinasi, che degradano i componenti principali delle strutture di semi oleosi. Inoltre, è stato valutato il miglior rapporto farina di semi: buffer.

Key words: aqueous enzyme extraction, pumpkin seeds oil, oilseeds, solvent-extraction.

1. Introduction

Enzymatic treatments that improve oil extractability from oilseeds represent a new and environmentally friendly way to produce oil for companies. It is well known that oilseeds are a good source of ω -3 and ω -6 fatty acids and α -tocopherol. These have significant and valuable benefits on human health that classify them as a nutraceutical oil. The oil extracted by oilseeds is a very good source of polyunsaturated fatty acid (Câmara & Schlegel, 2016). In particular, pumpkin seeds contain 27-49% of oil, which is an important source of unsaturated fatty acids, phytoestrogens and vitamins E, known to have potential pharmaceutical, nutraceutical, and cosmeceutical properties. The unsaturated fatty acid value was about 73% and consisting mainly of 18% oleic acid and 52% linoleic acid. In addition, pumpkin seeds contain a valuable amount of proteins (around 20-40%) (Merua *et al.*, 2018).

This study focuses on the development of enzymes processes that are able to increase the oil extractability. The commercial hexane used as the most common solvent for oil extraction is listed among hazardous air pollutants associated with neurological and respiratory disorders on prolonged exposure. Because of some safety considerations, it is necessary to develop aqueous oil extraction, but these methods are not successful, mainly due to the low oil yields. However, aqueous enzymatic extraction might be an alternative eco-friendly way to extract oil from oilseeds. The presence of certain enzymes

during extraction enhances oil recovery by breaking cell walls and oil bodies (Rosenthal *et al.*, 1996). Different study that used different source of edible oil, such as coconut, peanut, soybean, reported good results. In order to obtain more information about oil extractability, we used the different enzymes, such as protease, cellulase and pectinase, which degrade the principal components of the oilseed structures. The present study on pumpkin seeds can increase the possibility to use an environmental method to produce high quality oil from oilseeds using commercial enzyme.

2. Materials and Methods

Materials. Deshelled pumpkin seeds provided by the company were used for the investigation. Commercial enzymes (papain, acid cellulase and pectinase blend for oil) were obtained from Creative enzyme (USA). The other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Methods. Pumpkin seeds were ground to a fine powder by using a coffee grinder at high speed. The powder was then dispersed in sodium acetate buffer 0,05 M at pH 5 at different concentration of powder-to-buffer ratio followed by gentle up and down to mix the solution. Different kind of enzymes or blend of enzymes were added and in particular, the level of enzyme required was setted at 100 mg (w/w). The samples were then incubated at 50°C and 150 rpm in an orbital shaker for 16 h, followed by centrifugation at 8000 rpm for 30 min. A control (aqueous oil extraction) was also carried out for the above extraction during which no enzyme was added. The upper oil phase was carefully quantified by image analysis using Adobe Photoshop software (Yusoff *et al.*, 2016). Oil recovery was calculated as percentages of total oil present in each seed. Total oil was determined by solvent extraction using n-hexane in a Soxhlet apparatus. Pumpkin seeds oil is reportedly present in the range of 27–49 g oil/100 g pumpkin seeds (Merua *et al.*, 2018). A value of 49 g oil/100 g pumpkin seeds was taken as 100% recovery of oil when calculating the oil recovery under various conditions in aqueous enzymatic extraction.

3. Results and Discussion

3.1 Determination of enzymatic oil extraction parameters

In most of the aqueous enzymatic oil extraction procedures reported in the literature, commercial enzyme preparations containing varieties of enzyme activities, such as cellulase, pectinase and amylases, were used. For example, Yusoff *et al.* (2016), used a mixture of protease and cellulase to maximize the oil recovery from *Moringa oleifera*. In our experiments papain, acid cellulase and pectinase blend for oil were used to study wich enzyme works better to enzymatic oil extraction. As described in literature, proteases are the best enzymes for increasing the oil recovery, because of the high amount of proteins. Fig. 1 shows that papain is the most performant enzyme as it decreases the amount of trapped oil in the pellet, thus increasing the extraction yield.

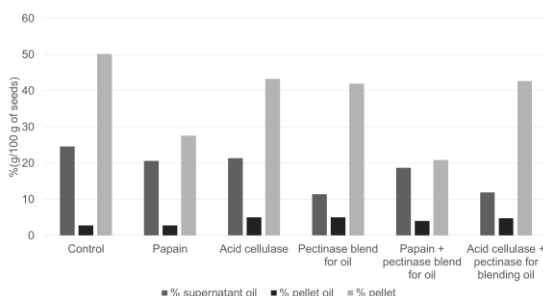


Figure 1 Percentage of recovered oil in supernatant and in pellet and percentage of pellet residue.

Thus, commercial papain was chosen as the best enzyme useful for the oil extraction in considered oilseeds and then, the incubation time and the ratio between buffer and sample were evaluated. As shown in Fig. 2, the ratio 1:2 and 1:3 powder-to-buffer was found to give the maximum oil recovery, that is almost the 50% of oil on the total weight of the sample after 20 and 24 hours of incubation respectively.

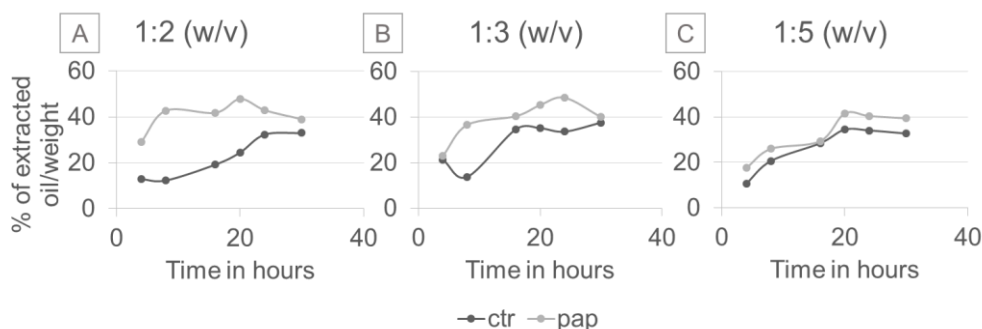


Figure 2 Amount of oil extract (%) at different ratio of flour: buffer. A, 1:2; B, 1:3; C, 1:5. The level of enzyme required was set at 100 mg (w/w). Slurry was made of 5 g powder of pumpkin seeds in ratio of 1:2, 1:3 and 1:5 (w/v) of sodium acetate buffer 0.05 M at pH 5. The incubation time was for 4, 8, 16, 20, 24 and 30 hours at 50°C and 150 rpm. All the experiments were run in triplicate, and the standard deviation was less than 5%.

This work showed that aqueous enzymatic oil extraction gave higher yield than the simple aqueous oil extraction. In that case, the proteases had an important role because of the high level of proteins of the matrix. Therefore, papain may help the recovery of oil. In addition, the other parameters studied, such as time and ratio also affect the level of oil extraction. In the end, these parameters are a good starting point to optimize the aqueous enzymatic oil extraction, with the aim to avoid as much as possible the oil extraction with solvent.

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Design of Delivery Systems for Probiotic Bacteria: the Case Study of Monoglyceride-Based Structured Emulsions

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One of the activities of this PhD thesis research is the design of probiotic bacteria delivery systems for food applications. Among different considered systems, monoglyceride-based structured emulsions resulted particularly promising to deliver probiotic bacteria into food and protect them during digestion. Following, the structural characteristics of designed emulsions will be described along with the viability of an encapsulated probiotic strain of *Lactobacillus rhamnosus* upon *in vitro* digestion.

Sviluppo di sistemi di veicolazione di microorganismi probiotici: il caso studio di emulsioni strutturate a base di monogliceridi

Una delle attività di questo progetto di tesi di dottorato è lo sviluppo di sistemi per veicolare microorganismi probiotici in campo alimentare. Tra tutti i sistemi considerati, le emulsioni strutturate a base di monogliceridi sono risultate particolarmente promettenti per veicolare microorganismi probiotici negli alimenti e proteggerli durante la digestione. Di seguito verranno descritte le caratteristiche strutturali delle emulsioni sviluppate e la vitalità durante la digestione *in vitro* di una coltura probiotica di *Lactobacillus rhamnosus* incapsulata.

Key words: structure, monoglycerides, probiotic bacteria, *in vitro* digestion, delivery system.

1. Introduction

In accordance with the PhD thesis project previously described (Melchior, 2018), this poster reports the main results of the activities concerning:

A1) the structural characterization of monoglyceride-based structured emulsions containing different water phases;

A2) the study of the effect of the designed emulsions on probiotic viability upon *in vitro* digestion.

2. Materials and Methods

The monoglyceride-structured emulsions were prepared in two replicates as previously described (Marino et al., 2017). In particular, three different ternary systems were prepared with the following composition: 36.4% (w/w) of a lipid phase made up of sunflower oil; 7.2% (w/w) of a mixture of saturated monoglycerides (MG), palmitic and stearic acids in 5:1:1 (w/w) ratio and 56.4% (w/w) of a water phase consisting on i) UHT skim milk, ii) 1 mM of NaOH aqueous solution, or iii) 0.2% (w/w) whey proteins aqueous dispersion. Briefly, the water phase and the lipid phase, previously heated at 70 °C, were mixed using a high-speed homogenisator at 1600 g for 20 s. During mixing 1 mL of UHT skim milk containing *Lactobacillus rhamnosus* cells was added to the mixture (about 10⁷ CFU/g in the final system). Samples were then cooled and stored at 4 °C for 24 h before analysis. Samples were characterized by pH measurement, rheological analysis (stress sweep and frequency sweep) and polarized light microscopy (PLM). The samples were

in vitro digested following the protocol proposed by Minekus *et al.* (2014). Probiotic viability was determined at the end of oral, gastric and intestinal phases. Viable counts were compared to those of controls made of free *L. rhamnosus* cells suspended in the water phase of each system. t-test or analysis of variance (one-way ANOVA) and Tuckey's comparison test were performed on collected data. These activities were conducted in collaboration with Prof. Marilena Marino and Prof. Nadia Innocente (University of Udine).

3. Results and Discussion

The MG-based emulsions were characterized by a white color, a self-standing behavior, and pH values suitable to guarantee probiotic viability (Tab. 1). Microscopic images showed that monoglycerides surrounded small oil droplets dispersed in the aqueous phase forming a network entrapping both oil and water phases (data not shown). Indeed, rheological measurement highlighted that all the structured emulsions presented a weak gel behavior characterized by G' and G'' parallel in the entire frequency domain, with G' higher than G'' . However, the presence of skim milk or whey proteins in water phase determined an oil droplet increase and a reduction of the mechanical parameters in comparison with water. This behavior can be attributed to the interactions between monoglycerides and milk components, such as proteins and salts, resulting in a weaker gel (Valoppi *et al.*, 2015).

Table 1 Visual appearance, storage modulus (G'), $\tan \delta$ and pH values of monoglyceride-based system made of milk (Gel milk), water (Gel water) and whey protein aqueous solution (Gel proteins) as water phase. Means in the same column indicated by different letters indicate a significant difference ($p < 0.05$).

System	Visual appearance	G' (Pa)	$\tan \delta$	pH
Gel water		10845 ± 162^a	0.45 ± 0.01^b	5.19 ± 0.02^b
Gel milk		3002 ± 5^b	0.48 ± 0.01^a	5.56 ± 0.02^a
Gel proteins		3772 ± 345^b	0.32 ± 0.01^c	5.52 ± 0.03^a

Furthermore, monoglyceride-based systems containing *L. rhamnosus* were *in vitro* digested. Observing Table 2 it is evident that gel structure affected the probiotic viability. Gel water and gel milk were able to guarantee a high bacteria protection during the different digestion phases in comparison to the relative controls, which showed a reduction of the viable count at the end of gastric and intestinal phases probably due to the reduced pH as well as the presence of enzymes and bile salts. Gastrointestinal conditions are claimed to be responsible for membrane damage, protein misfolding, and DNA injury by oxidative shock and low intracellular pH. Interestingly, the structured

emulsions containing both milk and water maintained at the end of digestion process a number of viable probiotic microorganisms higher than 10^6 CFU/g, which is the established limit to guarantee health benefits.

Different results were observed considering gel proteins. In this case, a viability loss of 3.03 ± 0.51 log CFU/g was detected after digestion, which could be attributed to the presence of whey proteins in the system affecting the gel network structure. In fact, whey proteins are reported to be able to displace MG from oil droplet surfaces leading to a reduction of system stability (Valoppi et al., 2015).

Table 2 Viability of *L. rhamnosus* as a function of digestion phase of Gel water, Gel milk, Gel proteins (log CFU/g) and relative controls (probiotic cells suspended in water phase; log CFU/mL). * indicate a significant difference ($p < 0.05$) between monoglyceride-based emulsions and relative controls.

Water phase	System	Viability (log CFU/g – log CFU/mL)			
		Digestion phase			
		Pre digestion	Oral phase	Gastric phase	Intestinal phase
Water	Control	7.39 ± 0.12	7.52 ± 0.05	3.90 ± 0.28	3.92 ± 0.22
	Gel	7.41 ± 0.10	$6.85 \pm 0.11^*$	$6.88 \pm 0.28^*$	$6.02 \pm 0.36^*$
Milk	Control	7.49 ± 0.06	7.00 ± 0.01	3.72 ± 0.33	4.19 ± 0.16
	Gel	7.27 ± 0.05	$6.76 \pm 0.01^*$	$6.68 \pm 0.23^*$	$6.38 \pm 0.03^*$
Proteins	Control	7.81 ± 0.72	7.43 ± 0.01	3.58 ± 0.36	4.41 ± 0.61
	Gel	7.37 ± 0.11	6.75 ± 0.16	$5.63 \pm 0.15^*$	4.34 ± 0.63

Results acquired clearly demonstrated that monoglyceride-based systems were able to ensure a high viability of *L. rhamnosus* during *in vitro* digestion. It could be supposed that probiotic cells were located prevalently in the aqueous phase near the monoglyceride bilayer where they can find essential nutrients and protection from the environmental stresses (Marino et al., 2017). This result is consistent with previous data highlighting that the microbial cells can act as Pickering-type stabilizers by residing at the oil–water interface (Firoozmand & Rousseau, 2016). Based on these promising results, the PhD thesis project will proceed investigating the role of formulation and structure of MG-based emulsions on both probiotic viability upon *in vitro* digestion as well as during food processing and storage. For this reason, MG-based emulsions will be exploited as delivery system of probiotic bacteria in different food prototypes.

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The Profile of Cyclic Proanthocyanidins in South Tyrolean Wines

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The aim of this study was to identify and characterize the profile of cyclic proanthocyanidins and non-cyclic congeners in Pinot Noir, one of the main wines produced in South Tyrol. Two classes of recently identified cyclic proanthocyanidins (cyclic procyanidins and cyclic prodelphinidins) were identified using HPLC-HRMS/MS. The effects of different winemaking practices were evaluated on the relationships between the chemical profile of these compounds and the sensory properties of eleven Pinot Noir wines produced in a single winery. A possible application as chemical markers of the wine quality was discussed.

Il profilo delle proantocianidine cicliche nei vini altoatesini

Gli obiettivi di questo studio sono stati l'identificazione e la caratterizzazione del profilo delle proantocianidine cicliche e dei congeneri non ciclici nel Pinot nero, uno dei maggiori vini prodotti in Alto Adige. Due classi di proantocianidine cicliche recentemente identificate (procianidine e prodelfinidine cicliche) sono state identificate tramite HPLC-HRMS/MS. Sono stati valutati gli effetti di diverse pratiche di vinificazione sulle relazioni fra il profilo chimico dei questi composti e le proprietà sensoriali di undici vini Pinot nero prodotti in una singola cantina. È stata anche discussa la loro possibile applicazione come marcatori chimici di qualità del vino.

Key words: Cyclic proanthocyanidins, sensory analysis, high resolution mass spectrometry, red wine, Pinot Noir.

1. Introduction

Cyclic B-type procyanidins share the identical elemental composition as non-cyclic A-type procyanidins. So, it is not possible to distinguish them by classical high-resolution mass spectrometry alone (Longo et al., 2018a; Zeng et al., 2019). However, these classes differ in the number of exchangeable protons. For this reason, H/D exchange was applied in the liquid chromatography method and the relative abundance of non-cyclic and cyclic B-type pentameric and hexameric procyanidins were determined in wine. Successively, prodelphinidins (the proanthocyanidins containing at least one group of (epi)galocatechin) were also studied in wine (Longo et al., 2018b). To summarize, these two classes of cyclic proanthocyanidins showed similar behaviors in comparison to their linear analogs: (1) the retention times were anticipated in RP-HPLC (more polar than the non-cyclic analogs); (2) they showed much fewer HPLC peaks (one main regio- and stereoisomer); (3) the MS/MS spectra showed less fragmentation at the same collision energy.

In accordance to the PhD project (Merkyte et al., 2018) proposed last year, this study is aimed at investigating the profile of these cyclic compounds in Pinot Noir, one of the main wines produced in South Tyrol, related to different winemaking practices.

2. Materials and Methods

2.1. Wine samples

Eleven red dry wines obtained from 100% Pinot Noir grapes at the Franz Haas winery (Montagna, Bolzano, Italy) were analysed. Grapes were harvested in 2016 in different vineyards (350 – 800 metres a.s.l. in Trentino-South Tyrol). Wines 1, 4, 5, 6, 7, 8 and 9 were produced under the same winemaking practices: mass of grapes 3.5 t each; 8 d maceration; fermentation at 25–26 °C. Other wines differed in the winemaking technology. For the wine 2, thermal maceration at 42 °C was applied for 8 h before the alcoholic fermentation. Wine 3 was re-inoculated with selected yeasts and a high dose of SO₂ was added after a stuck fermentation. Wine 10 was made with 20% of whole grapes that were left in the must during maceration/fermentation. Wine 11 was obtained with 100% raisin grapes.

2.2. HPLC-HRMS/MS analysis

The preparation of samples and the HPLC-HRMS/MS analysis were performed according to already published papers with some modifications (Longo et al., 2018b and its references). The ODS Hypersyl C18 LC column and the DAD detector (210 – 600 nm) were used. The phase A (0.1% v/v formic acid in 0.02 M ammonium formate in H₂O) and phase B (0.1% v/v formic acid in saturated ammonium formate in acetonitrile) at a flow rate of 1 mL min⁻¹ had the following HPLC gradient programme: from 5% (v/v) B at 0 min to 25% B at 21 min, then to 95% B at 22 until 27 min, to 5% at 28 min, followed by a re-equilibration step (5% B) at 32 to 35 min. Full MS was done in positive ion mode with normalized collision energy 15 and in negative ion mode where normalized collision energy was 30 – 60.

2.3. Sensory analysis

A panel was used to define a common vocabulary of the sensory descriptors for Pinot Noir wines. Then, 19 sensory descriptors were identified and evaluated using a 10-point scale with the procedure of the round table. The descriptors were hue, color intensity, clarity, olfactory intensity, floral, fruity, herbaceous, spicy, liquor, maderization, caramelized, solvent, alcoholic, softness, sweetness, acidity, sapidity, tannicity, and balance. At the end of the session, an overall judgement was also asked.

2.4. Statistical analysis

The principal component analysis was performed using XLStat (version 2016.02.28430, Addinsoft, Paris, France).

3. Results and Discussion

Eleven monovarietal Pinot Noir wines were obtained in a single winery with different winemaking practices. The results of the sensory panel were compared to the profile of non-cyclic and cyclic proanthocyanidins, because these recently discovered

compounds have not been investigated in relationship with other chemical, oenological or sensory data. The results showed (Fig. 1) a consistent clustering: the separation of the most different samples (wine 3 and 11) was evident in both plots (sensory data and non-cyclic and cyclic proanthocyanidins profile). Besides, cyclic proanthocyanidins were positively correlated with non-cyclic proanthocyanidins. Wine 3 had a stuck fermentation; this was associated with very low levels of proanthocyanidins (cyclic and linear). The sensory panellists identified this wine as highly maderized and with a lack of tannins. Wine 11, that was produced using raisins, contained the highest amount of cyclic tetrameric prodelphinidins. Wine 11 was also well accepted by the panellists and identified as a fine and balanced wine.

These results show that the profile of cyclic proanthocyanidins is a promising tool to distinguish some aspects related to the winemaking procedure. Although, further studies must be undertaken to establish a method sensitive enough to differentiate a monovarietal wine according to winemaking techniques.

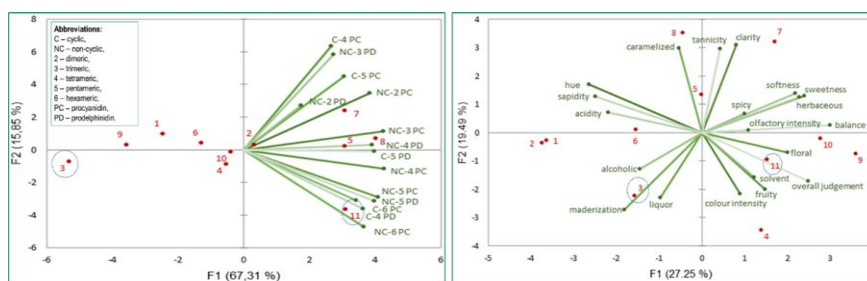


Figure 1 Principal component analysis (PCA) of proanthocyanidins (including the cyclic analogs) (left) and of the sensory perceptions (right) related to Pinot Noir wines. Wines 3 and 11 were circled.

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Development of a Mathematical Model to Predict Extra Virgin Olive Oil (EVOO) Shelf Life

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This PhD research project is addressed to develop a mathematical model able to predict the shelf life of extra virgin olive oil (EVOO). To this aim and to take into account the huge variability of environmental conditions suffered by EVOO during storage, accelerated shelf life tests will be performed by using both temperature and light as accelerating factors. This poster presentation reports data relevant to the use of temperature to speed up the shelf life assessment process.

Sviluppo di un modello matematico per la previsione della shelf life dell'olio extra vergine di oliva

Questo progetto di tesi di dottorato mira a realizzare un modello matematico capace di descrivere il comportamento dell'Olio Extra Vergine di Oliva durante lo stoccaggio. A tale scopo e in virtù della variabilità delle condizioni ambientali a cui l'EVOO è sottoposto durante lo stoccaggio, verranno effettuati test di invecchiamento accelerato considerando sia la temperatura che la luce come fattori acceleranti. In questo poster l'attenzione verrà posta sull'uso della temperatura per ridurre i tempi di determinazione della shelf life.

Key words: Shelf life, extra virgin olive oil, predictive model, accelerated shelf life test.

1. Introduction

The quality characteristics of EVOO are reported in the European regulation (CEE 2568/91 and Reg Deleg. UE 2015/1830) and must be guaranteed until the “best before” date reported on the label. Unfortunately, due to development of oxidative reactions, some of these compulsory indexes (e.g. peroxide value, absorbance at 232nm and 270nm) could change during storage leading to product non-conformity. Concomitantly, other quality indexes, such as polyphenol and tocopherol content, volatile compounds and pigments, are expected to be involved in the oxidative reactions. The complex interplay among these reactions could definitively affect the final product shelf life.

Producers have the hard task to correctly define the “best before” date. Thus, a reliable and easy to use tool to predict product shelf life is highly required. To this aim, in this research, EVOO was tested under different environmental conditions to generate kinetic data whose elaboration can finally allow to develop a shelf life predicting model. In particular, to speed up the shelf life assessment process both temperature and light will be exploited as accelerating factors in accelerated shelf life tests (Labuza and Schmidl, 1985; Fu and Labuza, 1997; Gomez-Alonso et al., 2004).

In accordance with the PhD thesis project previously described (Milani, 2018) the focus of the activities presented in this poster concerns mainly the effect of temperature on the EVOO stability.

2. Materials and Methods

The research was conducted by storing 15 bottles containing 250 mL of extra virgin olive oil each at three different ovens (Pol-Eco Aparatura SP.J. ST 500, Wodzislaw SI, Polska) setted up respectively at 40, 50 and 60 °C $\pm 0.1^\circ\text{C}$ in dark. Bottles were made by clear glass with a squared-base parallelepiped shape sealed with metallic caps with air in the headspace. The extra virgin olive oil used in this work has a total polyphenol concentration of 600 ppm. Samples were picked up and analysed deciding time after time, based on the results obtained, when performing the subsequent analyses. The data for the peroxide value, optical density at 232 and 270 nm were considered in this presentation. The results reported in this work are the average of at least two determinations. When possible, data were elaborated by linear regression analysis to obtain the zero order rate constants. The goodness of fitting was evaluated considering the coefficient of determination (R^2). To evaluate the temperature dependence of the rate of lipid oxidation, the Arrhenius equation in logarithmic form (Eq.1) was applied:

$$\ln k = -\left(\frac{E_a}{RT}\right) + \ln k_0 \quad (1)$$

Where k is the zero order rate constant, R is the molar gas constant (8.31J/Kmol), T is the absolute temperature (K), E_a is the thermal activation energy (J/mol) and k_0 is the oxidation rate at 0 K.

Then shelf life was computed by applying the following equation:

$$SL = \frac{A_{lim} - A_0}{k} \quad (2)$$

Where A_{lim} is the value of the index considered as acceptability limit; A_0 is the value at time zero and k is the rate constant at selected temperature.

3. Results and Discussion

Different oxidative indexes were followed during storage of EVOO at 40, 50 and 60 °C. Peroxide values as well as DO 232 nm highlighted slight changes even at the highest temperature, never exceeding the legal limits for EVOO. On the contrary, Absorbance at 270 nm evidenced a progressive increase as a function of storage time and the compulsory limit of this index (0.22) was exceeded in all the considered cases (Fig. 1A). Apparent zero order rate constants of absorbance changes (k) were calculated by linear regression of at least five points from the initial part of the curve corresponding to time “zero” of storage.

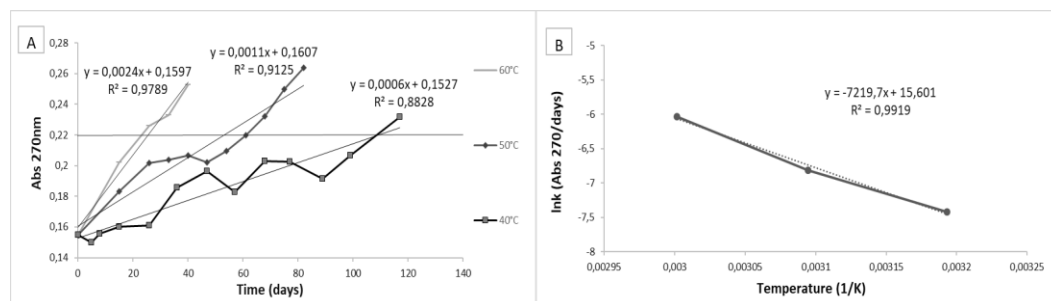


Figure 1 A) Changes of absorbance of EVOO during storage at 60, 50 and 40°C under dark. B) Arrhenius plot pseudo zero order oxidation rate constant (k) of extra virgin olive oil stored at increasing temperature.

The determination coefficient (R^2) resulted to be satisfactory, varying from 0.97 for sample stored at 60°C to 0.88 for samples stored at 40°C, with an intermediate value of 0.91 for 50°C stored samples. The application of Arrhenius model (Eq. 1), which is conventionally used to describe the effect of temperature on reactions rate (Labuza and Schmidl, 1985), allowed to define the temperature dependence of oxidation rate (Fig. 1B). From the obtained equation reported in Figure 1B, the values of activation energy (E_a) was calculated and we obtained a value of around 60kJ/mol that is comparable with those typically reported in literature for lipid oxidation (Frankel, 1993; Tan et al., 2001). Finally, based on results above described and using the general shelf life model reported in Eq.2, it was possible to estimate the product shelf life at different storage temperature, as reported on Table1.

For instance, considering a storage temperature of 20°C, that can be considered the average temperature in a supermarket environment, the proposed model estimated a shelf life of 19 months. Interestingly, by increasing temperature of about 5°C, the estimated shelf life was 6 months lower highlighting the strong effect of storage temperature on EVOO shelf life.

Based on these promising results, the PhD thesis project will proceed investigating other samples with different initial concentrations of antioxidant, to evaluate if this first developed model is valid also for EVOO with medium and low concentration of polyphenols. Moreover, the light dependence of EVOO oxidation development is under study.

Table 1 Estimated shelf life of extra virgin olive oil at different storage temperatures.

Temperature (°C)	Estimated shelf life (months)
20	19
25	13
30	9
40	4

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VOCs as Indices of Shelf-Life of Stracciatella Cheese Added with Natural Antimicrobial Compounds

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After setting up the method for volatile organic compounds (VOCs) evaluation by SPME-GC/MS, and early investigations on their formation in pasta filata cheeses, the study has continued focusing on their role on the sensory characteristics. Several products have been investigated, and the present report shows the results of a study on the possible role of VOCs as shelf-life indices of Stracciatella added with natural antimicrobial compounds (NAC).

VOCs come indice di shelf-life della Stracciatella addizionata di composti antimicrobici naturali

Dopo la messa a punto del metodo SPME-GC/MS, e dopo le prime indagini sul fondamentale ruolo dei VOCs nei formaggi a pasta filata, il lavoro è proseguito con particolare enfasi sul loro ruolo sensoriale. Sono stati condotti studi su diversi prodotti, e nella presente relazione si riportano i risultati concernenti lo studio dei VOCs come possibili indicatori di shelf-life della Stracciatella addizionata di sostanze antimicrobiche naturali (NAC).

Key words: VOCs, Stracciatella, sensory analysis, natural antimicrobial compounds, shelf-life.

1. Introduction

In accordance with the PhD thesis project previously described (Natrella, 2018), this poster reports the main results of the activities carried out. They are included in the following points:

A3) Evaluation of VOCs in raw materials, and assessment of their evolution during processing related to zootechnical, agronomical, compositional and technological variables;

A4) Evaluation of relationship between VOCs profile and sensory analysis.

2. Materials and Methods

Stracciatella is a mixture of cream and frays of mozzarella. It can be sold alone or included in Burrata cheese, of which it represents the inner core. For the experimentation, it was taken from a local dairy and 3 samples were prepared by adding different NAC: sorbic acid (SORB), olive leaf extract (OLE) and combination of the two (SIN). The samples were stored at 8°C for 16 days and analyzed each 4 days. A control sample without NAC (CTR), was included. VOCs were determined by SPME-GC/MS as reported by Faccia et al. (2018), organic acids by HPLC-DAD (Cane, 1990), sensory analysis (QDA) by a trained panel (Trani et al., 2016), microbial counts by culture-dependent approach.

3. Results and Discussion

3.1 Microbiologic and organic acids analyses

The results of the microbiological analyses showed that the best antimicrobial effect was found in the SIN sample. CTRL had the highest counts of Lactobacilli and Lactococci (5.61 ± 0.10 e 5.72 ± 0.13 log UFC/g, respectively), *Pseudomonas* ($\log 8.57 \pm 0.04$ log UFC/g) and Enterobacteriaceae (4.00 ± 0.06 log UFC/g). SIN and OLE had similar values for Lactobacilli and Lactococci (5.04 ± 0.03 e 5.83 ± 0.04 log UFC/g, respectively), whereas some inhibiting effect on LAB was observed in SORB (4.43 ± 0.06 e 5.15 ± 0.10 log UFC/g, respectively). The most relevant antimicrobial activity was exerted against Enterobacteriaceae, which were absent in all samples with NAC, except OLE at day 12.

The profile of organic acids further described the differences in the antimicrobial effect. The pattern was similar in the pair CTR-OLE: as a consequence of higher microbial activity, citric acid constantly decreased, and acetic and lactic acid increased. Differently, the concentration of all acids did not show any relevant variations both in SORB and SIN. The pH values (5.7 ± 0.10 in CTR-OLE and 6.09 ± 0.01 in SIN-SORB) confirmed these results.

3.2 Volatile organic compounds and sensory analysis

Fig. 1 shows the VOC profile of the samples. In total, 41 compounds were identified belonging to different classes: ketones, alcohols, esters, aldehydes, sulfur compound, acids and aliphatic hydrocarbons. The results matched with those of the microbiological analyses. In fact, the samples were rather similar until day 8, then ketones and alcohols started to increase in OLE and CTR due to microbial activity. Among them, the most abundant were acetoin, ethanol and 3-methylbutanol. They could derive from microbial citrate metabolism by Lactococci activities, alcoholic fermentations and Strecker degradation (Hassan et al., 2012; Heil et al., 2007; Chambers et al., 2013).

During storage, other compounds strongly increased in OLE and CTR such as ethylacetate, ethylbutanoate and ethyletanoate. They are connected to the activity of lactic acid bacteria's esterase (Wolf et al., 2010) in good agreement with the results of the microbiological analyses. Higher microbial activity also accounted for the presence of acetic acid, acetaldehyde and 3-methylbutanal (Molimard P. et al, 1996; Valero et al. 2001). Dimethyl sulfide formation was negatively linked to the presence of NAC, differently from CTR in which it constantly increased. The relevant concentration of butyric and hexanoic acid in CTR suggests the involvement of lipolytic activity when NAC were not used.

The sensory analysis matched with the chemical and microbiological results, even though some differences were already perceived at day 8. CTR and OLE always had the worst scores both for aroma and flavor, and off-flavours started to be perceived in correspondence with the increase of VOCs concentration.

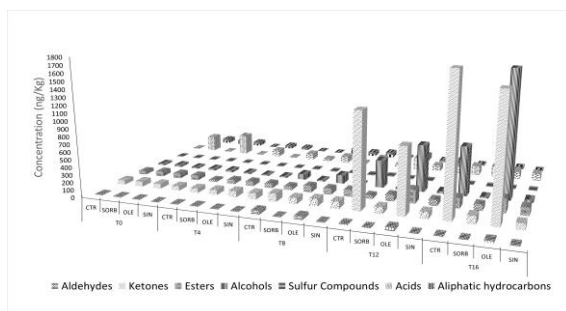


Figure 1 VOCs chemical classes of Stracciatella samples during storage.

4. Conclusions

In conclusion, the VOC profile proved to be a useful index for evaluating the shelf-life of Stracciatella, and some specific compounds seems to be good indices for monitoring cheese alteration. The tested antimicrobial substances could be useful for extending shelf life of the product. However, the leaf extract alone seems to have negligible effect, whereas its use in combination with sorbic acid is very promising.

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3D Food Printing: New Advances on Printing Speed and Predict Texture

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The experiments performed during these first activities have been focused on two main challenges for the real implementation of 3D Printing in food sector: a better control of print speed and non-printing variables aiming to speed up the food printing process, keeping the printing fidelity of high quality. In additions, as second activity, the study of textural characteristics of printing food, and the creation of food with novel textural perceptions has been performed.

3D Food Printing: studio della velocità di stampa e sviluppo di un modello di predizione della texture

Durante le prime attività di dottorato si è focalizzata l'attenzione su due importanti aspetti riguardanti l'applicazione della stampa 3D in campo alimentare: controllo e studio della velocità di stampa e dei movimenti di non stampa con l'obiettivo di velocizzare il processo, tenendo in considerazione la fedeltà di stampa dei campioni. La seconda attività ha riguardato lo studio delle caratteristiche di texture dei campioni stampati e la creazione di alimenti dalle nuove caratteristiche sensoriali.

Key words: 3D food printing, print speed, fidelity, texture, Young modulus.

1. Introduction

3D Printing is an emerging technology showing important potentialities when used in food sector. Some of these are: to create novel shapes and structures, to modulate sensorial properties, to create personalized food. In accordance with the PhD thesis, as previously described by Paolillo (2018), the main results of the experiments are reported:

A1) results on the quality of printed structures and the printing time by a workflow that analyzes the effects of some understudied variables (flow, filament diameter, travel speed, retraction speed and retraction distance).

A2) results on the increasing capability to create novel texture perceptions inspired by the microstructure properties of plant-based food, mainly apples.

2. Materials and Methods

A 3D Printer mod. Delta 2040 (Wasp project, Italy) equipped with the Clay extruder kit 2.00 (Wasproject, Italy) was employed for 3D printing experiments. The microCT images of apples tissues and printed samples were acquired by microtomography (Skyscan 1174, Bruker) with a resolution of 4.5 μm and 28.5 μm respectively with the scanning conditions reported by Derossi et al., 2017. The cross-sectional images were used to create a 3D virtual model saved as stl file while the software CURA ver 3.3.1. was used as slicing software for tuning the printing conditions.

Printable food formulations have consisted of (A1) wheat flour 00 61,5%, water 31%, extra virgin oil 6,1% and NaCl 1,4%; (A2) water 34% wheat flour 00 29,5% , rice flour 29,5%, extra virgin oil 6%, NaCl 1%. The samples have been weighed on balance (Gibertini, EU-C 7500) and then cooked in ordinary oven at 100°C. The image acquisition has been obtained by a photo-camera (Canon, Eos 1200D) and analysed with ImageJ

software to calculate dimension (A1) and to determinate porosity, applying threshold to the best operator conditions (A2).

Printing conditions for sample A1: nozzle size: 0,84 mm; layer height 0,7 mm; travel speed 30 mm/s; infill 20%. A full factorial design $3^{(k-p)}$ where k is the number of the independent variables ($k=3$) and $p=0$ was used to study the effects of the retraction distance (mm), RD , travel speed (mm/s), TS , retraction speed (mm/s), RS .

Printing conditions for sample A2: nozzle size 0,64 mm; layer height 0,6 mm; print speed 10 mm/s; infill 100%, flow 95%. Textural characteristics have been determined through 3 point bend test (Mudgil et al., 2017).

Finite element method (FEM) has been used to estimate the mechanical properties of the printed samples by using Comsol Multiphysics.

3. Results and Discussion

3.1 Printing speed and fidelity of printing

It is common to use a simple mathematical equation to link the print speed to the extrusion rate for 3D printing:

$$ER(mm^3/s) = Dn(mm) * hc(mm) * Vn (mm/s) \quad (1)$$

where ER is the volume of the extruded material for unit of time (mm^3/s), Dn is the nozzle diameter (mm), h is layer height and v_n is the desired print speed (mm/s) (Yang et al., 2018). However applying this formula in real printing test, the material deposited results to be not enough during printing movement. Indeed, Eq.1 does not consider the speed of the printer in E -axis that modulates the amount of material deposited for unit of time. For what concerning the printing time, the data exhibit a clear exponential trend: it is possible to observe a first and sudden reduction from ~ 700 s to ~ 270 s by increasing the print speed from 15 mm/s to 50 mm/s, while further increase did not reduce significantly the overall time. The reasons resides in the limitation of the acceleration factor that become more important for high print speed values until 100-150 mm/s. Concerning the printing fidelity at high print speed, the distance of retraction and the speed of retraction significantly influenced the quality of printed samples. In fact, in the case of cereal-dough printing, the fidelity of object was better with decreasing distance and increasing speed.

Figure 1 Time needed for 3D printing of sample for different print speeds

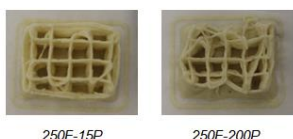
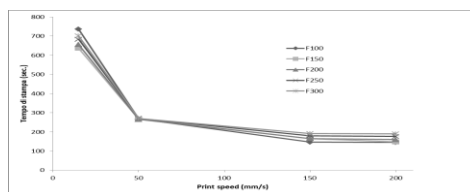


Figure 2 Representative samples printed at same flow (250%) and different speed (15-200 mm/s²).

3. Innovative sensorial features by using 3D printing

Under the aim of this second step of the PhD programme, the experiments have been focused on the creation of snacks with novel texture properties mediated by the microstructure properties of some plant-based products. Starting from the cross-sectional images of apple tissues, snacks with 3 different thicknesses have been created and

cooked until the same water content. The obtained products have shown porosity fraction of $19,0 \pm 1.2\%$ very close to apple porosity $19.7 \pm 1.1\%$ as reported by Nugraha et al., (2019). Instead, how it could be expected, the porosity value determined from microstructural analysis has been much higher ($49,2 \pm 4,2\%$). This was due to the several micro-porosity of printing material (cereal dough) that increased during the cooking. This aspect has been confirmed by the porosity distribution graph (Fig. 3). The probability to find pores < 150 μm has been of 20% while the others two peaks between 800-1000 μm and 1200-1400 μm indicated macro pores.

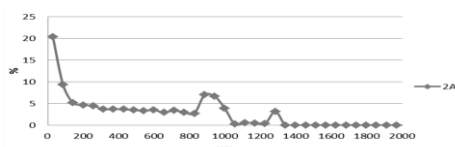


Figure 3 Distribution of Porosity of sample

The data of textures have confirmed that, by increasing the number of layers, the maximum force peak significantly increased (from $0,7 \pm 0,3$ to $8,1 \pm 1,8$ N). Later, Young modulus of samples was determined according to Baltsavias et al., (1997):

$$E = \frac{L^2}{4db^3} \left(\frac{dF}{dy} \right) y \rightarrow 0 \quad (2)$$

Young modulus of $24,8 \cdot 10^6$ N/m² was computed for this type of structure. Currently we are using the basic material properties of the printed snacks to model the mechanical behavior of the samples by FEM. After that, we will be able to create new structures alternating, in a single snack, layers with morphological properties coming from different vegetables (apples, eggplants, turnips, ecc.) with the aim to create novel multi-layered food product with uncommon textural perception personalized for different consumers groups (elder people, childs, tee-ager, etc).

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Improvement of the Transformation Process of Ancient Wheat Grains

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In recent years there has been a renewed interest for healthy and functional foods, and particularly, the interest for bread made with unrefined grains continuously increase. In this context, ancient wheat varieties have gained consumer attention since they have shown positive effects on human health (Dinu et al., 2018). However, unrefined doughs show worse technological performances, as well as breads show worse qualitative characteristics. Furthermore, technological processes were developed and optimized for white flours. Hence, in order to improve the poor technological properties of ancient wheat flours and the final product quality, new process strategies are necessary. The activities of the PhD thesis project focus on the improvement of the process for the obtainement of unrefined bread from ancient varieties. Firstly, a survey was conducted to report the bread making process with ancient wheats in Tuscany. Then, the effect of the main improvers used for bread production were evaluated on the most common ancient wheat flour of Tuscany using a Screening Design method followed by a Full Factorial Design study.

Miglioramento dei processi di trasformazione dei frumenti antichi

Durante gli ultimi anni si è riscontrato un rinnovato interesse nei confronti di alimenti salutari e funzionali, in particolare l'interesse per pane da cereali non raffinati è in continuo aumento. In questo scenario, le antiche varietà di frumento hanno catturato l'attenzione del consumatore, dimostrando di avere effetti positivi sulla salute umana (Dinu et al., 2018). Tuttavia, gli impasti da farine non raffinate mostrano peggiori performance tecnologiche così come i pani risultano di bassa qualità. Inoltre, i processi tecnologici attuali sono stati sviluppati per le farine raffinate. Quindi, nuove strategie di processo sono necessarie al fine di migliorare le scarse proprietà tecnologiche dei frumenti antichi e la qualità dei loro prodotti finiti. Le attività della tesi di dottorato sono incentrate sul miglioramento dei processi tecnologici per l'ottenimento di pane da farine antiche non raffinate. In un primo momento è stata condotta un'indagine al fine di riportare le strategie di panificazione da frumenti antichi diffuse in Toscana. Successivamente, l'effetto dei principali miglioratori utilizzati in panificazione è stato valutato sulla farina antica maggiormente diffusa in Toscana, secondo metodo dello Screening Design, seguito da un piano sperimentale Full Factorial.

Key words: Ancient wheats (AWs), ancient wheat flours (AWFs), bread making, farinograph, alveograph, texture analysis, sensory analysis.

1. Introduction

This poster reports the main results of the first two activities of the PhD thesis project:

A1) The survey about the bread making process with AWs in Tuscany, conducted using a qualitative semi-structured interview (Guerrini et al., 2019).

A2) The investigation of the best combination of common bread improvers able to optimize the technological performance of the most common ancient wheat flour in Tuscany, Verna.

2. Materials and Methods

A1) The survey study was conducted using qualitative semi-structured interviews following the systematic methodology described by Kallio et al. (2016). The sample was selected using the handpicked sampling method, trying to be representative of the AW bread production in Tuscany. The number of interviews collected (i.e. 20) was based on the saturation criterion (Marshall, 1996). An interview topic-guide was created on the basis of the research literature and it was firstly evaluated with an “internal testing”, secondly with both an “expert assessment” and “field-testing”. Analysis of the qualitative data was performed according to Elo et al. (2008) and Green et al. (2007).

A2) Experimental trials were carried out with wholemeal AWF (sp. *Triticum aestivum* L., cv. Verna). Screening Design trial (T1): 7 different improvers (i.e. extra virgin olive oil, EVOO; salt, NaCl; guar gum, GG; sugar, Suc; ascorbic acid, AH₂; gelatinized flour, GF; and Ice), included the addition of GF previously tested on unrefined flour (Parenti et al., 2019), were simultaneously evaluated on dough rheology and bread quality. Full Factorial Design (T2): the 3 variables (i.e. EVOO, Suc and Ice) that showed the greatest effect on bread specific volume in T1 were deeply evaluated in a Full Factorial Design. 2 levels of EVOO were tested, 0% – 3% (w/flour w), and 3 levels of Suc and Ice, 0% – 2% – 4% (w/flour w) and 0% – 10% – 20% (w/water w), respectively. Rheological analysis on doughs were performed with a Brabender Farinograph. In each trial the straight dough method was applied; bread doughs were prepared with the amount of water required to reach the reference consistency, 500 Brabender Units (BU). Bread quality evaluation included: bread specific volume, crumb specific volume, crumb and crust moisture, texture profile analysis (TPA). Moreover, a final sensory evaluation on the optimized bread sample (i.e., EVOO 3% and Suc 2%) was performed.

3. Results and Discussion

3.1 (A1) Results and Discussion

Ingredients: AWFs are generally stone milled and used as unrefined flour for bread production, mostly as brown flour (85% of extraction rate, 0.95g ash/100g d.m.) to optimize both nutritional and technological quality. The most common wheat specie used is *Triticum aestivum* L., but some bakers also add *Triticum durum* in the bread recipe. Few bakers work single variety flours, but the most of them prefer using blends, reporting it as a strategy to obtain a better technological performance. Bakers usually prepare highly hydrated doughs, as a mean to achieve a better balance between dough tenacity and extensibility (P/L), and use the sourdough as leavening agent. Bread making process: the main process strategies outlined by the survey are shown in Fig 1.

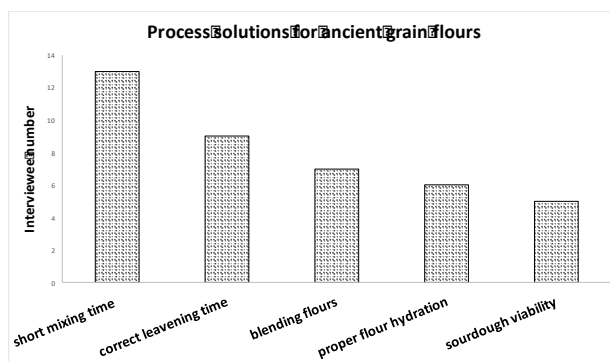


Figure 1 Process solutions for the bread making process with AWF

3.2 (A2) Results and Discussion

The main effects of 7 common bread improvers were tested simultaneously with a Screening Design method, considering 2 levels of addition for each variable: 0% and the highest amount reported in the scientific literature for the bread formula. Data about bread quality evaluation showed that the addition of EVOO (3%, w/flour w), Suc (6%, w/flour w), and Ice (20%, w/water w) had the best effect on bread specific volume. Hence, these improvers were selected to perform a Full Factorial trial. Suc (i.e. 4%) and EVOO significantly enhanced the dough stability (DS), as well as they decreased the dough weakening (DW) during the farinographic mixing. A significant effect of the interaction Suc x EVOO was observed on bread and crumb specific volume: the first was maximized with 3% EVOO (w/flour w) addition, while the combination of EVOO and Suc maximized crumb specific volume. The texture parameter hardness showed the best values with EVOO and Suc addition, too. The combination of improvers that maximized both bread/crumb specific volume and bread softness with the least ingredients' addition was identified as the optimized bread (3% EVOO – 2% Suc). The study also included a sensory evaluation on the optimized bread.

4. Future Perspectives

Future developments of the study include an extension of the present approach to other AWF varieties. Overcoming AW technological issues could promote the consumption of healthy products together with the safeguard of Triticum genus biodiversity.

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Non-Saccharomyces Yeast for Reduction of Ethanol Content in Wine

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This thesis is aimed to the study and set up of mixed starter cultures, consisting of *Saccharomyces* and non-*Saccharomyces*, in order to produce wines with a low alcohol content and increased aromatic complexity. The experimental activities have been addressed to a first screening of non-*Saccharomyces* yeasts, through the evaluation of parameters of technological interest. The enological aptitude of selected strains was investigated by micro-vinification in different conditions.

Lieviti non-Saccharomyces per la riduzione del contenuto alcolico del vino

Questa tesi di dottorato è incentrata sullo studio e messa a punto di colture starter miste, costituite da lieviti *Saccharomyces* e non-*Saccharomyces*, con l'intento di ottenere vini a ridotto tenore alcolico e maggiore complessità aromatica. Le attività sperimentali sono state indirizzate alla selezione di lieviti non-*Saccharomyces* attraverso la valutazione di parametri di interesse tecnologico. L'attitudine enologica di ceppi selezionati sulla base della prima fase di screening è stata valutata attraverso prove di microfermentazioni in diverse condizioni sperimentali.

Key words: Non-*Saccharomyces* yeasts, enzymatic activities, mixed fermentations.

1. Introduction

In accordance with the PhD thesis project previously described (Pietrafesa, 2018), this contribution reports the main results of the first two activities concerning:

A1) technological screening non-*Saccharomyces* isolates (resistance to antimicrobial compounds, killer activity, enzymatic activities);

A2) mixed fermentation in synthetic must: optimization of fermentation condition and analysis of experimental wines for ethanol content.

2. Materials and Methods

2.1 Screening of non-Saccharomyces yeast

Thirty three non-*Saccharomyces* strains, previously isolated from grape must of different origins and identified by restriction analysis of amplified ITS region, were tested. The strains, belonging to the following species: *Debaromyces polymorphus* (Dp), *Hanseniaspora uvarum* (Hu), *Starmerella bacillaris* (Sb) and *Zygosaccharomyces bailii* (Zb), were tested for parameters of technological interest, such as production level of hydrogen sulphide (H₂S), resistance to ethanol, SO₂ and copper. The production level of H₂S was determined by plating the yeasts onto Biggy Agar (Oxoid), whereas ethanol, SO₂ and copper resistance was tested on agarized media added with increasing doses of antimicrobial compounds, following the protocols reported by Mauriello et al. (2009). Screening of killer-sensitive pattern (killer, sensitive and neutral phenotypes) was carried out as described by Capece et al. (2013). The tolerance to ethanol and SO₂ was

evaluated also as strain growth in microplates (Englezos et al., 2015), evaluating the cell growth as the ratio (%) between the growth in broth with and without ethanol or SO₂ at the specific incubation times. Strains showing a percentage of growth ratio < 10% were considered not resistant. The strains were tested for enzymatic activities, such as the ester-hydrolase activity (Englezos et al., 2015), β -glucosidase and β -xylosidase activities (López et al., 2015).

2.2 Fermentation trials with mixed cultures

Selected non-*Saccharomyces* strains were tested during mixed fermentation performed 100 mL of synthetic must (pH 3.48, °Brix 21.2). Non-*Saccharomyces* strains were inoculated at 10⁷ CFU/mL, while EC1118 *S. cerevisiae* strain was inoculated at two different concentrations, 10⁷ and 10³ CFU/mL. Two inoculum modalities were tested, simultaneous inoculum (SiF) and sequential inoculum (SeF). Fermentation kinetics were monitored daily by gravimetric determinations until constant weight.

3. Results and Discussion

3.1 Screening of non-*Saccharomyces* yeast

As regards the evaluation of killer phenotype, three strains belonging to *D. polymorphus* showed the killer phenotype. The sensitive character was very spread among the strains (about 73%), while the neutral character was present in few strains (about 27%), in particular in *D. polymorphus*, *St. bacillaris* and *Z. bailii* strains.

β -Glucosidase activity was detected in 77% of the isolates, in particular the *H. uvarum* species exhibited the highest levels of this activity. Few strains gave positive results for β -xylosidase activity. As regards the technological characteristics, almost all the strains exhibited medium production level of H₂S and high tolerance to ethanol; in fact, the maximum doses tolerated for ethanol was 14% v/v, except some *H. uvarum* strains, which didn't grown at concentration higher than 12%. High variability among the strains was found for copper and SO₂ tolerance.

Ethanol and SO₂ tolerance were evaluated also as growth in liquid medium, at different concentrations of ethanol (10-16% v/v) and total SO₂ (50-200 mg/L) at 24 of incubation at 26 °C. The growth was determined by comparing the growth with and without ethanol or total SO₂ at pH 5.5 and pH 3.0, respectively, as shown in Fig. 1.

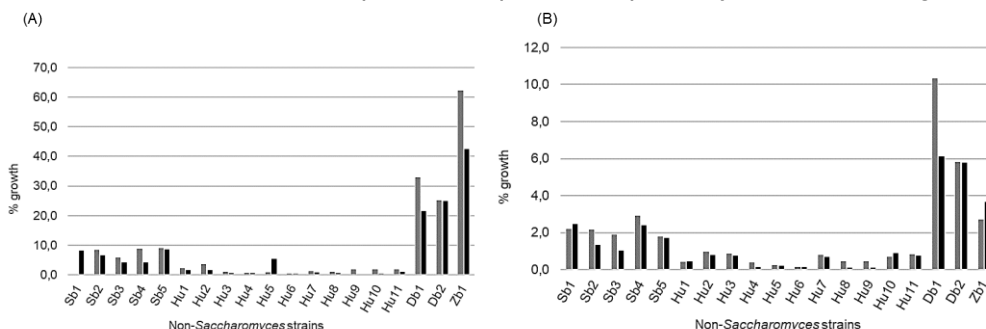


Figure 1 Strain tolerance to ethanol (A) and SO₂, expressed as the ratio (%) between the growth in broth with and without ethanol or or SO₂. ■ = 14% of ethanol and 150 mg/L of SO₂; ■ = 16% of ethanol and 200 mg/L of SO₂.

Both for ethanol (Fig.1A) and SO₂ (Fig.1B), Db and Zb strains were high tolerant, followed by Sb strains, whereas all the Hu strains were characterized by low tolerance level.

3.2 Fermentation trials with mixed cultures

The analytical parameters determined in experimental wines obtained by mixed fermentations, both for simultaneous and sequential inoculum, are reported in Table 1, in comparison to wine obtained from single starter. All trials gave complete fermentations in 14 days, and final wines contained less than 3 g/L of sugars (Glucose and Fructose). All the mixed fermentations produced wines containing lower ethanol content than wine from the pure culture of *S. cerevisiae* (EC). Generally, simultaneous trials produced the highest reduction of ethanol, and the starter including Zb1 presented the highest reduction level (0.80% v/v).

These results will be used for selection of mixed starter culture and fermentation conditions to be used in fermentation at pilot scale for producing wine with reduced ethanol content.

Table 1 Analytical parameters of experimental wines, produced by mixed starter cultures obtained with simultaneous (SiF) and sequential inoculum (SeF).

Sample	Alcohol % (v/v)		Fructose (g/L)		Glucose (g/L)		Reduction EtOH %	
	SiF	SeF	SiF	SeF	SiF	SeF	SiF	SeF
Sb1	11.8±0.06	11.4±0.01	1.4±0.28	1.4±0.07	0.9±0.00	1.1±0.14	0.5±0.11	0.4±0.21
Sb2	11.9±0.23	11.7±0.13	1.5±0.07	1.5±0.21	1.1±0.07	1.1±0.35	0.5 ±0.28	0.1±0.33
Sb4	11.9±0.21	11.5±0.09	1.4±0.14	1.4±0.07	0.9±0.21	1.0±0.14	0.5±0.25	0.3±0.29
Sb5	11.9±0.16	11.3±0.13	1.5±0.07	1.4±0.00	1.1±0.00	1.0±0.07	0.5±0.21	0.5±0.07
Hu2	12.0±0.01	11.6±0.07	2.5±0.49	1.5±0.07	1.4±0.00	1.1±0.07	0.4±0.06	0.2±0.27
Hu5	12.2±0.02	11.7±0.05	2.2±0.42	1.4±0.00	1.6±0.07	1.2±0.07	0.2±0.07	0.1±0.15
Db1	12.1±0.07	11.5±0.08	2.4±0.42	2.0±0.14	1.5±0.00	2.1±0.00	0.3±0.12	0.3±0.12
Zb1	11.6±0.20	11.5±0.00	1.8±0.00	1.8±0.07	4.2±1.41	1.8±0.28	0.8±0.25	0.3±0.20
EC	12.4±0.05	11.8±0.20	1.8±0.14	2.8±0.71	1.5±0.07	1.7±0.14		

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Geochemical and Spectroscopical (NIR) Fingerprinting for the Authentication and Traceability of Geographical Provenience of “Limone di Sorrento” PGI

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The first two different research approaches for my PhD are described. They are, firstly, the geochemical fingerprinting (GF) of lemon by multi-elemental composition of lemon juices and the cultivation soils determined by ICP-MS; secondly, the spectroscopical fingerprinting (SF) by means of Near Infrared (FT-NIR) spectra of intact lemons. Two different Chemometrics models have been developed for each approach, respectively: Linear Discriminant Analysis (LDA) and Soft Independent Modeling of Class Analogy (SIMCA), aimed to the authentication and discrimination of the geographical provenance of fruits.

Definizione di firme geochimiche e spettroscopiche per l'autenticazione e la tracciabilità dell'origine geografica del “Limone di Sorrento” IGP

Sono descritte le prime due attività del progetto di tesi di dottorato. La prima ha avuto come obiettivo quello di definire firma geochimica (GF) dei limoni tramite analisi multi-elemento sia del succo che del suolo di coltivazione con ICP-MS. La seconda, invece, quella di definire la firma spettroscopica (SF), del limone intero tramite analisi spettroscopica nel vicino infrarosso (FT-NIR). Per la trattazione chemiometrica dei due approcci analitici sono stati applicati due modelli discriminati differenti, rispettivamente: Linear Discriminant Analysis (LDA) and Soft Independent Modeling of Class Analogy (SIMCA), al fine di poter autenticare e determinare l'origine geografica dei frutti.

Key words: Mass spectrometry; Spectroscopy, Traceability, Lemon, geographical indication.

1. Introduction

In accordance with the PhD thesis project previously described (Ruggiero, 2018), this poster reports the main preliminaries results of the first two activities concerning:

A1) the determination of the GF of lemon juices and of cultivation soils at two depths (top soil 0-20 cm and sub soil 40-50 cm) was performed to investigate the relationship between soil and lemon and discriminate lemon fruits of only one cultivar, Ovale di Sorrento, from different origins areas in Campania region.

A2) the determination of the SF of intact lemons was performed to test this authentication for discriminating the geographical origins of Ovale di Sorrento fruits arising from different cultivation areas in Campania region.

2. Materials and Methods

Full ripening lemon fruits, cv “Ovale di Sorrento”, were collected (from Jan. to Mar. 2018) in four different sampling sites in Campania Region: Massa Lubrense (MS) and Piano di Sorrento (PZ), that are PGI Limone di Sorrento areas; and germplasm fields of the Dept. of Agricultural Sciences Univ. of Naples Federico II, Portici (PORT) and of the experimental farm Improsta, Eboli (EBO), that are NO-PGI areas. Also related sub soils,

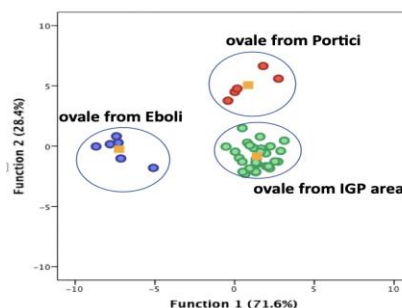
40-50 cm, and top soils, 0-20 cm, under the lemon trees were collected. An FT-IR PerkinElmer Frontier with NIRA accessory was used for SF: eight NIR spectra were acquired on lemons along their equatorial circumference at each eighth of turning. Spectra were acquired between 10,000 and 4000 cm^{-1} at 16 cm^{-1} spectral resolution. For GF lemon juices were mineralized by acid digestion (5 mL HNO_3 , 2 mL H_2O_2) in microwave. Potentially bioavailable elements in soils were extracted by EDTA 0.05 M with 1:10 soil/solution ratio. The elements were determined by ICP-MS Agilent 7900.

3. Results and Discussion

3.1 Geochemical fingerprinting and relationships lemons vs soils

The ICP-MS is a successful tool for determining the geographical authenticity of numerous food products (Luykx & van Ruth, 2008). In lemon juices 16 trace elements were determined. After Levene's test for equality of variance, 10 elements (Ti, Fe, Co, Cu, Zn, Se, Rb, Sr, Mo, Ba) were chosen to develop discrimination model. All data set was normalized in according to Pareto Scaling (Oefner et al., 2011). Exploratory analysis by PCA was satisfactory carried out (KMO test = 0.593; Bartlett sphericity test, $p < 0.001$). The PCA analysis evolved 10 principal components with the first two explaining 58.2% of the cumulate variance. The scatter plot of first and second PC showed a clear grouping of juices from Eboli, while not equally clear grouping was observed for PGI and PORT juices. The dominant variables for PC1 were Ti, Fe, Zn and Mo; for PC2, Fe, Co, Rb and Ba; for PC3, Co and Sr. Pearson Correlation analysis (soils vs lemon) carried out between lemon and soil mineral composition, showed highly significant correlations for Fe, Co, Rb, Sr and Ba. Then, LDA was applied (λ Wilks 0.017 and p -values < 0.0001). The best model, achieving 100% of correct classification, was based on the same 10 elements (Ti, Fe, Co, Cu, Zn, Se, Rb, Sr, Mo, Ba) showing an optimal discrimination between the 3 lemon groups with different geographical provenience. The model was cross-validated with satisfactory results (100% of correct reclassification). The LDA structure matrix showed that the variables with the highest discrimination ability were the elements characterized by significant correlations between their content in lemon and soil. Investigations with a more significant number of cases are in course to confirm these preliminary results.

Figure 1 Scatter plot of first (71.6%) and second (28.4%) discriminant functions of LD



3.2 Spectroscopical Fingerprinting of intact lemon

Near Infrared Spectroscopy (NIR) is rapid, non-destructive and low cost technique. Lemons from Portici and Sorrento PGI areas were considered. The mean spectra obtained from eight spectra recorded for each intact lemon were used to develop the SIMCA models. Before model development, all spectra were pretreated with MSC to remove the noise and, first order derivative) was applied to minimize shifts in the baseline. The performance of model classification was excellent (Fig.2), in terms of %recognition and %rejection (both 100). The inter-material distance for the two groups reached 5.1,

the inter-materials distances between the clusters are higher than 3.0, this indicates that the samples are well separated (Meza-Márquez et al., 2012). The Coomans plot (Fig.3) shows the degree of discrimination between spectra of individual materials in the model. Further investigations with a more significant number of cases are carrying out to confirm these preliminary results.

Figure 2 SIMCA hyperboxes based on Principal Component Analysis (PCA).

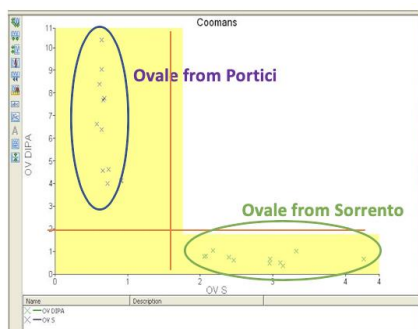
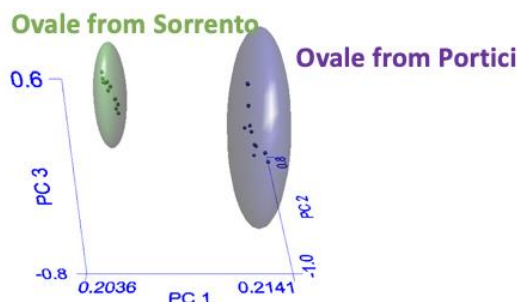


Figure 3 Coomans plot

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Use of Natural Extracts for the Improvement of Quality, Functional Properties and Stability of Food Products

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The first part of the PhD project was focused on the evaluation of quality, functional properties and stability of frozen carrots enriched by green tea extract. The enrichment of the vegetable with green tea extract alone or in combination with trehalose has been carried out by exploiting freezing pre-treatments i.e. blanching and vacuum impregnation. Quality and functional properties of the vegetable product was investigated both just after the pre-treatments, and after freezing and storage in frozen state at -18°C up to 60 days.

Impiego di estratti naturali per il miglioramento della qualità, delle proprietà funzionali e della stabilità dei prodotti alimentari

La prima parte del progetto di dottorato si è concentrata sulla valutazione della qualità, delle proprietà funzionali e della stabilità di carote congelate arricchite con estratto di tè verde. L'arricchimento di tale vegetale con estratto di tè verde da solo o in combinazione con trealosio è stato effettuato sfruttando pre-trattamenti al congelamento quali blanching ed impregnazione sottovuoto.

Le proprietà qualitative e funzionali del prodotto vegetale sono state studiate sia subito dopo i pre-trattamenti, sia dopo il congelamento e la conservazione in stato congelato a -18°C fino a 60 giorni.

Key words: *Daucus carota*, L., green tea extract, vacuum impregnation, quality, frozen storage, stability.

1. Introduction

In accordance with the PhD thesis project previously described (Santarelli, 2017), this poster reports the main results of the first activities on:

- *Production of minimally processed/semifinished frozen products:* (i) Evaluation of the effect of VI pre-treatments on the physical properties and enzyme activity of the plant matrices; (ii) study of the effect of the enrichment with functional ingredients on the technological functionality and on the functional and physical properties of the vacuum impregnation (VI) pre-treated products; (iii) study of the effect of the enrichment with functional ingredients on the functional, physical and sensory properties of the VI pre-treated products during frozen storage.

2. Materials and Methods

One batch (20 kg) of organic carrots (*Daucus carota* L., var. Carvejo) at the same ripening stage (i.e. length of 18-20 cm and diameter of 1.5-2 cm) were washed, peeled and cut into slices of 5-mm thick. Carrot slices (0.5 cm) were (i) pre-treated by blanching in water (BLw) or trehalose 4% w/v (BL_{4T}) at 90°C for 3 min and (ii) vacuum impregnated in water (VIw), trehalose 10% w/v (VI_{10T}), green tea extract (GTE) 0.25% w/v (VI_E) and trehalose 10% w/v in combination with green tea extract 0.25% w/v (VI_{10T-E}). VI treatments were performed by using a laboratory-scale equipment at 50 mbar for 10 min with a post-

vacuum time of 10 min. Samples were packed in BOPP bags (film thickness: 30 mm) in air and frozen at -40°C and stored at -18°C up to 60 days.

The following evaluations were carried on carrots before and just after the pre-treatments and after freezing and storage: colour, firmness, total carotenoids, total polyphenols content (TPC), antioxidant activity (AOA, ABTS assay), and enzymatic activity of peroxidase (POD) and pectinmetilesterase (PME).

Data are expressed as mean and standard deviation and additionally analysed by one-way ANOVA analysis. Significant differences between means were computed by LSD test at a significance level of 0.05.

3. Results and Discussion

3.1 Effect of pre-treatments on the enzymatic activity, physical and functional properties of sliced carrots

After blanching, the POD residual activity was lower than 10% and no residual PME activity was detected in accordance with the industry requirements.

Pre-treatments affected to a similar extent ($p > 0.05$) the carrots colour by determining a decrease of lightness (-18% on average) and a slight increase of the hue angle (+11.2% on average). No differences in total carotenoids were observed among fresh and pretreated samples, likely the thermos-induced degradation was balanced by a higher extractability related to the structure damage induced by the pre-treatments.

Blanching pre-treatments impaired the carrots firmness caused by the cell wall pectins gelification, solubilisation and degradation (Neri et al., 2014) occurring during heat treatment. On the contrary, the VI treatment did not induce any significant further change of carrots firmness. Samples blanched in trehalose solution and then vacuum impregnated with tea extract or tea extract in combination with trehalose evidenced a higher firmness compared to the other pre-treated samples. This result could be due the synergistic effect given by the thermoprotective effect of trehalose on the tissue structure and by the polyphenols adsorption to the carrots cell wall by VI.

BL_W and BL_{AT} showed a TPC significantly ($p < 0.05$) higher than the fresh vegetable (figure 1a). This result is probably due to the higher extractability of these compounds promoted by structural modifications induced by the thermal treatment. When the blanched samples were further treated through VI in water, no significant TPC variation was observed. VI treatment in green tea solutions determined a significant increase ($p < 0.05$) in polyphenols, which resulted three times higher than in the fresh vegetable. The addition of trehalose to GTE solution negatively influenced the TPC of VI processed carrots.

As regards the antioxidant activity (figure 1b) a positive effect of blanching in trehalose solution and of VI with GTE solutions was observed; in particular, AOA was higher when VI treatment was performed by using GTE solution in combination with trehalose, despite of its lower TPC; polyphenols oxidation which lead to the the formation of oligomers, which are characterized by a higher scavenging activity compared to their precursors (Lotito et al., 2000), could be responsible for this result.

3.2 Effect of frozen storage on the physical and functional properties of pre-treated sliced carrots

In general freezing and frozen storage for 60 days negatively affected the firmness and colour of carrots slices and this effect was observed to a higher extent on the not-

pretreated sample due to their enzymatic activity, and to a lower extent on all the samples which were blanched in trehalose solution. This result suggests that trehalose is able to exert a cryoprotective effect on the plant cell structure only when added by blanching or rather when its penetration at intracellular level is promoted by changes in cell wall and membrane permeability occurring during the heat treatment. After freezing and frozen-storage a significant ($p < 0.05$) TPC decrease was observed on samples blanched and then vacuum impregnated with GTE solutions while no TPC variation occurred for the other samples (figure 2a). These results could be dependent to the different sensitivity of endogenous and exogenous polyphenols towards oxidation reactions due to their different localization within the cells, and binding to cell wall constituents. Despite the TPC decrease, the samples impregnated with GTE solutions after freezing and frozen storage confirmed their highest polyphenols content, which was up to 2.9 times higher than the fresh vegetable.

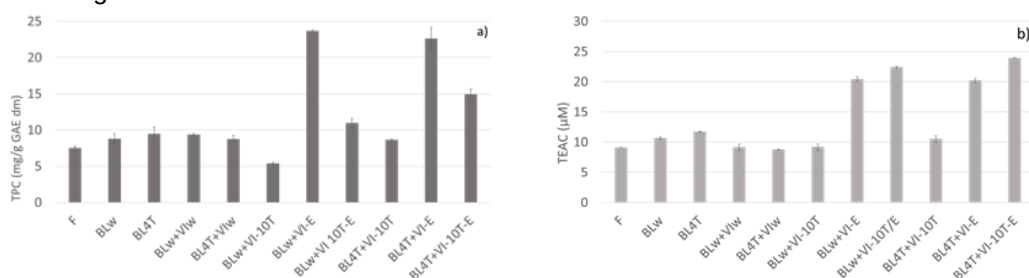


Figure 1 Total polyphenol content (a) and antioxidant activity (b) of fresh and pre-treated carrots.

As regards the antioxidant activity (figure 2b), samples showed in general a significant TEAC ($p < 0.05$) decrease due to freezing and frozen storage as an effect of both the carotenoid and polyphenols loss. The samples blanched and VI in GTE solutions after freezing and frozen storage showed a AOA doubled respect to fresh carrots.

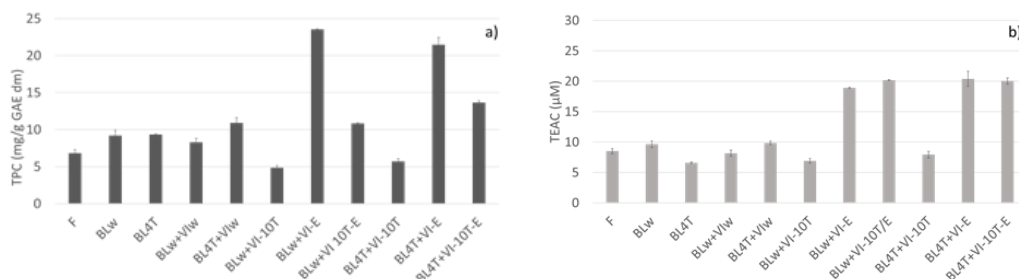


Figure 2 Total polyphenol content (a) and antioxidant activity (b) of not pre-treated (F) and pre-treated carrots after freezing and frozen storage.

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Rosehip Pericarps and Seeds as Possible Food and Natural Antioxidants in Food and Beverage

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The aim of the study is to determine the phytochemical composition of the rosehip pericarp and seeds of selected rose plants growing in some central Italy Regions (Marche, Lazio and Emilia Romagna) as possible ingredients in functional food formulations and/or dietary supplements to replace synthetic antioxidant (butylated hydroxyanisole, butylated hydroxytoluene, etc.) with natural alternatives. The PhD project includes the evaluation of the effectiveness of polyphenols extracted from rosehip pericarps as antioxidants in some vegetable oils (maize, sunflower and high-oleic sunflower) and the possibility to use rosehip seed oil as functional food, due to the particular fatty acid and phytochemical composition.

Pericarpi e semi di cinorrodo di rosa come possibili cibi funzionali e antiossidanti naturali in cibi e bevande

Lo scopo di questo studio è la determinazione della composizione in fitocomposti di alcuni tipi di rose selezionate in alcune regioni dell'Italia centrale (Marche, Lazio ed Emilia Romagna) da usare come ingredienti in cibi funzionali (semi) e antiossidanti in cibi e bevande (pericarpi). Il progetto di Dottorato prevede la valutazione dell'efficacia di polifenoli estratti dai pericarpi di cinorrod di rosa come antiossidanti naturali in alcuni oli vegetali (mais, girasole, girasole alto oleico) e la possibilità di uso di olio di semi di cinorrodo come alimento funzionale, data la sua particolare composizione in acidi grassi e in composti fitochimici.

Key words: antioxidants, rosehip

1. Introduction

Rosehips pericarps and seeds can be utilised in food, pharmacological and cosmetic applications as potential sources of bioactive compounds.

This paper reports the results of the experimental activities performed during the second year of the PhD project, which consisted in the determination of total polyphenolic fraction and fatty acid composition in 11 samples of *Rosa spp.* collected in three different sites of central Italy. The further aim of the research will be the tentative to use rose hips pericarps as natural antioxidants in some vegetal oils and seeds as functional food.

2. Materials and Methods

Rosehips of 11 *Rosa spp.* were collected in three sites of central Italy: two species in Maciolla, Urbino, Marche, one in the Natural Park of Simbruini Mountains in Camerata Nuova, Roma, Lazio and eight from the Antique Rose Collection of Faenza, Forlì-Cesena, Emilia Romagna. Pericarps and seeds were manually separated and a lyophilisation step was performed to allow comparison of samples with different water content.

Total Phenolic Content (TPC) was determined in pericarps and seeds following the Folin-Ciocalteu colorimetric method and results are reported as mg GAE (Gallic Acid Equivalents)/g dry weight. Lipid extraction from seeds was carried out by an automated Soxhlet apparatus (Soxterm - Gerhardt, Bonn, Germany). Fatty acid methyl esters

(FAME) were obtained by acid-catalyzed transesterification of extracted lipids, as described in Mozzon and Frega (2013). Gas chromatographic conditions and analyses were performed according to Tavoletti et al. (2018).

3. Results and Discussion

Polyphenolic fraction: The results of the total polyphenolic content (TPC) in the pericarps and seeds of the 11 rose samples are reported in Table 1. TPC in lyophilized pericarps resulted in a wide range, from 16.80 ± 0.92 mg GAE g^{-1} in *Rosa gallica versicolor* to 288.19 ± 4.70 mg GAE g^{-1} in *Rosa gallica damascena*. Some of the TPC values measured were in accordance with other reports (Barros et al., 2010; Fascella et al., 2019). The TPC values found for some varieties studied, such as *Rosa gallica belle sultane*, *Rosa gallica bella porpora violetta*, *Rosa gallica alba*, *Rosa gallica complicata* and *Rosa gallica damascena*, resulted high and suitable for the purpose of the PhD research, which is the use of pericarps as natural antioxidants to protect polyunsaturated vegetable oils against thermo-oxidative deterioration.

Fatty acids: The relative abundance of the main fatty acids found in the seeds of the 11 rose samples are reported in Table 2. The three unsaturated fatty acids, oleic, linoleic and alfa-linolenic, represented almost 90% of the total in all the samples. Linolenic acid was the most present, ranging around 50% of the total in all rose samples (from 43.79% to 55.91%). Oleic acid ranged between 12.32% in *Rosa canina* Camerata Nuova and 25.83% in *Rosa canina* Faenza, while alfa linolenic acid ranged from 13.46% in *Rosa gallica versicolor* to 23.51% in *Rosa canina* Camerata Nuova. Other fatty acids, i.e. C12:0, C14:0, C15:0, C16:1 n-9, C17:0, C18:1 D11, C20:1 n-11, were present in a percentage even lower than 1% and not reported in Table 1.

Table 1 Total Polyphenolic Content (mg g^{-1}) in the lyophilized rosehip and seeds of the 11 rose samples collected in central Italy sites (mean of three replicates; s.d. in parentheses); (nd = not detected).

	BS	BPV	C CN	CF	G	GA	GC	GD	GO	GR	GV
Pericarps	260.41 (8.13)	213.19 (9.34)	113.19 (9.11)	190.97 (9.31)	165.97 (18.54)	238.19 (16.90)	265.97 (12.43)	288.19 (4.70)	41.52 (2.92)	35.69 (0.81)	16.80 (0.92)
Seeds	30.81 (3.14)	47.50 (3.65)	45.08 (2.12)	21.25 (0.65)	17.50 (0.05)	27.25 (0.11)	20.16 (0.12)	50.41 (0.11)	20.35 (0.07)	nd (nd)	nd (nd)

BS = *Rosa gallica belle sultane*, BPV = *Rosa gallica bella porpora violetta*, (Urbino); CCN = *Rosa canina* (Camerata Nuova); CF = *Rosa canina*, G = *Rosa gallica*, GA = *Rosa gallica alba*, GC = *Rosa gallica complicata*, GD = *Rosa gallica damascena*, GO = *Rosa gallica officinalis*, GR = *Rosa gallica rugosa*, GV = *Rosa gallica versicolor* (Faenza).

The relative abundance of the main fatty acid in rose samples studied suggests a possible use of each one of them as natural functional foods, due to the high unsaturated fatty acid content, as also described by Fofana (2013), who demonstrated the best quality of Prince Edward Island wild roses with respect to other plant seeds (canola, olive and blueberries).

In conclusion, the nutritional composition and the presence of bioactive compounds make the rosehip pericarps and seeds a valuable source of phytonutrients and may be proposed as natural antioxidants and as ingredients in functional food formulations. The research program will continue next year with the laboratory studies on the effectiveness of extracted polyphenols in protecting some vegetable oils from

oxidation, measuring parameters as IC₅₀ (DPPH radical scavenging test) and PV (Accelerated Storage). Moreover, the composition of other phytochemicals (ascorbic acid, carotenoids, sterols) in the rosehip seed oil of the 11 *Rose spp.* will be determined in view of the use as functional food.

Table 2 Relative abundance (%) of the main fatty acid of rosehip seeds of the 11 rose samples collected in central Italy (mean of three replicates; standard error in parentheses).

FA	BS	BPV	C CN	CF	G	GC	GD	GO	GR	GV
C16:0	4.98 (0.11)	4.55 (0.24)	3.76 (0.13)	4.44 (0.20)	5.49 (0.33)	7.06 (0.07)	7.98 (0.15)	4.69 (0.17)	3.93 (0.12)	5.13 (0.24)
C18:0	1.89 (0.06)	2.35 (0.07)	2.67 (0.11)	3.29 (0.16)	2.51 (0.03)	2.61 (0.18)	2.84 (0.23)	1.85 (0.06)	1.61 (0.14)	1.73 (0.08)
C18:1 n-9	21.72 (0.52)	22.96 (0.16)	12.32 (0.24)	25.83 (0.85)	24.16 (0.57)	23.56 (0.47)	19.18 (0.36)	24.02 (1.03)	18.05 (0.21)	23.98 (0.77)
C18:2 n-6	47.58 (0.47)	53.52 (0.76)	55.91 (0.12)	46.89 (0.42)	44.46 (0.57)	43.79 (0.81)	48.40 (1.21)	50.67 (0.69)	52.45 (0.65)	52.72 (1.20)
C20:0	1.02 (0.11)	0.76 (0.04)	0.89 (0.04)	1.27 (0.13)	1.00 (0.04)	0.76 (0.05)	0.79 (0.07)	1.30 (0.08)	0.79 (0.05)	1.13 (0.10)
C18:3 n-3	20.99 (0.34)	14.57 (0.48)	23.51 (0.44)	17.20 (0.34)	20.77 (0.35)	19.65 (0.48)	17.96 (0.31)	15.75 (0.72)	21.41 (0.83)	13.46 (0.48)

BS= *Rosa gallica belle sultane*, BPV = *Rosa gallica bella porpora violetta*, (Urbino); C CN = *Rosa canina* (Camerata Nuova); CF = *Rosa canina Faenza*, G= *Rosa gallica*, GC = *Rosa gallica complicata*, GD = *Rosa gallica damascena*, GO = *Rosa gallica officinalis*, GR = *Rosa gallica rugosa*, GV = *Rosa gallica versicolor* (Faenza).

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Use of Lactic Acid Bacteria Fermentation and Non-Wheat Flours to Improve the Nutritional Quality of Pasta

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The state of the art shows that pasta can be used as carrier of valuable compounds such as fiber and proteins, enhancing functional and nutritional profiles. Here, lactic acid bacteria fermentation has been proposed as pre-treatment of hemp, chickpea and milling by-products as wheat substitutes to fortify experimental pasta. The fermentation led to a decrease of the anti-nutritional factors and increase of free amino acids as well as the protein digestibility. Moreover, the use of these ingredients allows the production of pasta containing more than 5 and 13% of fiber and proteins, respectively.

Impiego di batteri lattici in matrici alternative al frumento per migliorare la qualità nutrizionale della pasta

Lo stato dell'arte dimostra come la pasta possa essere vettore di composti come fibre e proteine, migliorando i profili funzionali e nutrizionali. In questo studio, la fermentazione dei batteri lattici è stata proposta come pre-trattamento di sottoprodotti della molitura, sfarinati di canapa e di cece come sostituti del frumento da utilizzare per la fortificazione di pasta sperimentale. La fermentazione ha portato ad una diminuzione dei fattori anti-nutrizionali e aumento degli aminoacidi liberi nonché della digeribilità delle proteine. L'utilizzo di questi ingredienti consente la produzione di pasta contenente oltre il 5 e il 13% di fibre e proteine, rispettivamente.

Key words: Lactic acid bacteria, fermentation, pasta, nutritional properties.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:

A1) Evaluation of the fermentation process on chickpea, hemp and bran and wheat germ as biotechnological process to fortify pasta;

A2) Evaluation of nutritional and functional characteristics of fortified pasta.

2. Materials and Methods

2.1 Raw materials, bacterial strains and dough fermentation

Commercial samples of hemp (H), chickpea (C), wheat germ (G) and bran (B), certified for mycotoxins levels were used. Strains of lactic acid bacteria identified by partial sequencing of the 16S rRNA and recA genes as *Lactobacillus plantarum* LB1 and *Lactobacillus rossiae* LB5 were used in this study. To be used as a mixed starter for sourdough fermentation of H, C and WGB, lactic acid bacteria cells were harvested by centrifugation. Milling by-products (WG and WB) were mixed (WGB, 1:4, wt/wt) prior dough preparation. In detail, 50 g of H (DY, dough weight $\times$ 100/flour weight, was 160), chickpea (DY, 160) and milling by-products (DY, 250) doughs were prepared mixing

31.25 g e 20 g of flour and 18.75g and 30 of tap water, respectively containing the cell suspension (final cell density in the dough of ca. $7.0 \log_{10}$ cfu/g). Fermentations were carried out at 30 °C for 24 h (H_F , C_F , and WGB_F)

2.2 Microbiological and biochemical characterization of fermented doughs

Proteolysis by means of total free amino acids (TFAA) and peptide profiles were evaluated. Water/salt-soluble extracts (WSE) from doughs were prepared according to the method originally described by Osborne (1907) and modified by Weiss et al. (1993). TFAA were analyzed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, United Kingdom), peptides profiles were investigated by Reversed-Phase Fast Performance Liquid Chromatography (RP-FPLC). Organic acids were determined by High Performance Liquid Chromatography (HPLC). Doughs prior bioprocessing were used as the controls (H , C , and WGB). pH values of H , C and WGB and H_F , C_F and WGB_F were determined as reported above. Presumptive lactic acid bacteria were enumerated using MRS agar medium (Oxoid) supplemented with cycloheximide (0.1 g/liter) as reported above. The IVPD of doughs was determined by the method proposed by Akeson and Stahmann (1964) with some modifications (Rizzello et al., 2014).

2.3 Pasta making

Experimental pasta was manufactured using a pilot plant *La Parmigiana SG30* (Fidenza, Italy). All the doughs were made with a DY of 130, corresponding to a 23% (wt/wt) water and 77% (wt/wt) flour mixture. H_F , C_F and WGB_F were fermented at 30 °C for 24 h with the mixed starters as described before. A reference pasta was made only using wheat semolina (pCT). Ingredients were mixed in three steps (1 min mixing and 6 min hydration). Then, the final dough was extruded at 45–50 °C, through a n. 76 bronze die. The extruded material was cut with a rotating knife for short pasta shapes to obtain grooved “macaroni”.

2.4 Chemical and nutritional profile of pasta

pH, TTA values, TFAA and peptides concentrations of pasta samples, were determined as reported above on pasta dough prior the extrusion. Raffinose and phytic acid concentrations were determined by using the Megazyme kit Raffinose/D-Galactose Assay Kit K-RAFGA and K-PHYT 05/07 (Megazyme International Ireland Limited, Bray, Ireland). Condensed tannins were determined using the acid butanol assay, as described by Hagerman (2002). IVDP and starch hydrolysis were determined on pasta samples cooked. IVPD was determined as described before. The analysis of starch hydrolysis was carried out with a procedure mimicking the in-vivo digestion of starch (De Angelis et al., 2009). Pasta obtained was evaluated by sensory analysis.

3. Results and Discussion

A significant decrease of the values was found as results of the fermentation (ca. 2 points of pH). Increases of both acids were found after 24 h of fermentation, reaching values in the range 180.7 ± 4.3 – 246.4 ± 6.2 mmol/Kg and 31.6 ± 0.3 – 43.9 ± 0.9 mmol/Kg, for lactic and acetic acid, respectively. The highest concentration of lactic acid was found in WGB_F , while H_F contained the highest amount of acetic acid.

When lactic acid bacteria were used as starters for fermentation, a more relevant increase of the TFAA concentration was observed. Indeed, concentrations up to two times higher as compared to the corresponding unfermented doughs were obtained after 24 h of fermentation. Overall, the highest concentration of TFAA was found in C_F (2457 ± 16

mg/Kg), however the highest increase was found in fermented milling by-products. According to the peptide profiles, two (H and C) out of three samples were characterized by lower (21 and 71%, in HF and CF respectively) total peak area when the fermentation was used as bioprocessing (2741 and 4216 mAU in HF and CF, respectively).

The highest decrease was found in C_F (three times), however, according to the TFAA concentration, the lowest value was found in H_F . Values of TFAA and peptides concentrations well reflected on the IVPD of the samples. Overall, fermented doughs were characterized by higher values of the IVPD as compared to the corresponding unfermented ones. In detail, increases ranging from 16 to 37% were found in fermented doughs, with WGB_F and H_F having the highest values.

Unfermented and fermented doughs were used to fortify wheat pasta.

The pH and TTA values and TFAA concentration were determined on pasta doughs before extrusion. The pH value of pasta doughs fortified with unfermented ingredients did not differ and was in the range of 6.37 - 6.42. When H_F , C_F and WGB_F were added, the pH of doughs before extrusion ranged from 5.06 to 5.16 and differed significantly from pCT . A similar trend was observed between pasta samples, as regard to the TFAA concentration. Indeed, increases up to two and eight times were found when the unfermented and fermented doughs, respectively were used to fortify pasta. Regarding the nutritional properties, as expected the fortification with H, C and WGB improved the content of protein and total dietary fibers up to 20 and 100%, respectively.

Fiber contents higher than 6% were reached with the fortification.

Although the fortification led to an increase of the anti-nutritional factors in all samples, pH_F , pC_F and $pWGB_F$ contained lower amounts compared to pH , pC and $pWGB$, respectively. Overall, decreases of ca. 10-times were determined in fermented doughs-containing pasta as regard to phytic acid and raffinose. pC_F and pH_F contained the lowest content of phytic acid and raffinose, respectively. Condensed tannins were from 23 to 59% lower in pH_F , pC_F and $pWGB_F$ as compared to the corresponding unfermented doughs-containing pasta. Relevant decreases (up to 79%) were found in term of HI. In detail, lower values were found in fermented samples as compared with pH , pC and $pWGB$. Similar trend was found as regard to the IVPD. Increases from 22 to 45% were found when H, C and WGB were used as ingredients in pasta making as compared to pCT . Values 43 - 64% higher than pCT were found in pH_F , pC_F and $pWGB_F$. The fermentation led to pasta having IVPD from 10 to 22% higher than the corresponding pH , pC and $pWGB$. Aiming at highlighting the effect of the fortification on the sensory profile of pasta, pH , pC , $pWGB$, pH_F , pC_F and $pWGB_F$ were subjected to sensory analysis and data were compared to commercial pasta produced with the same flour. No significant differences were found between pH and pH_F . On the contrary, seemed to significantly influence the sensory profile when chickpea and milling by products were used.

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Comparison between Organic and Sustainable Protocols in Food Production

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The comparison of the fulfilments between organic and integrated production methods and private certification schemes such as the Std. Globalgap IFA FV, shows that the compliance requirements of the latter concern not only questions relating to the cultivation methods but also aspects concerning the sustainability, safety in the workplace, respect for ethical and social principles for workers, including in some way and then expanding the research spectrum of the same organic production method that focuses mainly on campaign activities.

Confronto tra sistemi di produzione in Bio e sistemi a basso impatto

Il confronto degli adempimenti tra i metodi di produzione biologici e integrati e gli schemi di certificazione privati come lo Std. Globalgap IFA FV, mostra che i requisiti di conformità di quest'ultimo riguardano non solo questioni relative ai metodi di coltivazione ma anche aspetti inerenti la sostenibilità, sicurezza sul posto di lavoro, rispetto dei principi etici e sociali per i lavoratori, includendo in qualche modo e poi ampliando lo spettro di ricerca dello stesso metodo di produzione biologica che si concentra principalmente sulle attività di campagna.

Key words: Organic, Certification, Voluntary, Globalgap, Conformities, Control, Check, Sanctions.

1. Introduction

All farms are required to keep a notebook to record the cultivation operations carried out and the products used on the crops. The annotation of the treatments carried out and the phytosanitary products used, the quantity and the date is of considerable importance for the subsequent production collection operations and to be able to transmit information on food safety and product traceability to the final consumer. For this reason, the methods of management based on Integrated Production (IP), Organic Agriculture (OA), Good Agricultural Practices (GAP) are increasingly widespread and applied under the guise of private voluntary standards capable of guaranteeing an accurate control over the products obtained and on processes.

In accordance with the PhD thesis project, this poster reports the activities concerning:- the different methods of annotation envisaged between organic and conventional production methods and the obligations imposed by the respective regulations and the comparison with similar registrations provided by private certification schemes, such as the FV Globalgap IFA; - the Control and Supervisory Systems operating by public or private authorities and the sanctioning systems.

2. Materials and Methods

In the period January 2017 - January 2019 interviews were conducted with ICQRF officials (Central Inspectorate for the Suppression of Fraud) to understand the biological products inspection (OP) process in Italy, (Chiappara and Fallico, 2008) the objectives of the program, its organization and how auditing takes place. With a structured

questionnaire, 4 inspectors from the certification bodies and the technical manager of a biolo producer organization were interviewed. The survey recorded detailed information on pesticide use, checklists and inspection reports.

3. Results and Discussion

3.1 Voluntary certification process

Regulated product certifications such as the organic production method.

- The production of organic food in Italy is subject to a double surveillance system: the accredited and authorized private control body (OdC) chosen by the company and the public authority (ICQRF and regions) that have the task of supervising it. The CB carries out at least one annual inspection (both of the sites and of the registrations and documents), even without warning and with sampling to be subjected to a multi-residual analysis, which can be increased in case of presence of crops included in a high risk class. Unregulated product certifications such as the IFA FV Globalgap

- The accredited private certification body chosen to obtain the certification of primary productions according to the Std. Globalgap IFA FV carries out an inspection verifying the correct application of the requirements such as the registration of product traceability, the application of integrated defense and good practices agricultural, food safety and environmental sustainability obligations. 3.2 System of Controls by public authorities and the applicable Sanctions

- Conventional and / or integrated production companies: the controls are delegated to the health structures of the Regions and autonomous provinces, to the Carabinieri Command which operates with the ICQRF. The inspection includes inspection and verification of documents. The applicable law provides for administrative and financial penalties.

- Organic farms: the controls are coordinated by the Ministry of Agricultural, Food and Forestry Policies for Tourism (Mipaaff) which assigns them to the private CBs authorized by it, in turn controlled by the ICQRF, the regions and by Mipaaff itself. The control of organic operators by the CBs takes place with access to the sites and documents and provides for sanctions up to exclusion. - GlobalGap IFA FV Standard: the control is conducted by the chosen certification body without any intervention by the Public Authority. Compliance is subject to compliance with the control points established by the Std; the verification of Non-Conformity requires the management with adequate corrective actions, under penalty of the suspension of the company or one or more products covered by the certificate (from Globalgap General Rules - Part I Requirement General Version 5.1).

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Globalgap General Rules - Part I General Requirements Version 5.1.

Innovative Technologies for the Production and Preservation of Natural Leavening Products with High Nutraceutical Content

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The research has been focused on the study of the effect of fortification of a sourdough bread with a flour obtained from lyophilized purple potatoes (cv. Vitelotte), with and without lyophilized albedo of *Citrus aurantium* L. The chemical composition of raw materials, dough and final fortified breads has been evaluated together with changes in nutraceutical properties of the products, estimated analyzing anthocyanin contents, phenolic composition as well as antioxidant capacity. Sensory profile of fortified breads has also been evaluated. All the results have been compared with those obtained from the analysis of a traditional white sourdough bread.

Tecnologie innovative per la produzione e conservazione di prodotti a lievitazione naturale a elevato valore nutraceutico

Scopo della ricerca è stato lo studio della fortificazione di un pane a lievitazione naturale con una farina ottenuta da patate viola liofilizzate (cv. Vitelotte), con e senza aggiunta di albedo liofilizzato di *Citrus aurantium* L. E' stata valutata la composizione chimica delle materie prime, degli impasti e dei pani fortificati insieme con i cambiamenti nelle proprietà nutraceutiche dei prodotti, stimati analizzando il contenuto di antocianine, la composizione fenolica e il potere antiossidante dei campioni. È stato anche valutato il profilo sensoriale del pane fortificato. Tutti i risultati sono stati confrontati con quelli ottenuti dall'analisi di un pane bianco tradizionale a lievitazione naturale.

Key words: Sourdough, antioxidant capacity, albedo, Vitelotte potato, purple bread, food fortification.

1. Introduction

This PhD thesis is aimed at developing new bakery products combining all the benefits connected with the use of sourdough with the beneficial effects of natural antioxidants extracted from natural matrices. This poster reports the main results of the first activities, concerning: i) the evaluation of chemical composition of raw materials (plain flour, lyophilized purple potatoes, lyophilized albedo), utilized for the fortification of a traditional white sourdough bread in order to assess their potential as functional ingredients; ii) the characterization of fortified breads obtained by the enrichment of dough with lyophilized purple potatoes (purple bread) and lyophilized purple potatoes with lyophilized albedo (purple bread+albedo) with their chemical-compositional, nutraceutical, technological and sensory evaluation.

2. Materials and methods

Concerning sourdough bread production, the starter dough maintainment and baking procedure were performed according to Venturi et al, 2016. Plain flours type "0" Pane Toscano Consortium were used for refreshments and for the following bread making tests. Potatoes (*Solanum tuberosum* cv. Vitelotte) were supplied by Azienda Agricola Ravotto Mauro (Mombarcaro, Cuneo, Italy). After washing, potatoes were blanched in boiling water, peeled and freeze-dried (Telstar-Lyoquest-55). The dried chips were then ground, sieved and the resulting powders stored at 4 °C for further analysis.

The albedo was obtained from *Citrus aurantium* L. cultivated at the Certosa of Calci (Pisa). After washing, it was freeze-dried, ground, sieved and the resulting powder stored at 4 °C for further analysis.

Chemical analyses were performed following ICC standard methods for moisture and pH, while total titratable acidity was performed following Gélinas et al. (1995). The concentration of the main fermentative metabolites (ethanol, D/L-lactic acid, acetic acid) was determined by using specific enzymatic kits (Megazyme), after pre-extraction with HCl 0.1 N (Venturi et al., 2016). The analysis of total phenols, anthocyanins and antioxidant capacity were performed according to Sgherri et al. (2016), with some modifications, on extract samples of lyophilized purple potatoes, lyophilized albedo, dough and bread. The extraction was carried out starting from 3 g of sample and using 70% ethanol with 1% HCl as solvent, maintaining the samples in the dark, in continuous stirring and in inert atmosphere (N₂) for 24 h. The extracts obtained were finally centrifuged (10000 rpm, 15 min) and filtered by Sartorius filters (Minisart 0,45 µm), stored inside coded tubes saturated with N₂ and immediately analyzed. Sensory profiles of the bread samples were determined by descriptive analysis by a panel of trained assessors, which evaluated bread samples 2 hours after to be taken out of the oven. The crumb were tasted separately from bread crust to better identify the specific contribution of these two bread fractions, using a sensory sheet specifically developed for this purpose. The chemical and sensory evaluations were performed in triplicate. Statistical analysis of data was performed by one-way ANOVA (CoStat, Cohort 6.0), and means separation by the Tukey's HSD test at P≤0.05 of significance.

3. Results and Discussion

Table 1 shows the concentrations of the main antioxidant compounds evaluated in the matrices used to carry out the fortification of the doughs and then of the breads.

Table 1 Total anthocyanins, flavonoid and non flavonoids phenols of plain flour, lyophilized purple potatoes and lyophilized albedo extracts. Within the same row, parameters sharing the same letter do not have a significantly different mean value.***: P<0.001.

	Total anthocyanins (g cyanidin-3-O-glycoside/kg dw)	Flavonoid phenols (g gallic acid/kg dw)	Non flavonoid phenols (g gallic acid/kg dw)
Plain Flour	0,080 ± 0,004 ^b	0,63 ± 0,11 ^b	1,31 ± 0,03 ^c
Lyophilized purple potato	2,613 ± 0,012 ^a	n.d.	6,04 ± 0,21 ^b
Lyophilized albedo	n.d.	17,55 ± 1,11 ^a	12,27 ± 0,18 ^a

Total titratable acidity, pH, moisture and total concentration of ethanol, D/L-lactic acid and acetic acid were determined in samples collected during the whole bread-making (data not shown). In order to put in evidence if and how the fortification could influence the fermentation processes, two chemical indexes able to describe the metabolic activity of homo/heterolactic bacteria fermenting strains (fermentation ratio, FR) as well as the metabolic activity of Lactic Acid Bacteria (LAB)/Yeast strains (Microbial Ratio, MR) were calculated. Furthermore, the total organic acidity (TOA) value was utilized as marker of the whole fermentation activity carried out by the sourdough microorganisms. As shown in Table 2, traditional dough shows a prevalence of yeast activity compared to lactic bacteria, with a significantly lower value of MR compared to enriched doughs, which in any case exhibit average values typical of sourdoughs. Results suggest a possible selective action of the ingredients used, rich in phenolic compounds with a bacteriostatic

action, which could have induced a selection/inhibition of some populations favoring others. The nutraceutical properties of obtained breads were also investigated (Table 3): the fortified breads show higher antioxidant capacity, with a good total anthocyanin content in purple bread, which further increased by adding albedo; moreover, the addition of the albedo has also almost doubled the total phenol content already increased in purple bread.

Table 2 *Technological Indexes of traditional, purple dough and purple dough + albedo. Data are expressed as means $\pm$ confidence intervals ($p=0,05$).*

Technological Index	Traditional dough	Purple dough	Purple dough + albedo
FR= $\frac{[(D+L)-Lactic\ acid]}{[Acetic\ acid]}$	3,77 $\pm$ 0,17	2,57 $\pm$ 0,13	1,98 $\pm$ 0,14
MR= $\frac{[(D+L)-Lactic\ acid]}{[Ethanol]}$	0,57 $\pm$ 0,02	1,04 $\pm$ 0,07	1,01 $\pm$ 0,10
TOA=[(D+L)-Lactic acid]+[Acetic acid]	0,09 $\pm$ 0,00	0,13 $\pm$ 0,00	0,10 $\pm$ 0,00

Table 3 *Total anthocyanins, flavonoid and non flavonoids phenols and antioxidant capacity of fortified breads compared to traditional bread. Within the same row, parameters sharing the same letter do not have a significantly different mean value. ***: $P<0.001$.*

	Total anthocyanins (g cyanidin-3-O- glycoside/kg dw)	Flavonoid phenols (g gallic acid/kg dw)	Non flavonoid phenols (g gallic acid/kg dw)	Antioxidant capacity (mmol Trolox/kg dw)
Traditional bread	0,019 $\pm$ 0,005 ^c	0,014 $\pm$ 0,005 ^c	0,537 $\pm$ 0,025 ^c	0,63 $\pm$ 0,02 ^c
Purple bread	0,290 $\pm$ 0,005 ^{ab}	0,213 $\pm$ 0,150 ^b	1,910 $\pm$ 0,190 ^a	1,74 $\pm$ 0,01 ^b
Purple bread + albedo	0,300 $\pm$ 0,001 ^a	1,200 $\pm$ 0,020 ^a	1,540 $\pm$ 0,020 ^{ab}	2,05 $\pm$ 0,08 ^a

Fortified breads were finally positively evaluated by the sensory panel, with high values with regard to frankness. In particular, despite the addition of various fortifying elements and the perception of hints related to the presence of potato flour, the breads obtained have continued to exhibit, albeit in a less marked manner, the peculiar notes of sourdough bread, such as hints of yeast and acetic acid as well as the notes of hay and wheat typical of flour.

In conclusion, the preliminary results indicate that the fortification with purple potatoes flour and citrus albedo greatly influences nutritional and chemical characteristics of sourdough bread suggesting how the partial substitution of refined wheat flour could turn out to be a viable alternative for the preparation of new bakery products with higher nutraceutical capacity and with an interesting sensory profile.

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Effects of Modified Atmosphere Packaging and Rapid Refrigeration Treatment on the Quality of Fruits of Mulberry (*Morus alba* L.)

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The first two activities of the PhD thesis project are described. It was evaluated the effect of different types of Modified Atmosphere Packaging on the evolution of the quality parameters of mulberry fruit (*Morus alba* cv. Kokuso 21) and the impact of Rapid Refrigeration on the quality of fruits stored in passive atmosphere by comparing them with those with Modified Atmosphere Packaging. In both cases, the secondary aim was to evaluate physical-chemical parameters during storage at low temperature.

Effetti del confezionamento in Atmosfera Modificata e del trattamento di Crioconservazione sulla qualità di frutti di gelso (*Morus alba* L.)

È stato valutato l'effetto di diverse tipologie di *Modified Atmosphere Packaging* sull'evoluzione dei parametri qualitativi dei frutti di gelso (*Morus alba* cv. Kokuso 21), la successiva shelf-life e l'impatto di tecniche di *Rapid Refrigeration* sulla qualità al consumo. I frutti sono stati conservati sia in atmosfera passiva che in atmosfera modificata. In entrambi i casi, obiettivo secondario dell'esperimento è stato quello di valutare i parametri fisico-chimici durante la conservazione a bassa temperatura.

Key words: Modified Atmosphere Packaging, Rapid Refrigeration, mulberry, quality, *Morus alba*, shelf life.

1. Introduction

The first objective of the PhD thesis project is to test the effects of Modified Atmosphere Packaging (MAP) and Rapid Refrigeration (RR) in the extension of post-harvest life, evaluating the quality of the fruits subjected to these techniques. Secondary objective, functional to the principal objective, is identification of the optimal harvesting time to optimise the organoleptic, merceological and nutraceutical quality of the fruit. MAP and RR will be applied to fresh and IV gamma fruits and for all treatments will be examined the evolution of the main chemical-physical parameters, the content of bioactive compounds and their possible role in the prevention of degenerative diseases through in-vitro tests. All phenomena of senescence, such as physiological and infectious alterations, will also be examined. In Gastec-Vesta S.r.l. the research activity will focus on food safety, nutraceutical value and the use of alternative plastic films in order to reduce environmental contamination. In accordance with the PhD thesis project, this poster reports methodologies used and the most important results of the first two activities concerning:

A1) technologies used to achieve the objectives of the PhD thesis project;

A2) the evolution of qualitative parameters and subsequent shelf-life of mulberry fruits subjected to MAP, and the impact of RR on fruit quality.

Mulberry is a climateric fruit (Calabrese, 1993) and thanks to their good taste, nutritional value, and biological activity, the production and consumption of mulberry fruit has rapidly increased in recent years. Due to rapid ripening processes and deterioration in quality, the fruit easily loses commercial value after harvest (Hu *et al.*, 2014). Therefore, there is much interest in developing preservation methods for the mulberry fruit. MAP is a dynamic system in which the respiration of the packaged product and the permeation of gases through the packaging film takes place simultaneously. Variation in the composition of the product's storage atmosphere involves changes in respiratory activity, ethylene production and enzymatic reactions (Ahvenainen, 1996). MAP offers two advantages: it modifies the atmosphere inside the package and maintains a high humidity environment (Kader *et al.*, 1989). It is important that for each type of product are used gas concentrations and types of gases appropriate to the physiology of the fruit, as the tolerance to low concentrations of O₂ and/or high concentrations of CO₂ can vary the respiratory metabolism of the fruit with the production of undesirable compounds and consequent tissue alterations (Zagory and Kader, 1988). The most commonly used gases for MAP are: O₂, fixed at the lowest level, to inhibit the growth of aerobic bacteria thanks to the prolongation of the latency phase (Day, 1998); CO₂ as it has shown excellent ability to inhibit bacterial growth; N₂ used to prevent oxidative phenomena; Ar, an innovative gas still little used on fruit, which manages to reduce microbial growth (Jamie and Saltveit, 2002), has inhibitory effects on tyrosinase and malic dehydrogenase activity (Zhang *et al.*, 2001) and improves the efficiency of sanitizing (Qadir and Hashinaga, 2001). The most important factor affecting lifetime of fruits is the temperature, which directly influences the rates of chemical and enzymatic reactions. The conservation of fruits by refrigeration is based on the partial or total suppression of the main agents responsible for the changes in this food group: microbial activity and growth, tissue metabolic activities after harvest, and chemical and enzymatic reactions (Ordóñez, 2005). Vegetables products are characterized by the susceptibility to undesirable changes in temperature and humidity in the surrounding environment. Sometimes these changes cannot be noticed immediately, but they will be observed throughout the commercial chain through changes of taste, odor, firmness, and other product characteristics (Chitarra and Chitarra, 2005). RR is the fast reduction of temperature of freshly harvested product to the optimum temperature for storage, being cooled usually by 7/8 or 88% of the difference in temperature (Gast and Flowers, 1991). A product, even quickly refrigerated, will be well preserved only if its heat content is maintained until the final consumption (Dùssan-Sarria and Honòrio, 2005).

2. Materials and Methods

Mulberries were harvested using the epicarp colour as a harvest index. The fruits were pre-treated in Ox-Virin (peroxyacid-based detergent) and packed in high-density polyethylene bags into which two different gas mixtures were placed: MAP1 (N₂, CO₂, O₂); MAP2 (Ar, CO₂, O₂) using the vacuum compensated packaging technique by a bell-shaped packaging machine (VM 16 Orved). The fruits in MAP1 and MAP2 were stored at 0°C (UR 95%), the fruits in RR were subjected to a cryogenic bath in liquid nitrogen (-196°C) and chilled to -20°C. The analyses concerning the chemical-physical and sensory characteristics were carried out after 4, 8 and 12 days from the start of the experiment. Moreover, during the entire storage period, the evolution of the fruit shelf-life was evaluated: after 4 days (I SL), after 8 days (II SL) and after 12 days (III SL). Mulberries stored in RR were only opened after 12 days of storage. The analyses conducted on the samples were destructive and non-destructive. Statistical significance was defined as $p \leq 0.05$.

3. Results and Discussion

MAP treatment has proved effective in inhibiting browning, preventing anaerobic fermentation reactions and inhibiting aerobic and anaerobic microbial growth of mulberry fruits. The combination of MAP technology with storage at low temperatures (5°C) allowed to keep the fruits with good chemical-physical and organoleptic characteristics during 12 days (Table 1). In addition, the MAP2 treatment, as can be seen from the analysis of the data, has exalted the organoleptic characteristics of the fruit. In II SL in MAP2 optimal results were recorded in terms of juiciness, colour and SST/TA ratio. These results show that the use of Argon in the gaseous mixture, allows a longer shelf life for a quality product that, in a few days, could reach the European markets. Packages containing mulberry fruits treated with RR, in the experiment, were only opened to III SL. The results obtained have shown that even if the fruit remains intact in the structure it tends to lose colour while maintaining its lustre. These fruits also showed high SST content and a low TA content, therefore, the treatment maintain the SST/TA ratio, fundamental characteristic that determines the taste of the fruit.

Table 1 *Evolution of the SST content (Brix°) of fruits. Data are the means $\pm$ S.E. of five replications.*

	T0	T4	T8	T12	r ²
CTR	14.55	18.05 $\pm$ 0.01	18.95 $\pm$ 0.06	16.65 $\pm$ 0.03	0.865
MAP1	14.55	17.85 $\pm$ 0.02	18.65 $\pm$ 0.02	19.45 $\pm$ 0.05	0.9673
MAP2	14.55	19.25 $\pm$ 0.03	19.45 $\pm$ 0.05	18.35 $\pm$ 0.06	0.9984
RR	14.55	-	-	17.85 $\pm$ 1.2	0.9672

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Very Low Protein Diet and Mediterranean Diet Modulate Intestinal Microbiota and Reduce Serum Levels of Total and Free Indoxyl Sulfate and P-Cresyl Sulfate in Chronic Kidney Disease

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The first activities of the PhD thesis project mainly concerned dependent and independent culture methods on faecal samples. Secondly, the study concerned biostatistic elaborations of data obtained from 16S-rDNA metagenetic analysis and bibliographic study of the pathology in question, aimed to detecting correlations with the intestinal microbiota.

Una dieta fortemente ipoproteica e la dieta mediterranea modulano il microbiota intestinale riducendo i livelli sierici totali di Indoxil solfato e P-Cresyl solfato liberi nella malattia renale cronica

Le prime attività del progetto di tesi di dottorato hanno riguardato principalmente, analisi microbiologiche coltura dipendenti e indipendenti su campioni fecali. Successivamente, lo studio ha riguardato elaborazioni biostatistiche di dati ottenuti da analisi metagenetiche 16S-rDNA e studio bibliografico della patologia in oggetto, finalizzato a rilevare correlazioni con il microbiota intestinale.

Key words: CKD, microbiome, Very Low Protein Diet, and Mediterranean Diet.

1. Introduction

For several decades, the interest of nephrologists for nutritional therapy in not-dialysis chronic kidney disease (CKD) has focused mainly on the possibility of delaying the progression of the disease. There are studies showing that nutritional therapy induces favorable metabolic changes and can also delay the need for dialysis by preventing signs and symptoms of renal failure. The Mediterranean diet (MD), which covers the daily needs of nutrients and proteins (protein intake 0.8 g/kg/day) recommended for the general population, is useful in the early stages of CKD (19-20). However, with the progression of CKD it is necessary to use a lower protein intake (0.6 g/kg/day) up to regimens that include a vegan diet or a very low protein diet (0.3-0.4 g/kg/day) supplemented with essential amino acids and ketoacids (VLPD) (Kopple & Fouque, 2018; Garneata et al., 2016).

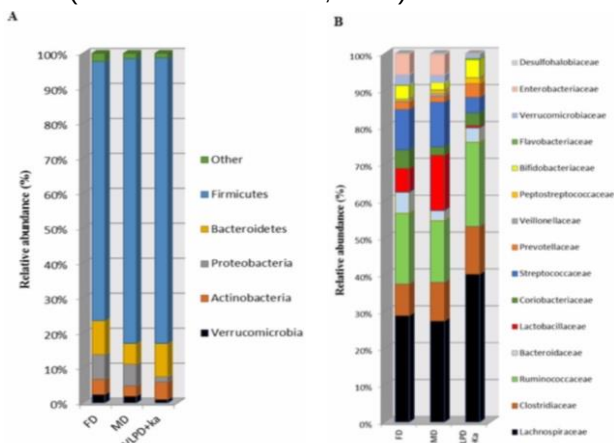
2. Materials and Methods

The study design has established a prospective, randomized, crossover-controlled trial. The 60 CKD grades 3B-4 patients who were randomly assigned (1:1) to 2 different dietary regimens: (i) 3months of free diet (FD), 6months of very low protein diet (VLPD), 3months of FD and 6months of Mediterranean Diet (MD); and (ii) 3months of FD, 6months of MD, 3months of FD and 6months of VLPD. The local Ethics Committee (Campania Nord Ethics Committee, Avellino, Italy) approved this study. All participants signed the informed consent. The study protocol was registered on ClinicalTrials.gov, with the identifier number NCT02302287. 16S Metagenomic Sequencing and statistical methods were described by De Angelis et al. (2014).

3. Results and Discussion

The three different dietary regimens did not affect the microbial alpha diversity values (dns). At baseline under FD, CKD patients exhibited a lower relative abundance Bacteroidetes and a strong gut colonization of Firmicutes (Figure 1A). Further, increased amounts of Proteobacteria characterize the dysbiosis of CKD patients (Castillo-Rodriguez et al., 2018). The VLPD strongly decreased the relative amount of Proteobacteria compared to the other diets ($p < 0.012$). The gut microbiome of CKD patients was also characterized by lowest level of Actinobacteria (Castillo-Rodriguez et al., 2018). Interestingly, VLPD diet improve the gut colonization of Actinobacteria in CKDs if compared to MD ($p = 0.028$). VLPD harbored the highest levels of Lachnospiraceae ($p < 0.006$), Ruminococcaceae ($p < 0.013$), Prevotellaceae ($p < 0.029$) and Bifidobacteriaceae (4.24%, $p < 0.009$) (Figure 1B). Compared to FD, MD showed a strong increase of Lactobacillaceae ($p = 0.047$). While, Enterobacteriaceae decreased in VLPD if compared to FD and MD diets ($p < 0.030$). Ruminococcaceae and Lachnospiraceae were positively correlated to healthy state, producing butyric acid. The positive effect of butyric acid on colonic inflammation is well known (Corrêa-Oliveira et al., 2016).

Figure 1 Bacterial phyla and families associated to dietary regimens in intestinal microbiota of CKD patients. Average of relative abundance (%) of bacterial phyla (A) and families (B) found in the fecal samples of CKD patients after consumption of free diet (FD), Mediterranean Diet (MD), and very low protein diet supplemented with alphaK (VLPD+Ka). Only phyla and families showing statistical differences ($p < 0.05$) in at least one dietary regimen were reported.



Moreover, MD and, especially, VLPD diet resulted in a strong decrease of Enterobacteriaceae. At colon level, some species of Enterobacteriaceae are considered pathobionts due to their competitive efficiency and capacity to opportunistically adhere and invade host tissues (Servin, 2014) and promote metabolic shifts in intestinal lumen, potentiating the emergence of pathobionts. At genus and species levels, VLPD harbored the highest relative amount of *Blautia* (*B. coccoides*, *B. obeum*, and *B. wexlerae*), *Bifidobacterium* spp., *Faecalibacterium prausnitzii*, *Coprococcus* (*C. eutactus*), and *Roseburia* (*R. faecis*) (Figure 2). Compared to FD, both MD and VLPD resulted in a decrease of *Ruminococcus* (*R. callidus*), *Collinsella* (*C. aerofaciens*) and *Bacteroides* (*B. uniformis* and *B. vulgatus*). Compared to FD and/or MD, VLPD showed a lower relative amount of *Lactobacillus* (e.g., *L. gasseri*, *L. salivarius*), *Akkermansia muciniphila*, *Streptococcus* spp. and *Escherichia* (*E. albertii*). The increased level of *Blautia*, *Faecalibacterium*, *Coprococcus*, and *Roseburia* spp. in VLPD was positively associated to dietary fibers, vegetal proteins, potassium, and ketoanalogues and it was negatively correlated with serum toxins (Figure 3). A positive anti-inflammatory effect of *Blautia* was described in other clinical settings (Jenq et al., 2015), similarly to *F. prausnitzii* (Sokol et al., 2008). *Roseburia* and *Coprococcus* species were reported as able to hydrolyze starch and other sugars producing SCFAs. Whereas, high fecal *Bacteroides*, *Ruminococcus*, and *Streptococcus* abundance was positively associated to animal-protein and -fat diet

(Johnson et al., 2017). Moreover, genera such as *Escherichia* and *Proteus* were previously found in the blood of CKD patients (Wang et al., 2012). Accordingly, this study shows positive association between *E. albertii* and the free and total IS and PCS serum levels.

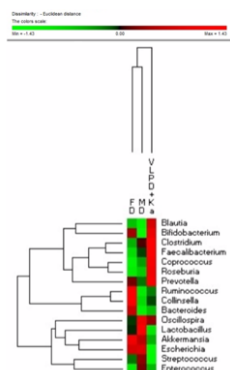


Figure 2 Bacterial genera associated to dietary regimens in intestinal microbiota of CKD patients. Average of relative abundance (%) of bacterial genera found in the faecal samples of CKD patients after consumption of free diet (FD), Mediterranean Diet (MD), and very low protein diet supplemented with α -ketoglutarate (VLPD+Ka). Only genera showing statistical differences ($p < 0.05$) in at least one dietary regimen were reported.

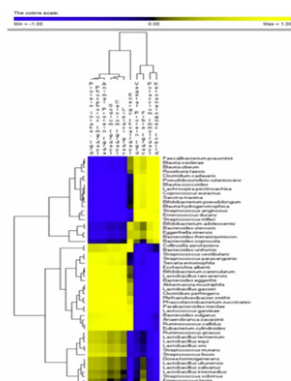


Figure 3 Correlations between bacterial species and dietary intake of CKD patients. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (yellow) and -1 a perfectly negative correlation (blue) between species and dietary intake. Only significant correlations (FDR 0.05) are shown.

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ORAL COMMUNICATIONS

Chemometric and Computational Approaches to Improve Quality and Increase Market Share of Fresh and Long-Ripened Italian Cheeses

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In this PhD project, different chemometric and computational approaches were used to estimate the effect of technological processes on characteristics of fresh and long-ripened Italian cheeses. The effects of different freezing processes and methods along with frozen storage was studied to improve shelf-life of Mozzarella cheese. Computational and photogrammetric models were also developed to better understand and control the freezing process of an irregular shape product as Mozzarella cheese. NIRs and image analysis were used as process quality control tools to estimate particle size and percentage of rind on grated Parmigiano-Reggiano cheese.

Approcci chemiometrici e computazionali per favorire la qualità e aumentare il mercato di formaggi italiani freschi e a lunga stagionatura

In questo progetto di dottorato, diversi approcci chemiometrici e computazionali sono stati usati per stimare l'effetto di processi tecnologici sulle caratteristiche di formaggi italiani freschi e stagionati. È stato studiato l'effetto di processi e metodi di congelamento, unitamente alla conservazione congelata, per prolungare la shelf-life di Mozzarella. Sono stati sviluppati modelli computazionali e fotogrammetrici per il controllo e una migliore conoscenza del processo di congelamento di un prodotto a geometria irregolare come la Mozzarella. NIRs e analisi d'immagine sono stati applicati come strumenti di controllo di processo per stimare la granulometria e la percentuale di crosta in Parmigiano-Reggiano grattugiato.

Key words: freezing process, grated cheese, rheology, computational modeling.

1. Introduction

Globalization of food markets and the increasing demand for some highly perishable and traditional Italian cheeses have forced the dairy sector to investigate in new methods to improve export and/or to control compliance with PDO regulations or internal quality schemes. Despite its high energy consumption, freezing of fresh dairy products can be considered a sustainable solution for supply chains: because of the improved storability and convenience, frozen products have a lower impact on the carbon footprint if compared with fresh, refrigerated products (Martindale, 2014). Considering distant markets, freezing can help to lower the costs of transport (maritime vs air) and decrease waste by decreasing the amount of expired product. The feasibility of high moisture cheese freezing and frozen storage may be valuable, but attention about their sensitivity in relation with the freezing process and frozen storage must be considered and the process must be closely controlled to preserve original properties (Alvarenga et al., 2013). Considering dairy products regulated by European Protected Designation of Origin (PDO), dairies need fast and reliable methods to control their processes and to verify compliance with both legal limits or more severe internal quality programs. In the case of Parmigiano-Reggiano (P-R) cheese, grated P-R cheese accounted for 13.5% of the overall market of P-R cheese in 2017 and this percentage continuously increased in the last years. Grated P-R PDO cheese must comply with the maximum percentage of rind and particles having diameter less than 0.5 mm (<18% w/w and <25%, respectively)

(DOOR, 2018), because an excessive amount of rind can have a negative impact on the sensory characteristics (lower degree of moisture and proteolysis and higher degree of salt concentration and oxidation) of the product (Zannoni, 2010).

In this context, the work was divided into four main activities, as follows:

A1) Mathematical modelling of Mozzarella cheese freezing process by applying a photogrammetric procedure to reconstruct the geometrical domain of the product;

A2) Evaluation of the effects of different freezing and thawing conditions and the presence or absence of covering liquid during freezing and thawing process on Mozzarella cheese characteristics.

A3) Evaluation of frozen and subsequently refrigerated storage of Mozzarella cheese, over several physical, chemical and sensory characteristics.

A4) Applicability of Near Infra-red spectroscopy (NIRs) and Image Analysis (IA) coupled with multivariate techniques to characterize particle properties and estimate rind's percentage of grated P-R cheese in relation with textural characteristics.

2. Material and Methods

2.1 Mathematical modelling of Mozzarella cheese freezing process

To validate and build the models, experimental freezing processes were carried out using fresh citric Mozzarella cheeses of 100g, kindly provided by Nuova Castelli S.p.A. (RE, Italy); cheeses were frozen using an air blast freezer (MF 25.1, Irinox, TV, Italy) until a temperature of -20°C in the core of the product was reached. Three freezing conditions (Fc), differentiated by air temperature and velocity were applied: C1) -40°C , $4.1 \pm 0.6\text{m/s}$; C2) -31°C , $2.5 \pm 0.4\text{m/s}$; C3) -25°C , $1.3 \pm 0.2\text{m/s}$. Temperature of the product was measured in 2 different positions using thermocouples type-K. Frozen cheeses were photographed in different positions and angles using a turntable and the 3D spatial model was built. The spatial domain was used to create models based on the apparent specific heat method using COMSOL Multiphysics v.5.1.

2.2 Effect of different freezing and thawing methods and procedures on Mozzarella cheese characteristics

To study the effect of freezing and thawing on Mozzarella cheese, four batches of fresh Mozzarella cheeses were frozen according to 2.1. Also, two different freezing and thawing methods (M) were considered: samples were frozen with (W) or without (D) covering liquid (0.4% w/w NaCl in water). Frozen samples were stored at -18°C for a short time (up to 10 days) to minimize the additional effects of frozen storage on cheese properties, and then were thawed applying two thawing conditions (Tc): S1) 4°C , $1.3 \pm 0.2\text{m/s}$; S2) 4°C , $4.1 \pm 0.6\text{m/s}$. After thawing, D treatments were immersed into covering liquid; all samples were stored at 4°C for 1 day prior to be analysed. The following cheese properties were measured: moisture content (drying method at 102°C until constant weight); Texture Profile Analysis (TPA) using a TA.XT2i (Stable Microsystems, Godalming, UK); rheological measurements with an ARES rheometer (TA instruments, Delaware, USA) equipped with a 25 mm sanded plate geometry; expressible serum (ES%) applying centrifugal separation (Guo & Kindstedt, 1995) and as the relative amount of serum separated by TPA compression; pH and electrical conductivity of ES%; Colour measurements in CIEL*a*b* colour space using a CR-2600d spectrophotometer (Minolta Co., Osaka, Japan); sensory descriptive analysis performed by 8 trained panellists (6 training sessions of 1 hour). Cheese weight before and after freezing was also evaluated. Trials were performed according to a complete randomized block design; split-split plot

ANOVA models were applied to highlight significant effects of the factors (M, Fc, Tc), as follows:

$$Y_{ijkl} = \mu + M_i + B_j + \delta_{ij} + Fc_k + (Fc \times M)_{ik} + \gamma_{ijk} + Tc_l + (M \times Tc)_{il} + (Fc \times Tc)_{kl} + (Fc \times Tc \times M)_{ikl} + \varepsilon_{ijkl} \quad (1)$$

2.3 Evaluation of frozen storage and subsequent refrigerated storage on Mozzarella cheese characteristics

Three batches of fresh Mozzarella cheese were frozen according to 2.1 and 2.2 by applying C3 and S2 freezing and thawing procedures and D freezing/thawing method. Frozen cheeses were stored in frozen conditions at -18°C for 1, 3, 4 months (Ft). After thawing and an overnight period at 4°C in covering liquid to equilibrate the samples, a refrigerated shelf life period (Rt) of 0, 2, 7 days was also considered. Fresh, non-frozen cheeses were analysed and considered as the control. Analyses were carried out according to 2.2; moreover, to investigate proteolysis, SDS and Urea PAGE, RP-HPLC and Fluorescamine assay were carried out according to Andrews (1983), Poulsen et al., (2012) and Dalsgaard et al. (2007), respectively. To investigate the state of water from a molecular point of view, low field NMR analyses were carried out by measuring longitudinal and transverse relaxation curves (T_1, T_2) with a 20 MHz MiniSpec NMR spectrometer (Bruker, Milano, Italy). Trials were performed according to a complete randomized block design; split-plot ANOVA models were applied as follows:

$$Y_{ijkl} = \mu + Ft_i + B_j + \delta_{ij} + Rt_k + (Ft \times Rt)_{ik} + \gamma_{ijk} \quad (2)$$

2.4 Application of NIRs and IA coupled with multivariate techniques to characterize particle size and estimate the rind percentage of grated P-R cheese

P-R PDO cheese samples (≈ 1 kg) were collected from three different cheese dairies (Nuova Castelli Spa, Reggio Emilia, Italy); five cheese batches with five different ripening times (12, 18, 21, 24 and 30 months) were considered. The rind of each cheese was cut away at 6 mm of depth, according to the definition of rind provided by the PDO regulation; the rind and the cheese were separately grated. To simulate the variability occurring in industrial plants where whole wheels or pieces of cheese with different ages are grated, rind and inner part were combined at different ratios and accurately mixed. The presence of different amounts of rind was set from 0 to 36% w/w for a total of 13 different mixtures, described in Alinovi et al., (2019). One-hundred and thirty-one and fifty samples were analyzed by NIRs using a FT-NIR Tango spectrometer (Bruker, MA, USA) in the spectral range of 1000 – 2500 nm and IA by acquiring images of grated cheese using a HP Scanjet 8200 scanner (Palo Alto, CA, USA), respectively. Textural analyses and moisture content were measured to observe relations with particle size and with the grating behavior of the cheese zones. Partial Least Square (PLS) models were built for: 1) the estimation of rind percentage based on NIRs data; 2) the determination of particle size properties based on NIR data; 3) the estimation of rind percentage based on IA measurements.

3. Results and Discussion

3.1 Mathematical modelling of Mozzarella cheese freezing process

The photogrammetric reconstruction of sample's geometrical domain gave good results, as absolute differences between calculated and predicted volume of samples

were lower than 3.5%. The developed finite element models were in accordance with the experimental data obtained from the three freezing rates (Figure 1). The irregular shape of Mozzarella cheese caused temperature differences among the spatial points of the domain, and in particular in the outer surface (Figure 2). Moreover, temperature differences in the surface were higher with faster freezing rates as differences between maximum and minimum values at 40 min were 8.8°C, 7.9°C and 4.4°C for C1, C2 and C3, respectively.

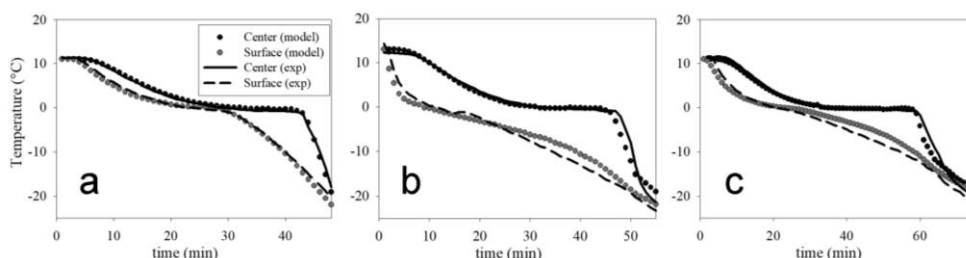


Figure 1 Time-temperature profiles of simulated and experimental freezing processes of Mozzarella cheese; a) C1: -40°C, 4.1 ± 0.6 m/s; b) C2: -31°C, 2.5 ± 0.4 m/s; c) C3: -25°C, 1.3 ± 0.2 m/s.

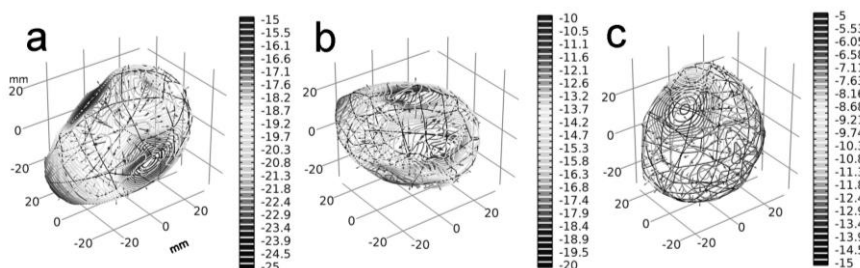


Figure 2 Temperature line plots of Mozzarella cheese surface during the simulated freezing process ($t=40$ min); a) C1: -40°C, 4.1 ± 0.6 m/s; b) C2: -31°C, 2.5 ± 0.4 m/s; c) C3: -25°C, 1.3 ± 0.2 m/s.

3.2 Effect of different freezing and thawing methods and procedures on Mozzarella cheese characteristics

Results highlighted the significant main effect of the freezing/thawing method (M) over many measured parameters. On the contrary, freezing and thawing rates (F_c , T_c) did not determine important statistical differences other most parameters considered, except for ES% and sensory translucency. W-Mozzarella cheeses showed a significant weight increase after the processes of about 7% while D-Mozzarella cheeses did not show important weight variation. The weight increase of W-Mozzarella cheese was associated to water absorption during the thawing process, as also the moisture content of this cheese was significantly higher. Freezing/thawing method showed influence also for the ES%, as W-Mozzarella cheese determined a higher amount of unbound water than control and D-cheeses, respectively; the lower water holding capacity was probably also related to a higher freezing damage, as the freezing time was longer than in the case of D-cheeses. Because of the different moisture, rheological parameters showed significant effects of freezing/thawing method, with D-Mozzarella cheese that showed a higher elastic behavior than the control and W-cheese, respectively. D-Mozzarella cheeses were perceived significantly less juicy by the panellists, despite the moisture content was similar to the control, and this can be related to re-arrangement of unbound

3.3 Evaluation of frozen storage and subsequent refrigerated storage on Mozzarella cheese characteristics

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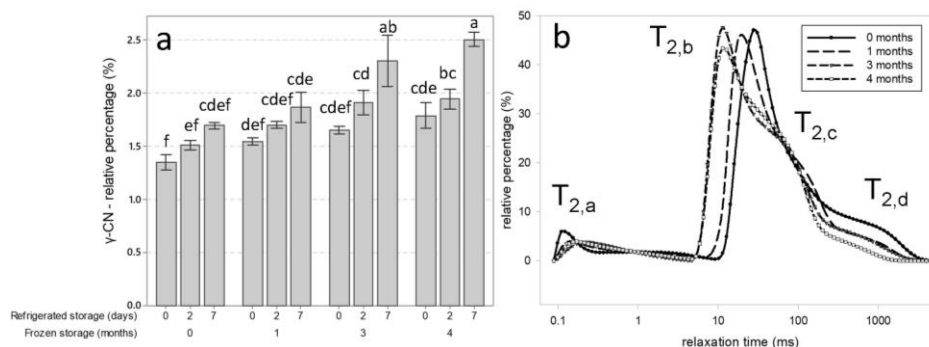


Figure 4 a,b a) Variation of γ -caseins relative percentage during frozen and refrigerated shelf life of Mozzarella cheese; b) T_2 NMR relaxation curves of Mozzarella cheese at different frozen storage times.

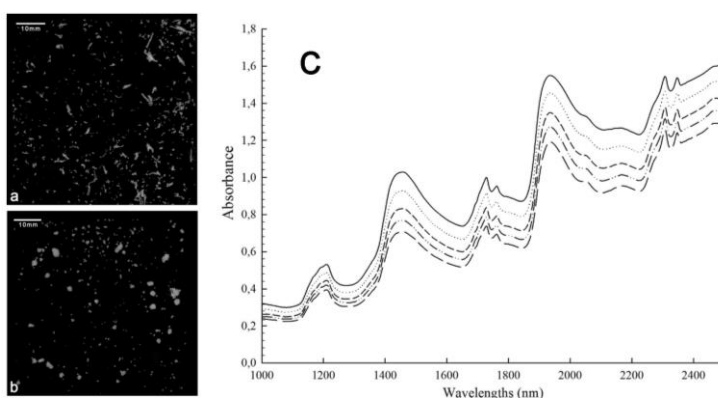


Figure 5 a,b a,b) Details of image acquired for rind (a) and inner part (b) of grated P-R cheese after 24 months of ripening; b) Averaged raw spectra of grated cheese having different rind percentages (w/w), namely 0% (—), 9% (.....), 18% (— — —), 27% (— . .), 36% (— — —).

3.4 Application of NIRs and IA coupled with multivariate techniques to characterize particle size and estimate the rind percentage of grated P-R cheese

P-R cheese rind showed greater textural hardness and lower moisture content than the inner parts of the cheese. Textural properties affected size and dimensions of the particles deriving from the grating procedures. A significantly strong positive relation was found between hardness and total number of particles and the number of finer particles (diameter $<0.5\text{mm}$) in grated P-R cheese; thus, a lower particle size should be expected in the case of cheese portions with higher hardness, as in the case of rind. Moreover, also circularity of particles showed a decrease in cheeses with high percentages of rind, as rind's particles diameter bigger than 0.5 mm were characterized by a more elongated, fibrous shape (Figure 5a). Considering these relations, a PLS model for the determination of rind percentage based on IA parameters was built and showed good results ($R^2_{cv}=0.86$, $RMSECV=4.17\%$). NIRs measurements showed the decrease of the spectrum's baseline at increasing amount of rind's percentage (Figure 5b); this was a consequence of the increasing number of finer particles and the subsequently increase of light scattering phenomena. PLS models based on NIRs measurements showed the possibility to predict particle size properties as the total number of particles ($R^2_{cv}=0.85$,

RMSECV=1045) and percentage of rind ($R^2_{cv}=0.94$, RMSECV=2.86%) with a good accuracy.

4. Conclusions and Future Perspectives

The present research enabled to improve the knowledge about factors and phenomena that rule the behavior and the modification of fresh and long ripened cheeses during freezing and grating processes. Computational freezing models highlighted surface temperature differences related to the irregular geometry of the cheese that can also cause modification of the characteristics in the external layer. Freezing and thawing rates did not cause significant modifications of Mozzarella as the applied temperatures and rates were enough low; on the contrary, the presence of covering liquid during freezing and thawing caused an absorption of water and it was less suitable to freeze the cheese. Frozen storage slowed down but did not stop proteolytic reactions that showed a higher rate after thawing; oxidative reactions were also present. Milk quality and cheese composition should be accurately controlled in order to obtain good results after prolonged frozen storage. Grating behavior of P-R cheese was related to the textural properties of its different zones; rind's particles had a different and lower particle size. According to these relations it was possible to successfully predict particle size and rind percentage thanks to NIRs and IA measurements; these techniques can be successfully applied by dairies as internal quality control measurements to comply with both PDO regulation and quality programs.

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Functional Food Development Through Novel Sustainable Processing and Formulation

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This Ph.D. thesis aimed at studying the impact of technological interventions, combinations with other foods or with drugs, and digestion on the functionality of different food matrices by an integrated approach. Based on acquired results, this approach seems promising to drive functionality through targeted interventions.

Sviluppo di alimenti funzionali mediante interventi di processo e formulazione innovativi e sostenibili

Nell'ambito di questa tesi di dottorato si è studiato l'effetto di interventi tecnologici, della combinazione con altri alimenti o farmaci, e della digestione sulla funzionalità di diverse matrici alimentari mediante un approccio integrato. Sulla base dei risultati acquisiti, questo approccio risulta promettente per orientare la funzionalità attraverso interventi mirati.

Keywords: food functionality; type 2 diabetes; apple juice; apple pomace; phenolic compounds.

1. Introduction

In accordance with the Ph.D. thesis project previously described (Alongi, 2017), this oral communication reports the main results of the following activities directed to:

A1) evaluate the effect of conventional processing on the functionality of foods naturally containing bioactive components;

A2) evaluate the effect of unconventional processing on the functionality of foods naturally containing bioactive components;

A3) evaluate the effect of formulation on the functionality of foods, by applying different ingredients among which are vegetable by-products rich in bioactive components;

A4) evaluate the effect of food-drug and food-food combinations on α -glucosidase inhibition and glycemic index;

A5) validate in vivo the glycemic response predicted in vitro.

2. An integrated approach to design functional food

Nowadays consumers are increasingly aware of the potential of some foods to prevent chronic diseases and are exponentially intensifying their demand for health-promoting products. Thus, the research interest towards these products has experienced a steep increase in the 21st century (from 500 to 5,000 published papers per year between 2000 and 2018). Foods which promote health beyond providing basic nutrition are generally referred to as "functional foods". Still, there is a lot of confusion on this topic, as the so-called functional foods are not recognized as an independent category and no dedicated regulation exists. Such regulatory gap jeopardizes the effective operability of the functional food development cycle. This requires considering several steps (Figure 1)

and involves many stakeholders, among which are legislator, clinicians, nutritionists, food technologists, and marketing experts.

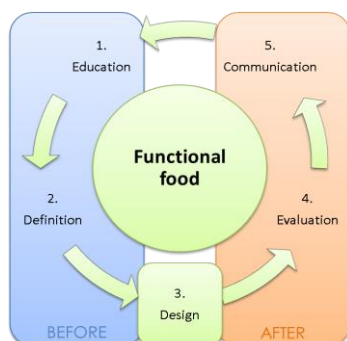


Figure 1 Functional food development cycle.

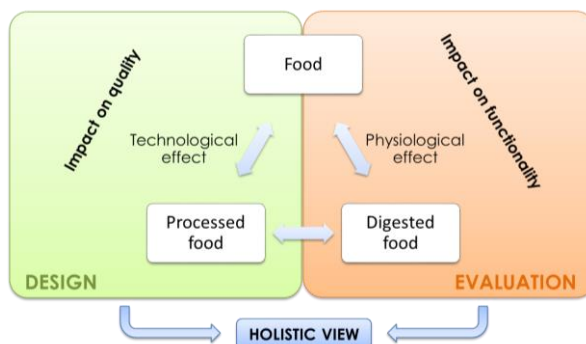


Figure 2 Holistic approach matching the design and evaluation of functional foods.

Although public and insider's education, definition and regulation, and consumer communication are essential steps in the development cycle, the key technical issues to deliver a successful functional food are its design and functionality evaluation. Regarding these crucial points, it is noteworthy that the food design is typically carried out by food technologists, while the assessment of functionality generally relies on the activity of nutritionists and clinicians. By analyzing current literature, a gap between the technological and the nutritional viewpoints can be identified. Most functionality studies are carried out on purified molecules instead of considering the whole food matrix and its digestion; conversely, when the whole matrix is considered, only bioaccessibility is assessed, without testing the targeted bioactivity. This approach seems disjoint as it does not consider the complexity of the food matrix, the potential interactions occurring among food components, the effect of technological interventions, nor the actual bioactivity upon digestion. To bridge this gap, a holistic strategy to design functional foods and assess their functionality is here proposed (Figure 2). Such integrated approach was applied to different study cases, including differently roasted and formulated coffee (Alongi & Anese, 2018; Alongi et al., 2019), lipid structures carrying lipophilic bioactive compounds and apple derivatives (Alongi et al., 2019; Alongi et al., 2018). The interventions were divided into three categories, namely formulation, processing, and diet/drugs. Functionality was in turn assessed by applying *in vitro* models, cell cultures, as well as *in vivo* trials.

Table 1 Food matrix, applied intervention, and evaluated functionality.

Food matrix	Intervention			Functionality		
	Formulation	Processing	Diet/drugs	<i>In vitro</i> model	Cell culture	Animal model
Apple juice		Untreated			Phenolic compound bioavailability	
		Thermal pasteurization		Phenolic compound bioaccessibility; α -glucosidase inhibition		
		Ultrasound pasteurization		Phenolic compound bioaccessibility; α -glucosidase inhibition		
Apple pomace			Acarbose	Interaction towards α -glucosidase inhibition		Glycaemia
	Biscuit reformulation			Glycemic index		Glycaemia
			Soft drink			Glycaemia
			Apple juice			Glycaemia

Table 1 shows the study cases relevant to apple juice and the by-product deriving from its production, *i.e.* apple pomace. The functionality evaluation focused on the ability to

reduce or prevent type 2 diabetes, which is one of the most prevalent chronic diseases worldwide. Although apples are known to negatively affect diabetes, neither the compounds responsible for the antidiabetic properties nor their mechanisms have been elucidated yet. Moreover, apples are often consumed as derivatives, such as apple juice, and technological interventions may modify food composition and thus its anti-diabetic properties. Different stabilization interventions (i.e. thermal and ultrasound pasteurization) were thus applied to apple juice and their effect towards the antidiabetic properties was assessed. In addition, apple juice production yields more than 20% by-product, namely apple pomace, which is mainly composed of dietary fiber and still contains phenolic compounds, potentially affecting type 2 diabetes. Apple pomace was therefore recovered and used as an ingredient to reduce the glycemic index of short dough biscuits while enhancing sustainability. The combination with other food matrices (i.e. soft drinks, juice), as well as with a drug commonly used to treat type 2 diabetes (i.e. acarbose) was studied to assess the effect of a dietary or pharmacological intervention on the glycemic response.

3. Materials and methods

3.1. Food matrices preparation

Apple juice was extracted from *Golden delicious* apples at 4 °C, centrifuged at 5,000 g for 5 min at 4 °C and filtered. The juice was kept refrigerated and was subjected to technological treatments within 10 min after preparation.

Apple pomace, consisting of peel, pulp, seeds, and stem, was recovered during the extraction of apple juice and dehydrated by using a vacuum oven (75 °C and 0.1 MPa). Apple pomace was milled, and the obtained flour was stored at 20 °C and 5% RH under dark conditions until use.

3.2. Technological interventions

3.2.1 Formulation

Short dough biscuits (Control) were prepared by mixing 51.6% wheat flour, 20.7% egg, 17.2% sucrose, 8.6% sunflower oil, 1.7% baking powder and 0.2% NaCl with a kneading machine and the dough was left to stand for 30 min at 4 °C. The dough was rolled out into a 2 mm layer and 50 mm diameter discs were obtained. Reformulated biscuits (R20) were produced by replacing wheat flour with 20% (w/w) of apple pomace. Samples were baked at 140 °C for 15 min, removed from the oven, cooled to room temperature and stored under vacuum in flexible poly laminate pouches in dark conditions until analysis.

3.2.2 Processing

Thermal pasteurization was carried out in a silicone oil bath and provided a sterilizing effect equivalent to 14.8 min at 90 °C, able to reduce by 2 Log *Alicyclobacillus acidoterrestris* (D90=7.4 min and $z=12$ °C) (Silva & Gibbs, 2001). Ultrasound pasteurization was carried out for 10 min using an ultrasonic transducer equipped with 22 mm probe, working at 20 kHz. Juice temperature was maintained at 65 °C by using a jacketed beaker connected with a thermostat (Saeeduddin et al., 2015). Immediately after treatments, juice samples were cooled in an ice bath and kept refrigerated until further analysis.

3.2.3 Diet or drug combination

Apple juice was combined with acarbose to test their interactive behavior towards α -glucosidase inhibition (in vitro test) and glycemic response (in vivo test). Control biscuits were tested in combination with a sugar solution (65.5 mg/mL fructose, 30.1 mg/mL glucose, 14.4 mg/mL sucrose), mimicking the composition of apple juice, hereafter called "soft drink", or with a commercial thermally pasteurized apple juice to investigate the effect of these combinations on the glycemic response.

3.3 Functionality evaluation

In vitro digestion was carried out according to Minekus et al. (2014). Phenolic compounds were analyzed by HPLC-ESI-MS as reported by Alongi et al. (2018). Bioaccessibility (BAC) was determined as the ratio between the concentration of the compound in apple juice after in vitro digestion and that in the undigested sample. Bioavailability (BAV) was assessed in a human colon adenocarcinoma (Caco-2) cell line. Digested apple juice was applied on the apical side of a Transwell insert, on which Caco-2 cells were previously cultured, while Hank's balanced salt solution (HBSS) was added to the basal chamber. After 1 h incubation at 37 °C, apical, basal and cell solutions were collected. Phenolic compounds were analyzed and BAV was expressed as the ratio between the concentration of the compound in the sample collected from the basal chamber and that in the digested sample.

The α -glucosidase inhibitory activity was determined spectrophotometrically (Alongi & Anese, 2018) and plotted against apple juice concentration. A logarithmic model was used to fit data and to compute the IC₅₀, i.e. the half maximal inhibitory concentration. The interaction between apple juice and acarbose towards α -glucosidase inhibition was investigated in vitro by computing the Combination Index (CI) (Chou & Talalay, 1984).

Control, R20 and a reference food (i.e. white bread) were analyzed for glucose content by HPLC after 20, 60, 90 and 120 min during in vitro intestinal digestion. The glycemic index was estimated as the ratio between the glucose curve of Control or R20 and that of white bread (Alongi et al., 2019).

The glycemic response was assessed in vivo on young adult male Wistar rats. They were administered with glucose, Control, R20, Control + soft drink, or Control + apple juice, respectively, after 15 hours fasting. Glycaemia was measured immediately before administration and after 15, 30, 45, 60, 90 and 120 min, respectively, and plasma glucose concentration was plotted against time.

4. Results and discussion

4.1 Apple juice: processing and drug combination study

Raw, thermally (T) and ultrasound (US) pasteurized apple juice was analyzed for the phenolic content before and after *in vitro* digestion, and BAC was computed (Table 2).

Table 2 Concentration (C) and bioaccessibility (BAC) of phenolic compounds in apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization.

Compound	Raw		T		US	
	C (mg/L)	BAC (%)	C (mg/L)	BAC (%)	C (mg/L)	BAC (%)
Chlorogenic acid	1.12±0.02	5.07±0.43	16.26±0.15	0.00±0.00	22.21±0.25	13.02±0.00
p-Cumaroylquinic acid	0.89±0.05	78.96±5.32	4.35±0.15	126.91±0.28	5.79±0.20	28.61±2.05
Hydroxycinnamic acids	2.01±0.07	37.80±2.59	20.61±0.30	26.78±0.06	28.00±0.05	16.24±0.43
Phloretin Xyloglucoside	0.27±0.02	333.03±9.13	2.30±0.02	20.90±0.26	3.19±0.09	44.17±0.46
Phloridzin	0.13±0.00	430.32±3.24	1.18±0.01	82.79±2.08	1.71±0.08	74.45±3.21
Phloretin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dihydrocalcone derivatives	0.40±0.02	365.50±13.84	3.47±0.01	68.33±1.84	4.89±0.17	54.74±0.82
Epigallocatechin Gallate	0.21±0.01	14.36±1.71	n.d.	n.d.	n.d.	n.d.
Procyanidin B2	n.d.	n.d.	0.20±0.00	0.00±0.00	0.51±0.08	0.00±0.00
(-) Epicatechin	n.d.	n.d.	3.79±0.13	0.49±0.03	2.08±0.02	0.00±0.00
(+) Catechin hydrate	n.d.	n.d.	n.d.	n.d.	0.22±0.02	0.00±0.00
Flavan-3-ols	0.21±0.01	14.36±1.71	3.99±0.13	0.47±0.03	2.81±0.08	0.00±0.00
Quercetin-3-O-galactoside	0.85±0.02	2.58±0.39	1.42±0.06	145.29±4.41	1.29±0.01	15.81±1.76
Quercetin-3-O-hexoside	0.08±0.01	654.87±18.40	0.97±0.04	29.50±0.92	0.72±0.02	19.64±0.79
Quercetin-3-O-xyloside	0.12±0.00	0.00±0.00	0.23±0.00	510.92±21.78	0.33±0.01	16.80±2.70
Quercetin-3-O-rhamnoside	1.45±0.02	29.65±3.41	1.65±0.02	8.47±0.30	1.64±0.02	62.59±0.55
Quercetin-3-O-arabinoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Others quercetin-pentoside	0.21±0.01	32.54±0.97	0.31±0.01	0.00±0.00	0.35±0.01	33.90±1.44
Flavonols	2.72±0.05	38.78±2.32	4.59±0.14	80.08±2.80	4.33±0.01	35.67±0.69
Total phenolic compounds	5.33±0.15	62.06±3.27	32.66±0.58	35.47±0.56	40.03±0.31	21.91±0.27

n.d.: not detected.

Total phenolic concentration (TPC) increased when raw apple juice was submitted to thermal pasteurization, probably due to polyphenol oxidase inactivation and

to the release of monomers and dimers upon thermal hydrolysis of heat-labile compounds. US produced a further increase in TPC. Despite a lower temperature was applied during ultrasound pasteurization, the combination of cavitation with heat could have induced tissue disruption, promoting the release of phenolic compounds from the vegetable matrix. An overall decrease in bioaccessibility was observed after *in vitro* digestion of all samples, which was more pronounced in the order Raw<T<US, indicating a higher susceptibility of phenolic compounds to digestion after exposure to technological interventions. Since TPC were more abundant in pasteurized samples as compared to the raw juice, they could be more easily available as a substrate for esterase activity, oxidative degradation, polymerization and complexation at intestinal level, resulting in a greater depletion. Besides producing an overall decrease in TPC, both processing and digestion also resulted in a prominent modification in their profile. As raw apple juice presented the highest BAC, phenolic BAV was also assessed in this sample. TPC presented a BAV of about 12%. However, only a few compounds were found in the basolateral chamber, namely *p*-cumaroylquinic acid, phloretin xyloglucoside, and quercetin-3-*O*-hexoside. It is noteworthy that, based on their concentration in the undigested sample and their BAC, these compounds were the most abundant of their category in the digested sample, suggesting that epithelial transport is concentration-driven. To this regard, it is noteworthy that phenolic compounds are mainly absorbed by paracellular diffusion (Konishi & Kobayashi, 2004). In addition, none of the flavan-3-ols was detected in the basolateral side, suggesting that their metabolism mainly occurs at gastrointestinal level. However, further investigation is required to elucidate these results. To understand if and how different pasteurization processes affected not only phenolic compound BAC and BAV but also the antidiabetic potential of apple juice, digested samples were analyzed for their ability to inhibit α -glucosidase (Figure 3).

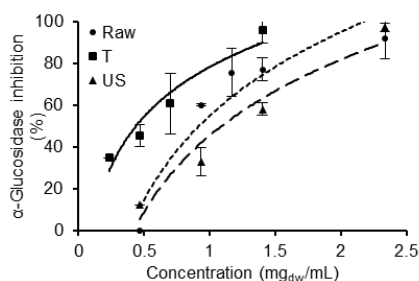


Figure 3 α -Glucosidase inhibitory ability of digested apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization, as a function of total solid concentration. Data fitting: lines, estimates; symbols, experimental data.

Digested samples inhibited α -glucosidase in a concentration-dependent manner but to a different extent. The IC_{50} accounted for 0.92, 0.44 and 1.09 mg_{dw}/mL for Raw, T and US samples, respectively. Thermal pasteurized apple juice was thus more effective than raw apple juice in inhibiting α -glucosidase upon *in vitro* digestion, while ultrasound pasteurization reduced the inhibitory capacity of apple juice. It should be noticed that the most effective sample (T) also presented the highest TPC upon digestion. However, no correlation was found between the phenolic concentration and the IC_{50} , suggesting that not only phenolic compounds but also other molecules, such as those formed upon the thermal treatment, may be involved in the antidiabetic effect of apple juice.

As apple juice presented a promising capacity to inhibit α -glucosidase even after digestion, it was combined with acarbose, to study the potential synergistic behavior paving the way for drug dosage reduction, thus limiting undesired side effects.

The *CI* showed that a synergistic effect between apple juice and acarbose occurred when the concentration in the combined system provided up to 40% α -glucosidase inhibition (Figure 4a). However, considering higher inhibition extents

(produced by higher concentrations of both apple juice and acarbose) the interaction turned to antagonistic.

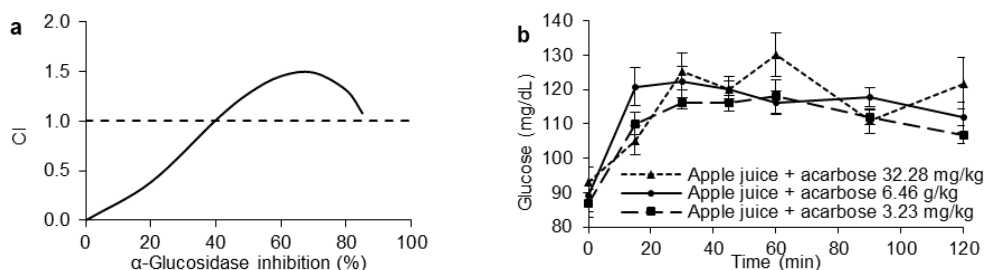


Figure 4 **a** Combination index (CI) relevant to apple juice-acarbose combined system as a function of the α -glucosidase inhibition percentage. $CI > 1$: antagonistic effect; $CI < 1$: synergic effect; $CI = 1$: additive effect. **b** Glycaemic response of apple juice combined with different dosages of acarbose.

These data were partially confirmed by *in vivo* trials. When apple juice was administered in combination with acarbose, a decrease in the glycemic response was observed as acarbose dosage was reduced (Figure 4b). Further research is thus required to identify the combination leading to a synergistic effect also *in vivo*.

4.2. Biscuit: reformulation and dietary intervention

Short dough biscuits were reformulated by using apple pomace as a functional ingredient to reduce the glycemic index. Biscuits were *in vitro* digested, and the glycemic index was estimated. Conventional biscuit (Control) presented a value of 70.4 ± 0.2 and was thus classified as high glycemic index food. Replacing flour by 20 (R20) percent significantly reduced the glycemic index to 60.8 ± 1.9 , thus ranking the product within the intermediate glycemic index foods. The glycemic response was also tested *in vivo* (Figure 5). The reformulation was confirmed to positively affect the glycemic response, as the glycemic peak was shifted at 60 min and the AUC slightly decreased, accounting for $13,997 \pm 138$, as compared to the Control one ($14,232 \pm 429$).

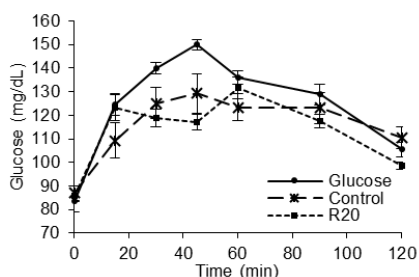


Figure 5 Glycaemic response of Control and R20 biscuits, as compared to glucose.

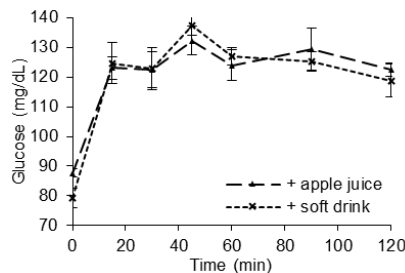


Figure 6 Glycaemic response of the conventional biscuit consumed combined with a soft drink or with apple juice.

To understand whether the glycemic response of biscuits could be modulated not only by reformulation with apple pomace but also through the concomitant consumption of apple juice, due to its antidiabetic potential, Control biscuits were administered together with apple juice or with a soft drink mimicking apple juice sugar composition. As shown

in Figure 6, different glycemic responses were observed. The soft drink induced a higher peak as compared to apple juice, which in turn produced a lower and flatter glycemic curve.

5. Conclusions and Future Perspectives

The approach followed in this Ph.D. thesis presented some innovative features as compared to the current literature relevant to the study of functional foods, in particular:

- the research focused on the whole matrix instead of on purified bioactive compounds;
- *in vitro* digestion was used to assess not only bioaccessibility but also bioactivity;
- the interaction between foods and drugs was studied to identify potential synergies;
- results acquired by *in vitro* experiments were compared to data deriving from *in vivo* trials.

Overall, acquired results highlighted how food functionality, and in particular the antidiabetic properties of some food matrices, is dramatically affected by food matrix, by the technological interventions, intended as formulation and processing, as well as by co-ingested components and by the digestion process. Still, the molecules and the mechanisms underlying this functionality are partially unrevealed. This knowledge would provide a tool to drive functionality by targeted interventions, based on a holistic approach. The latter requires a teamwork encompassing all the actors involved in the functional food development cycle.

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Poultry Meat Quality as Affected by Growth-Related Muscular Abnormalities and Early *Post-Mortem* Metabolism

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This PhD thesis project is divided into two principal research lines. The first aims at carrying out a comprehensive investigation by evaluating the effect exerted by the three emerging poultry growth-related abnormalities (i.e., White Striping, Wooden Breast and Spaghetti Meat) on early *post-mortem* carbohydrate metabolism and poultry meat quality. The second research line involves the study of *post-mortem* acidification pattern in chicken muscles characterized by a different *in vivo* energy metabolism, in order to evaluate the existing intra-specie variations and possible implications on meat quality.

Metabolismo *post-mortem* e anomalie muscolari delle carni avicole

Il progetto di tesi di dottorato è suddiviso in due linee di ricerca principali. La prima riguarda la caratterizzazione e la valutazione dell'effetto esercitato dalle principali anomalie che attualmente colpiscono le carni avicole (White Striping, Wooden Breast and Spaghetti Meat) sul metabolismo energetico *post mortem* e sulla qualità della carne di pollo e tacchino. La seconda linea di ricerca riguarda invece lo studio del metabolismo *post-mortem* di muscoli di pollo caratterizzati da un diverso metabolismo energetico *in vivo*, al fine di valutare le eventuali variazioni intra-specie esistenti durante l'acidificazione *post-mortem* e le possibili implicazioni sulla qualità della carne.

Key words: Poultry meat; meat quality; muscular abnormalities; *post-mortem* metabolism.

1. Introduction

In accordance with the PhD thesis project, this oral communication reports the main results of the following activities aimed at evaluating:

A1) implications of the three main growth-related muscular abnormalities (white striping, wooden breast and spaghetti meat) on both chicken and turkey meat quality;

A2) acidification patterns of different chicken muscles characterized by a different *in vivo* energy metabolism;

A3) *post-mortem* metabolism and glycolytic metabolites of wooden breast affected chicken muscles.

2. Material and Methods

2.1 Sampling

Muscle samples were obtained from birds farmed and harvested using standard commercial procedures. Carcasses and individual muscles were directly selected from the line of commercial processing plants based on experimental design adopted for each trial as reported in Table 1.

Table 1 Experimental groups, type of samples and post-mortem sampling times adopted in the experiments conducted for each activity during PhD thesis project.

Activity	Experiment	Experimental groups	Samples	Post-mortem sampling time
A1	Effect of White Striping (WS) on turkey breast meat quality	Control Moderate WS Severe WS	Turkey breast (n= 12/group)	24h
	Comparison of quality traits of chicken breast meat affected by WS, Wooden Breast (WB) and Spaghetti Meat (SM) abnormalities	Control WS WB SM	Chicken breast (n= 12/group)	24h
A2	Post-mortem acidification patterns in chicken breast and leg muscles	<i>Pectoralis major</i> <i>Ext. iliotibialis lateralis</i> <i>Peroneus longus</i>	Chicken breast and leg (n= 12/group)	15 min, 2,4,8, 24h
A3	Post-mortem metabolism of broiler <i>P. major</i> muscle as affected by WB myopathy	Control WB	Chicken breast (n= 12/group)	15 min and 24h

2.2 Meat quality

The qualitative characteristics of poultry meat samples have been assessed through the evaluation of meat ultimate pH, color, tenderness and both functional and structural properties. Ultimate pH was assessed by homogenizing 2.5g of meat in a sodium iodoacetate solution (Jeacocke, 1977), while meat color measurement was performed on the bone-side surface of breast fillets using a Chroma Meter CR-400 (Minolta Corp., Milan, Italy). Tenderness was assessed through a 40% compression force test achieved with a TA-TX2i Texture Analyser (Stable Micro Systems Ltd., Godalming, Surrey, UK) as previously described by Soglia et al. (2017). Advanced techniques such as Time Domain nuclear magnetic resonance (TD-NMR) and differential scanning calorimetry (DSC) were applied to investigate meat functional and structural properties, respectively.

2.3 Chemical composition and intramuscular collagen analysis

Proximate composition (moisture, protein, fat and ash) was determined following standard methods (Folch et al., 1957; Townshend, 2003), while intramuscular collagen analysis as well as its cross-link concentration were evaluated through HPLC as reported by Woessner (1961). Moreover, ¹H-NMR was also applied to evaluate the concentrations of meat histidine-containing compounds.

2.4 In vitro post-mortem acidification and glycolytic metabolites

Glycolytic metabolites (i.e. glucose, glucose-6-phosphate, glycogen, lactate and phosphofructokinase) were determined using enzymatic methods as described by Hammelman et al. (2003), while *post-mortem* acidification was studied through an in vitro glycolytic model as proposed by England et al. (2014).

2.5 Histological evaluations

Chicken breast's sarcomere length was assessed using a microscope equipped with an oil-immersion 100x objective by the calculation of the ratio between the total length of the myofibril and the number of sarcomeres on longitudinal sections previously stained with haematoxylin and eosin. Fiber typing was also evaluated on serial cross-sections stained for α -GPD and NADH-TR activity.

3. Results and Discussion

3.1 Implications of growth-related muscular abnormalities on poultry meat quality

3.1.1 Effect of White Striping on turkey breast meat quality

White Striping (WS) is a widespread muscular abnormality frequently observed in both chicken and turkey breast muscle and characterized by the presence of white striations parallel to the direction of muscle fibers (Soglia et al., 2018). While there are several studies reporting the effect of WS on broiler meat, there is a lack of information regarding its impact on turkey breast meat quality. The results obtained from this study showed that turkey *P. major* muscles affected by WS displayed 20% higher weight compared to unaffected ones. However, the presence of WS just slightly affected meat composition and qualitative traits, in contrast to what previously observed in broilers. Therefore, it is reasonable to hypothesize a specie-specific physiological response to the pressure in muscle tissue induced by the genetic selection.

3.1.2 Comparison of quality traits of chicken breast meat affected by White Striping, Wooden Breast and Spaghetti Meat abnormalities

Poultry industry has been facing up the occurrence of WS, WB and SM abnormalities, whose incidence has reached alarming levels. However, no investigations have been carried out to simultaneously examining these myopathies. Therefore, this study aimed at evaluating the impact of WS, WB and SM abnormalities on the main breast meat qualitative characteristics, chemical composition and functional properties throughout traditional and innovative techniques that allowed the survey of unexplored aspects such as non-reducible cross-links of muscle collagen and thermal properties of affected meat. The main results concerning chemical composition, tenderness and protein denaturation enthalpy are reported in Table 2. Results showed that the manifestation of WB and SM is associated with a significant increase in moisture content to the detriment of proteins, especially when the superficial section is considered. Regardless the sampling position, the 40% compression test showed profound variations in relation to the presence of WB condition ($P < 0.001$).

Table 2 Effect of muscular abnormalities on moisture and protein content, compression force, intramuscular collagen (IMC) and stromal protein denaturation enthalpy (SPDE) assessed on both superficial (S) and deep (D) sections of *P. major* muscle. ^{a-d} means within the same parameter, considering both the abnormality (same column) and sampling position (same row), followed by different superscript letters are significantly different. ***= $P < 0.001$; *= $P < 0.05$

	Moisture (%)		Protein (%)		Compression force (kg)		IMC (μg/mg)		SPDE (ΔH, %)	
	S	D	S	D	S	D	S	D	S	D
N	75.0 ^d	74.9 ^d	22.9 ^a	23.0 ^a	2.95 ^{bc}	2.01 ^c	14.7 ^b	13.8 ^b	16.4	19.0 ^b
WS	75.3 ^{cd}	75.1 ^{cd}	22.0 ^{abc}	22.5 ^a	3.53 ^{ab}	2.88 ^{bc}	14.0 ^b	13.4 ^b	16.9 ^b	18.2 ^b
WB	77.1 ^a	75.8 ^c	20.5 ^d	21.9 ^{abc}	4.45 ^a	3.41 ^{ab}	18.2 ^a	13.3 ^b	27.0 ^a	23.3 ^{ab}
SM	76.7 ^{ab}	75.9 ^{bc}	21.0 ^d	21.6 ^{bcd}	3.55 ^{ab}	2.92 ^{bc}	13.8 ^b	12.9 ^b	16.3 ^b	19.8 ^{ab}
sem	0.10		0.13		0.11		0.14		0.15	
Abnormality (A) Position (P)	<i>P</i> -value		<i>P</i> -value		<i>P</i> -value		<i>P</i> -value		<i>P</i> -value	
	***		***		*		***		***	
	***		***		ns		***		ns	

Indeed, WB superficial samples showed remarkably higher IMC content ($P < 0.001$) and, accordingly, the differential calorimetric analysis identified a significant increase in

stromal proteins denaturation enthalpy ($P < 0.001$), probably due to the collagen accumulation which is a distinctive trait of WB myopathy. However, WB affected muscle didn't exhibit higher collagen cross-linking.

Overall, results obtained from this study showed that the manifestation of myopathies mainly impacts the superficial layer of *P. major* muscle, while the deep section is just poorly affected. Moreover, the occurrence of WB seems to exert a more prominent damage on meat quality rather than the mere presence of WS and SM.

3.2 Post-mortem acidification pattern in chicken breast and leg muscles

The aim of the study was evaluating the acidification kinetics of three broiler chicken muscles, characterized by a different *in vivo* energy metabolism (breast: *Pectoralis major*; drumstick: *Peroneus longus*; thigh: *Extensor iliobtibialis lateralis*). Histological evaluation showed that selected breast and drumstick muscles are mainly composed of glycolytic and oxidative fibers, respectively, while thigh muscle presents intermediate fiber composition. The extent of acidification of the pectoral muscle was remarkably higher if compared to drumstick ($\text{pH}_u = 5.79$ vs. 6.18, respectively; $P < 0.001$) (Fig. 1), while thigh muscle presented intermediate pH_u values (5.99).

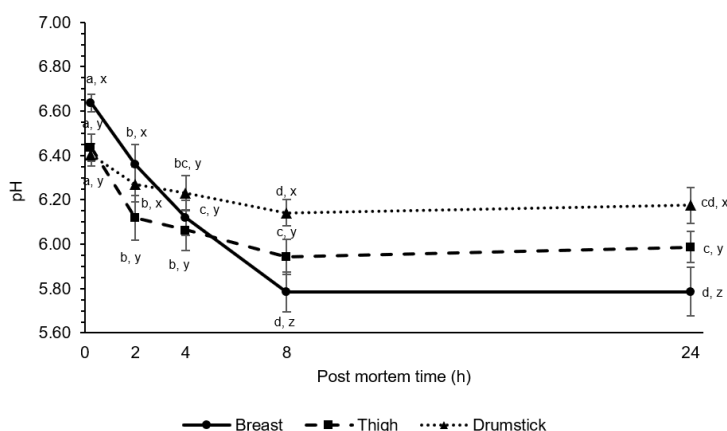


Figure 1 Average pH values in broiler breast, thigh and drumstick muscles during post-mortem time. ^{a-d} mean values followed by different letters significantly differ among the sampling times within the same muscle ($P < 0.05$); ^{x-z} mean values followed by different letters significantly differ among the muscles within the same sampling time ($P < 0.05$).

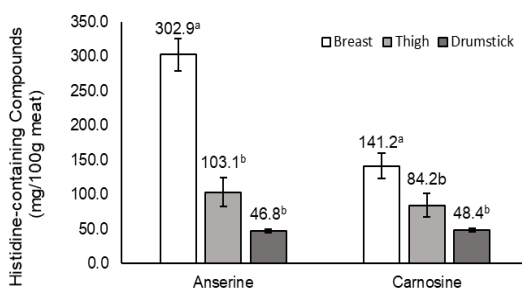


Figure 2 Concentration of anserine and carnosine (mg/100g meat) in broiler breast, thigh and drumstick muscles. ^{a-b} means within the same parameter followed by different superscript letters are significantly different ($P < 0.05$).

Moreover, breast muscle, if compared to drumstick, presented a considerably higher concentration of histidine-containing compounds (i.e. anserine and carnosine) ($P < 0.05$; Fig. 2).

Anserine and carnosine are characterized by a great buffering capacity and act as a buffer in the muscle, in order to mitigate an excessive acidification *in vivo*. Indeed, *P. major* muscle, in virtue of its marked glycolytic metabolism, should have exhibited a lower pH_u . Furthermore, chicken breast, if compared to meat cuts of other species with a similar fiber composition (i.e. turkey breast, rabbit loin), exhibited higher ultimate pH values, therefore suggesting that the remarkable content of histidine compounds could justify the differences in ultimate pH found intra- and inter-species.

3.3 Post-mortem metabolism of broiler *P. major* muscle as affected by wooden breast myopathy

Although one of the main hallmarks of WB abnormality is the elevated pH_u of meat, no studies on *post-mortem* acidification of WB affected muscles have been conducted so far. Thus, this study aimed at evaluating the effect of this myopathy on muscle *post-mortem* metabolism and glycolytic metabolites. As expected, WB samples showed a significantly higher ($P < 0.001$) pH_u , while no differences were detected at 15 minutes *post-mortem*. Results concerning glycolytic metabolites are shown in Fig. 3. Accordingly, patterns of lactate formation followed pH decline and resulted to be significantly lower in WB muscle at 24h *post-mortem* ($P < 0.001$). Residual glycogen found in WB samples at 24h *post-mortem*, along with unaltered phosphofructokinase activity, suggested that neither glycogen nor a deficiency of phosphofructokinase activity were responsible for arresting glycolysis prematurely. Thus, in order to test if a deficiency of muscle enzymes could be the reasons for the higher pH_u usually found in affected samples, an *in vitro* system was used to simulate muscle acidification with or without excess ATPase. Results showed that excess ATPase

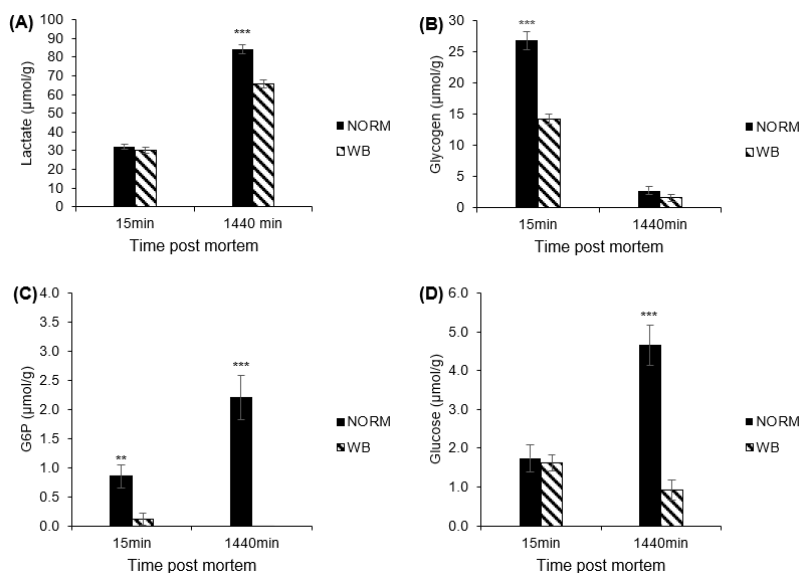


Figure 3 Average lactate (A, $\mu\text{mol/g}$), glycogen (B, $\mu\text{mol/g}$), G6P (C, $\mu\text{mol/g}$) and glucose (D, $\mu\text{mol/g}$) of NORM and WB broiler *P. major* muscle at 15 min and 24h *post-mortem*. Data represent means $\pm$ SEM. Asterisks indicate significant difference within the time point (***= $P < 0.001$; **= $P < 0.01$).

can extend *post-mortem* glycolysis in WB meat. Indeed, WB+ATPase samples reached an ultimate pH of 5.64, significantly lower if compared to WB samples without ATPase (5.80) ($P<0.05$) (Fig. 4).

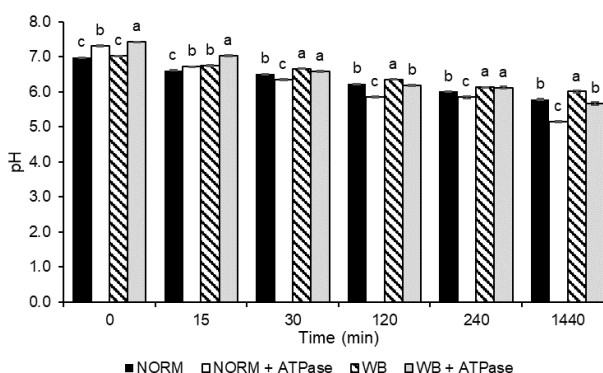


Figure 4 Mean pH of the *in vitro* model. Data represent means $\pm$ SEM. a, b, c, d means without a common letter significantly differ within a time point ($P<0.05$).

Thus, given the overall impaired muscle structure and the myodegenerative and necrotic processes associated with WB condition, the higher pH_u of WB meat might be the outcome of a drastically reduced glycolytic potential combined with a deficiency and/or a dysfunction of muscle ATPases.

Moreover, since the rate and the extent of *post-mortem* muscular acidification influence the degree of myofibrillar contraction, it was assessed whether the extreme stiffness of WB pectoral muscles could be partially due to a hypercontraction of sarcomeres caused by an abnormal *post-mortem* muscle acidification. In this regard, sarcomere length was assessed on both affected and unaffected samples. Unexpectedly, sarcomere length of WB muscles was significantly higher compared to the NORM group (1.87 vs 1.66 μ m; $P<0.001$). Therefore, the hardness of breast affected by WB does not seem to be related to the contractile status of the myofibrils, but rather to the considerable deposition of collagen, as also found in A1 research project.

4. Conclusions and Future Perspectives

In recent years, the artificial selection for fast-growing and high-breast-yield hybrids in meat-type chickens and turkeys considerably marked up the pressure on breast muscle development, leading to the appearance of a new group of muscular abnormalities that represent a growing concern for the scientific community. Growth-related breast meat abnormalities negatively impact both visual aspect and technological properties of raw and processed meat, causing relevant economic damages for the poultry industry (Kuttappan et al., 2017).

The studies carried out during the present PhD project aimed at deepening the current knowledge about muscular abnormalities by investigating unexplored aspects through traditional and innovative techniques. The results obtained permitted to highlight the peculiar effect of each muscular abnormality on meat quality and provided information that might be useful in managing abnormal meat for further processing. Moreover, the study of *post-mortem* metabolism of WB muscles allowed to investigate the mechanism underlying the biochemistry controlling *post-mortem* acidification of muscle affected by muscular abnormalities.

However, the causative mechanisms responsible for the occurrence of these abnormalities are still uncertain and further investigation aimed at inhibiting the onset of myopathies or at least alleviating the symptoms and consequences on meat quality should be carried out.

Furthermore, the present PhD thesis permitted to examine the intra-specie variations existing during *post-mortem* acidification and sharpen the current knowledge by defining the role of buffering compounds (i.e. anserine and carnosine) in defining ultimate pH of muscle characterized by different *in vivo* energy metabolism.

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Circular Economy Strategies for Management and Valorisation of Some By-Products of the Wine Industry

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This Ph.D. project focused on the development of solutions for the valorisation of some minor by-products of the wine industry, such as grape stalks and distillation scrap fractions. Grape stalks are made of lignocellulose biomass and represent a source of sugars, phenolic compounds, and fibre. In addition, a possible use as filler in bio-plastic formation was explored. Distillation fractions contain high concentrations of ethanol and methanol and their application in the fuel cell technology for the production of electricity was investigated.

Strategie di economia circolare per la valorizzazione di alcuni sottoprodotti dell'industria del vino

Questo progetto di dottorato si è focalizzato sullo sviluppo di soluzioni per la valorizzazione di alcuni sottoprodotti dell'industria enologica, ancora poco considerati, come raspi d'uva e residui di distillazione. I raspi d'uva, sono costituiti da biomassa lignocellulosica e rappresentano una fonte di zuccheri, composti fenolici e fibre. Inoltre, è stato esplorato un possibile utilizzo come filler nella formazione di bioplastiche. Le frazioni di scarto della distillazione contengono elevate concentrazioni di etanolo e metanolo ed è stata studiata una loro applicazione nella tecnologia delle celle a combustibile per la produzione di elettricità.

Key words: circular economy; by-products; grape stalks; distillation fractions; bio-plastic filler; fuel cells

1. Introduction

Among the activities of the food industry, winemaking process generates a great variety of by-products that can be used in many fields (Lucarini et al., 2018). Grape seeds and pomace represent the portion of winemaking leftovers with the highest concentration of bioactive compounds. Apart from oil, grape seeds constitute the part of the berry with the highest antioxidant activity, followed by the peels and the pulp (Pastrana Bonilla et al., 2003). Therefore, grape seeds can be used for the extraction of bioactive compounds, which can be conveniently used for cosmetic, nutraceutical, and pharmaceutical purposes. However, some leftovers of the wine industry, such as grape stalks, are not very considered. On the contrary, they could be an important source of lignocellulosic biopolymers. In fact, lignocellulosic biomass is a renewable and complex raw material composed of cellulose, hemi-cellulose and lignin (Brandt et al., 2013). Cellulose is composed of glucose units linked together by β -1,4-glycosidic bonds. Hemi-cellulose is a complex polymer consisting of pentose sugars, such as xylose and arabinose; hexoses such as glucose, mannose, galactose; and sugar acids (glucuronic and galacturonic acids). Finally, lignin is formed by units of phenylpropanoids bound together by ether and carbon-carbon linkages. Lignin is covalently linked to the hemi-cellulose through ferulic acid units (Brandt et al., 2013; Vanholme et al., 2010). In this complex structure, lignin and hemi-cellulose cover the surface of cellulose, thus making it difficult to separate and isolate.

Distillation allows the concentration of ethanol up to about 95%. Higher concentrations require more complex techniques such as the use of entrainer or the use of water-combining substances. Distillation plants are composed by many fractionation columns where wine is purified from other volatile congeners. These substances are concentrated in a minimum volume of ethanol and contain, in turn, methanol, fusel oils, esters, etc.

As these compounds derive from an alcoholic fermentation of sugar-containing juices generally obtained from food grade fruits, these substances are natural and they can be valuable ingredients for food flavouring. Moreover, methanol, residual ethanol, and other fusel oils can be conveniently used in fuel cells. The fuel cells are electrochemical energy converters, in which a fuel and an oxidant agent react for the conversion of chemical energy into electrical energy (Bahramietet al., 2013). In the direct methanol fuel cells, methanol and other alcohols are oxidised in the anode to release an electron flow (Li et al., 2013).

For these reasons, the partition of the different volatile molecules in an industrial plant was carried out.

1.1. Pre-treatment of lignocellulosic biomass

Since lignocellulosic biomass is a very complex material, a fractionation of these components and the breaking of their interactions are necessary. The different pre-treatment methods allow breaking down the lignocellulosic biomass into its fundamental units and increasing the accessibility for the subsequent treatments on these biopolymers (Lee et al., 2014). The pre-treatment methods can be divided into the following categories:

- physical pre-treatment methods (milling, sieving, use of microwaves, heating, steam explosion, cavitation);
- chemical and bio-chemical pre-treatments (acids, bases and enzymes).

2. Materials and methods

2.1. Grape stalk samples preparation

Grape stalks were obtained from some wine companies. They were first cleaned to remove gross residues and then dried at 65°C for 24h. This slow drying process was carried out to remove water and limit degradation reactions. Subsequently, dried grape stalks were subjected to milling to obtain a fine powder. The powder was sieved in order to separate the finest fractions from the coarsest ones. For this purpose, a set of stacked sieves with the following meshes were used: 850 µm, 500 µm, 212 µm, and 63 µm. Only the finest fractions ($\leq 63 \mu\text{m}$; $63 \geq 212 \mu\text{m}$) were used for subsequent treatments and analyses.

2.1.1. Application of the enzymes to the lignocellulosic biomass

An enzymatic approach (laccase, hemicellulase, and cellulase) was carried out to hydrolyse and isolate phenolic compounds using two different times (24 and 48 h) and different units of enzymatic activity (U). The release of phenolic compounds was monitored with UV-vis spectrophotometry at 280 nm.

2.1.2. Extraction tests on grape stalks powder

Grape stalk powder with size between 63 and 212 µm was used for lignocellulosic fractionation process. Samples of grape stalks powder were first treated with deionised water at 100 °C. At the end of each extraction step, the supernatants were recovered and

pooled together in a separate vessel, while the dried solid residues were subsequently extracted with sulphuric acid (2%) at 121°C. The acidic supernatants were separated and pooled together, as well. The yield of the solid residue was calculated for both the extractions. The supernatants obtained from the aqueous and acidic extractions were analysed to determine sugars and furanic compounds with HPLC and total phenolic compounds.

2.1.3. Extrusion process

The suitability of fraction with a size between 63 and 212 μm as reinforcements and fillers for polyesters was studied. Two kinds of derivatization reactions were carried out on the grape stalk powder in order to evaluate the effects on the mechanical properties of the different extruded materials. In particular, an acetylation and a silylation by refluxing with acetic anhydride and 3-methacryloxypropyltrimethoxysilane, respectively, were performed. Each grape stalk sample, including the non-derivatized grape stalk powder, was subjected to extrusion process with a polyhydroxyalkanoate-type polymer.

2.2. GC-MS analysis of the distillation fractions

The distillation fractions were classified as follows: distillation heads and tails deriving from lees, distillation heads and tails deriving from grape pomace, fraction from demethylation column, fraction from epuration column, and fractions containing fusel oils.

The distillation fractions were analysed by gas-chromatography coupled to mass spectrometry (internal standard, 2-octanol). Identification was carried out by comparing the retention times and the qualifying ions of all the available pure standards. Quantification was carried out by measuring the relative peak area of the quantifying ion in relation to that of the internal standard.

3. Results and discussion

3.1. Experiments on grape stalks

The figures 1A and 1B show the absorbance as a function of the bio-catalytic activity during 24 h and 48 h experiments. For all the samples, the absorbance at 280 nm increase during higher units of laccase activity as a consequence of phenolic free moieties. In the 24 h experiments (1A), the enzymatic systems that provided the highest absorbance values and hence a higher release of phenolic compounds were: the combination of 100 U of laccase and 200 U cellulase, and the combination of 100 U laccase, 200 U hemicellulase, and 100 U cellulase.

As for 48 h experiments (1B), a higher effect in comparison with the previous experiment was observed for the binary system 100 U laccase and 200 U cellulase. However, the LC-MS analyses of the extracts did not show the presence of the most common phenolic compounds. Due to the structural complexity of lignocellulose, it was hypothesized that solely oligomeric structures were released from the original matrix.

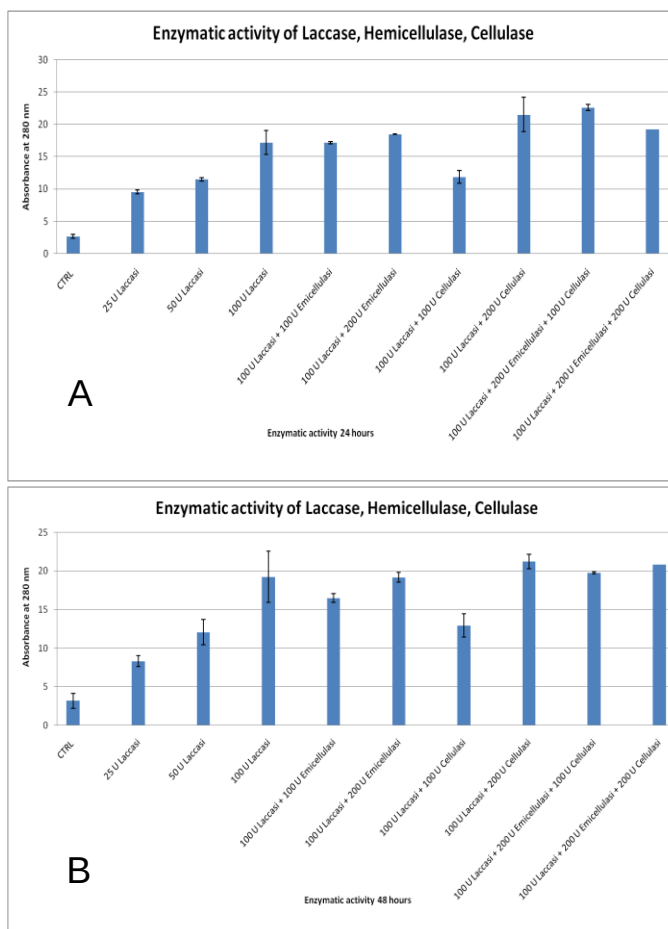


Figure 1 The absorbance as a function of enzymatic activity in 24 h(A) and 48 h(B).

The samples obtained from the aqueous extraction showed the presence of monosaccharides, such as glucose and fructose, while in the samples obtained from the acid extraction glucose and xylose were found in quite abundant amount. Xylose is one the product of the hydrolysis of the hemicelluloses, which are sensitive to acid hydrolysis. In the latter samples, some furanic compounds were detected, such as 5-hydroxymethyl-2-furfural and furfural. They are artefacts originating during the extraction process through heating in an acid medium. However, their formation could be explored with a pyrolysis process in order to obtain higher amount of these compounds that could be used as chemical building blocks.

The acetylated grape stalks were assessed with Fourier Transform Infrared Spectroscopy in comparison with the control material (not subjected to acetylation reaction). Figure 2A and 2B shows the FT-IR spectra of these samples:

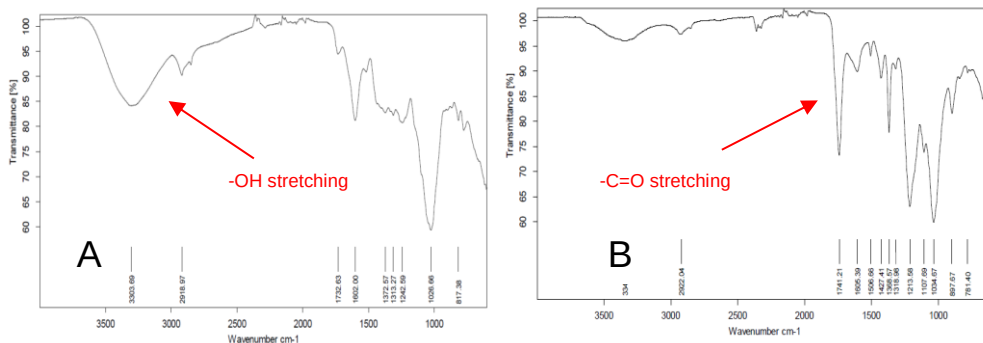


Figure 2 FT-IR spectra of raw(A) and acetylated (B) materials.

In the FT-IR spectrum of acetylated material (B) there is the disappearance of the absorption band around 3343 cm^{-1} relative to the stretching frequency of the hydroxyl group. To confirm the acetylation, we have the absorption band relative to the stretching frequency of the carbonyl group at about 1741 cm^{-1} .

3.2. Experiments on distillation leftovers

The results of the GC-MS analyses on the distillation fractions are shown in Table 1, together with the alcohol by volume of each sample. Heads and tails deriving from grape pomace (HT G) showed very high concentration of methanol (around 38 g/L) with an alcohol by volume around 75%. However, epuration waste (EW), demethylation waste (DW), and lees head and tails (HT L) had high values of alcohol by volume, as well. All these samples showed promising characteristics for fuel cell applications.

In the upper lipophilic phase of the fusel oil fraction, high concentrations of medium-chain esters and alcohols were observed. These volatile compounds can find interesting applications in flavour industries.

Table 1 Results of the GC-MS analyses on the distillation fractions. HT G: grape pomace heads and tails; DW: demethylation waste; EW: epuration waste; HT L: lees head and tails; n.d.: not detected.

	HT G	DW	EW	HT L	Fusel oils (upper phase)	Fusel oils (lower phase)
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Acetone	82.58	n.d.	16.57	12.45	14.26	19.16
Ethyl acetate	7898.66	47.33	5908.86	7272.11	4070.55	1385.34
Methanol	38253.91	8337.42	518.3	4526.69	2025.27	2960.14
Isobutyl acetate	31.08	n.d.	55.69	n.d.	99.63	7.32
2-Butanol	8116.08	52.377	19208.34	16005.11	26623.34	10434.73
1-Propanol	3887.05	n.d.	3559.8	6226.87	13420.95	5673.66
Ethylisobutyrate	31.04	4.796	13.83	14.7	302.58	22.45
Ethylvalerate	137.79	n.d.	71.23	85.74	4020.03	105.89
Isobutylalcohol	1078.45	1.616	n.d.	998.42	11264.78	2509.66
Isoamyl acetate	37.29	n.d.	260.85	69.97	1312.35	33.08
1-Butanol	26.5	n.d.	48.5	182.34	3340.56	1025.47
1-Pentanol	n.d.	n.d.	4.62	n.d.	1604.33	228.58
Isoamylalcohol	149.4	8.438	995.35	735.26	73176.77	14229.01
Ethylhexanoate	12.55	n.d.	107.85	63.62	4311.22	50.59
Hexyl acetate	n.d.	n.d.	3.03	n.d.	150.92	n.d.
Ethylactate	n.d.	n.d.	19.98	n.d.	6078.56	3626.55
1-Hexanol	0.47	0.57	40.68	0.6	11828.5	1083.4
Ethyl octanoate	n.d.	1.068	246.62	3.11	15338.23	263.81
Acetic acid	927.67	n.d.	79.56	36.77	722	525.6
Acetoin	1058.26	n.d.	n.d.	n.d.	n.d.	228.47
Butanoic acid	46.57	n.d.	8.28	n.d.	4064.72	1038.47
Furfurylalcohol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyldecanoate	n.d.	n.d.	295.11	n.d.	36684.82	541.43
Phenethylalcohol	n.d.	n.d.	1.41	n.d.	196.99	27.18
Benzylalcohol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl dodecanoate	n.d.	n.d.	84.386	n.d.	18191	197.64
Alcohol by Volume (%)	74.6	73.76	82.01	73.76	25.13	66.11

4. Conclusions and future perspectives

This investigation has been very useful for studying and evaluating circular economy methods applicable to the wine industry. Matrices with little value such as grape stalks and distillation leftovers have been evaluated for products with high added value. Some experimental tests are still in progress to obtain from the grape stalks reinforcing matrices for bio-composite materials and to test the potential applications of distillation heads and tails fractions in the fuel cell technology. Furthermore, the possibility of improving the enzymatic approach applied to grape stalks is being evaluated in order to fractionate the lignocellulosic biomass and depolymerize lignin.

This Ph.D. project could therefore expand the circular economy chain applied to the wine industry, giving a strong value to the part concerning biomaterials and to the energy sector for fuel cells.

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Functional Exploitation of the Metabolism of Lactic Acid Bacteria during Fermentation of Plant Matrices

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Fermentations may cause significant changes to the health-promoting features of plant foods. The lactic acid fermentation is the most widespread. This project aimed to exploit species or strain-specific metabolic features of selected lactic acid bacteria to increase the content of bioactive compounds and/or decrease the amount of antinutritional factors in plant matrices. More in depth investigations were addressed towards fructophilic lactic acid bacteria (FLAB). FLAB are a special group of lactic acid bacteria with specific nutritional and environmental requirements. In light of their peculiar metabolic traits, FLAB were successfully exploited in food applications to enhance the functional features of common purslane (*Portulaca oleracea* L.), to improve the nutritional value of bee-collected pollen, and to produce bread with low FODMAPs content.

Valorizzazione Funzionale del Metabolismo dei Batteri Lattici Durante la Fermentazione di Matrici Vegetali

I processi fermentativi sono in grado di influenzare significativamente le proprietà funzionali delle matrici vegetali. L'impiego della fermentazione lattica è uno degli approcci biotecnologici più seguiti. Questo progetto ha la finalità di valorizzare le caratteristiche metaboliche specie- o ceppo-specifiche di batteri lattici selezionati per incrementare il livello di composti bioattivi o ridurre il contenuto di fattori antinutrizionali nelle matrici vegetali. In particolare sono state studiate le potenzialità dei batteri lattici fruttofilici (FLAB). I FLAB sono un gruppo di batteri lattici con particolari requisiti nutrizionali. Grazie ai loro peculiari tratti metabolici, i FLAB sono stati impiegati con successo per migliorare le caratteristiche funzionali della *Portulaca oleracea* L., per migliorare il valore nutrizionale del polline raccolto dalle api e per produrre pane di frumento con ridotto contenuto in FODMAPs.

Key words: fermentation; plant matrices; bioactive compounds; functional food; fructophilic lactic acid bacteria

1. Introduction

In accordance with the PhD thesis project previously described (Cantatore, 2017), this oral communication reports the main results of the following three activities directed to:

A1) Exploitation of fermented *P. oleracea* juice as novel functional beverages;

A2) Set up of a novel solid-state fermentation protocol suitable for a scaled production of shelf-stable bee-collected pollen;

A3) Metabolic characterization and functional exploitation of FLAB in order to select FLAB strains capable of fully degrading FODMAPs in plant-derived products.

In a first experimental part, lactic acid fermentation was investigated as a tool to enhance the functional features of *P. oleracea* juice, a suitable raw substrate for novel functional foods, due to the inherent high level of nutrients and biologically active compounds (Filannino et al., 2017). A diversified pool of lactic acid bacteria species was

used, providing an exhaustive framework of potential biotechnological applications. In particular, novel functional attitudes of the fructophilic *L. kunkeei* strain were highlighted. Therefore, the subsequent experimental activity focused on the biotechnological exploitation of fructophilic lactic acid bacteria (FLAB). FLAB are found in fructose-rich habitats associated with flowers, fruits, fermented foods, and the gastrointestinal tract of several insects, and are gaining increasing interest as a newly discovered bacterial group. This interest relies on two main features of fructophilic lifestyle: (i) aptitude to colonize unusual niches, and (ii) capability to metabolize fructose preferentially. Recently, there has been an increasing demand for bee pollen and bee bread as dietary supplement for human consumption (Kieliszek et al. 2018). Nevertheless, marketing of bee bread is rather stunted, since its collection from the hive is very labor intensive and detrimental to the hive. To overcome these limitations, alternatives for pollen processing were considered to obtain fermented pollen comparable to bee bread. Thus, the use of autochthonous acid resistant and osmotolerant FLAB to ferment bee-collected pollen was investigated. Fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) have received increasing attention in the dietary management of irritable bowel syndrome (IBS), a common intestinal disorder causing abdominal pain, bloating, gas, diarrhea, constipation, and altered gut microbiota. Low FODMAPs diet is the most efficient dietary therapy for IBS (Halmos et al. 2014), aiming at excluding or reducing consumption of fruits and vegetables particularly rich in FODMAPs. Fermentation is a sustainable and valuable biotechnology for decreasing the FODMAPs content below cutoff values (0.3 g per 100 g of sample) in fruit-, vegetable-, and cereal-based products, which currently are classified as high FODMAPs. Thus, a novel biotechnological process, based on a consortium of targeted commercial enzymes and FLAB, was developed to reduce FODMAPs levels in wheat-derived products.

2. Lactic Acid Fermentation Enriches the Profile of Biogenic Compounds and Enhances the Functional Features of Common Purslane (*Portulaca oleracea* L.)

2.1 Materials and methods

Eight strains of lactic acid bacteria (*Lactobacillus plantarum* POM1, *L. plantarum* EnFIII3, *Lactobacillus brevis* POM4, *Lactobacillus rossiae* 2MR8, *Pediococcus pentosaceus* CILSWE5, *Leuconostoc mesenteroides* OP9, and *L. kunkeei* B7), previously isolated from plant matrices and identified, were used as starter in *P. oleracea* purea (POP) fermentation. Strains growth rate was evaluated through plate count determination on MRS agar (Oxoid); pH was measured with Foodtrode electrode. Kinetics data were modelled through Gomperts equation, modified as by Zwietering et al. (1990). Based on kinetics data, a fermentative bio-technological protocol was set up. Samples were singly inoculated (7 Log UFC/g) and incubated at 30°C for 36 h. Samples were taken before (raw POP) and after fermentation. POP, without bacterial inoculum and chemically acidified with lactic acid (final pH of ca. 4.0), was incubated under the same conditions and used as control (CA-POP). Lactic acid bacteria, enterobacteria and yeast cell density were counted through plate count (Filannino et al., 2017). Total titratable acidity (TTA) was determined on 10 g of POP homogenized with 90 mL of distilled water and expressed as the amount (mL) of 0.1M NaOH to achieve a pH of 8.3. The pH was measured as previously described. Moisture content was measured by a moisture analyzer (Sartorius MA35M, Sartorius AG, Germany). Sugars and organic acids concentration were detected as described by Di Cagno et al. (2017), through HPLC. Free aminoacids determination was performed through a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England) as described by Di Cagno et al. (2017). Volatile compounds were analyzed as described by Coda et al. (2011). Alkaloid concentration was determined in freeze-dried ethanol extracts through an UHPLC-MS technique

(Filannino et al., 2017). Fatty acids were extracted using petroleum ether in a Soxhlet system, and then their profile was evaluated through a gas chromatographic technique, as described by Trani et al. (2016). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of POP was evaluated through the method described by Yu et al. (2003). Also, POP anti microbial activity was evaluated, as described by Di Cagno et al. (2016). For each condition, samples obtained from three independent experiments were analysed in triplicate.

2.2 Results and discussion

Different lactic acid bacteria were used as single starter. *P. oleracea* fermented with *L. kunkeei* B7 and *L. plantarum* POM1 resulted in decreased levels (ca. 30%) of oxalic acid, which is commonly recognized as an anti-nutritional compound. *L. brevis* POM4 enhanced by ca. 400% the concentration of γ -amino butyric acid, a non-protein amino acid with various physiological functions. Fermentation of *P. oleracea* led to a marked increase of α -linalool, a monoterpene compounds with various pharmacological properties. Bioconversion of oleraceins by *L. kunkeei* B7 is likely responsible for the increased antioxidant activity (Fig. 1.) The decrease of polyunsaturated fatty acids potentially underlined the synthesis of various derivatives with potential health or nutritional effects. Hence lactic acid fermentation of *P. oleracea* is a powerful tool to achieve standardized functional foods.

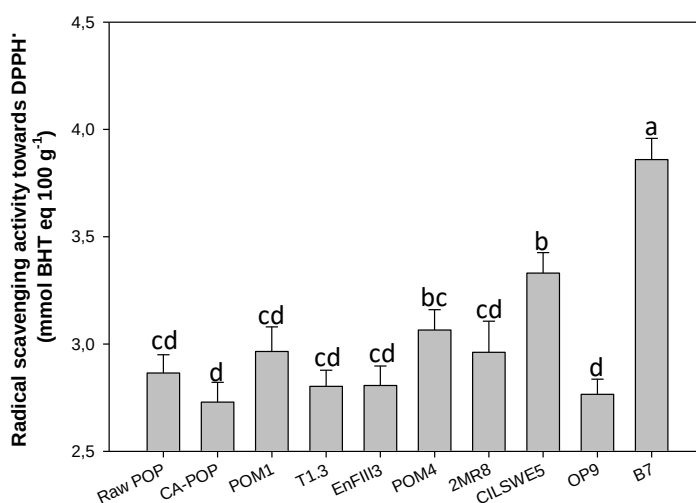


Figure 1 DPPH radical scavenging activity (mmol BHT eq per 100 g) of raw *Portulaca oleracea* puree (POP), CA-POP, or POP fermented at 30 °C for 36 h by *L. kunkeei* B7 and *L. plantarum* POM1 strains. Data are the means ($\pm$ SD) of three independent experiments analyzed in triplicate. Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $P < .05$. Bars with different superscript letters differ significantly ($P < .05$).

3. Solid-State Fermentation of Bee-Collected Pollen as a Novel Biotechnology Emulating the Natural Fermentation of Bee-Bread

3.1 Materials and methods

Ivy flours (*Hedera helix* L.), bee-collected ivy pollen (BCP), and 15 and 30 days hive stored bee bread (BB) were collected during September - October 2016 from organic fields of the Apulia region (Italy). Fructophilic lactic acid bacteria enumeration was performed on FYP agar plates, incubated at 30°C for 48-72h (Filannino et al., 2016).

FLAB isolation, typing and identification were performed as indicated by Filannino et al., (2016). FLAB strains, previously isolated from *Apis mellifera* gut, were also considered in this work (Filannino et al., 2016). FLAB strains selection was based on their growth and acidification rate and their ability to produce anti-microbial compounds. Growth and acidification kinetics were investigated on pollen extract obtained through multiple sequential extractions using solvents with different polarity (Di Cagno et al., 2019). Antimicrobial activity assay was carried out with fermented pollen extracts against 20 different pathogenic or alternative bacterial strains (Shillinger and Luke 1989). Three FLAB strains were selected based on growth and antimicrobial performances, and were used during BCP fermentation trials aiming to set up a fermentation protocol. The addition of pectinolytic enzymes Endozym ICS 10 Rouge or the mixed fermentation with the yeast *Hanseniaspora uvarum* AN8Y27B were evaluated. *H. uvarum* had been reported as pectinolytic yeast and pollen inhabitant (Di Cagno et al., 2019). The final fermentation protocol we set up included: (i) mixed inoculum of *L. kunkeei* PF12, PL13, and PF15 strains (7 Log CFU/mL) and *H. uvarum* AN8Y27B at the final density in fresh BCP of ca. 8 Log CFU/g; (ii) addition of sterile water to reach the final water content of 40% (w/w); (iii) and fermentation of BCP into sealed tubes at 30°C for 216 h. BCP treated under the same conditions, except for the use of microbial starters, was used as control (unstarted-BCP). Microbial pollen profile was evaluated both on fermented sample and control, during the fermentation and during a subsequent storage period of 50 days both at 25 and 4 °C. Bacteria, moulds and yeast were evaluated using selective media (Di Cagno et al., 2019). To further investigate the microbiological stability of BCP, a conidial germination assay was performed on BCP, inoculated with *Aspergillus parasiticus* CBS971.97, as described by Coda et al. (2008). BCP moisture content, TTA and pH were measured as previously described. Carbohydrates, polyols and organic acids were determined on extracts obtained as reported by Di Cagno et al., 2019 and detected through HPLC, as described by Di Cagno et al. (2017). For pollen peptides and free aminoacids determination, ethanol extracts were prepared as described by Di Cagno et al. 2019. Total and individual FAA were analyzed as previously described. Peptides concentration was determined by the ophthalaldehyde (OPA) method (Church et al., 1983). Peptides profiles were analyzed as described by Di Cagno et al., 2019. Total phenolics in water and methanol extracts was quantified according to Folin-Ciocalteu method and expressed as g gallic acid equ/kg dry weight of BCP (Singleton and Rossi, 1965). In vitro protein digestibility was performed following the protocol of Akeson and Stahmann (1964). For each condition, samples obtained from three independent experiments were analysed in triplicate.

3.2 Results and discussion

Structure of lactic acid bacteria biota in ivy flowers, fresh bee-collected pollen (BCP), hive-stored bee bread, and honeybee gastrointestinal tract was investigated. Although a large microbial diversity characterized flowers and fresh BCP, most of lactic acid bacteria species disappeared throughout the bee bread maturation, giving way to *L. kunkeei* and *Fructobacillus fructosus* to dominate long stored bee bread and honeybee crop. Adaptation of lactic acid bacteria was mainly related to species-specific, and, more in deep, to strain-specific features. Bee bread preservation seemed related to bacteria metabolites, produced especially by some *L. kunkeei* strains, which likely gave to lactic acid bacteria the capacity to outcompete other microbial groups. A protocol to ferment BCP was successfully set up, which included the mixed inoculum of selected *L. kunkeei* strains and *H. uvarum* AN8Y27B, almost emulating the spontaneous fermentation of bee bread. The strict relationship between lactic acid bacteria and yeasts during bee bread maturation was highlighted. The use of the selected starters increased the digestibility and bioavailability of nutrients and bioactive compounds naturally occurring in BCP (Fig.

2). Our biotechnological protocol ensured a product microbiologically stable and safe. Conversely, raw BCP was more exposed to the uncontrolled growth of yeasts, moulds, and other bacterial groups.

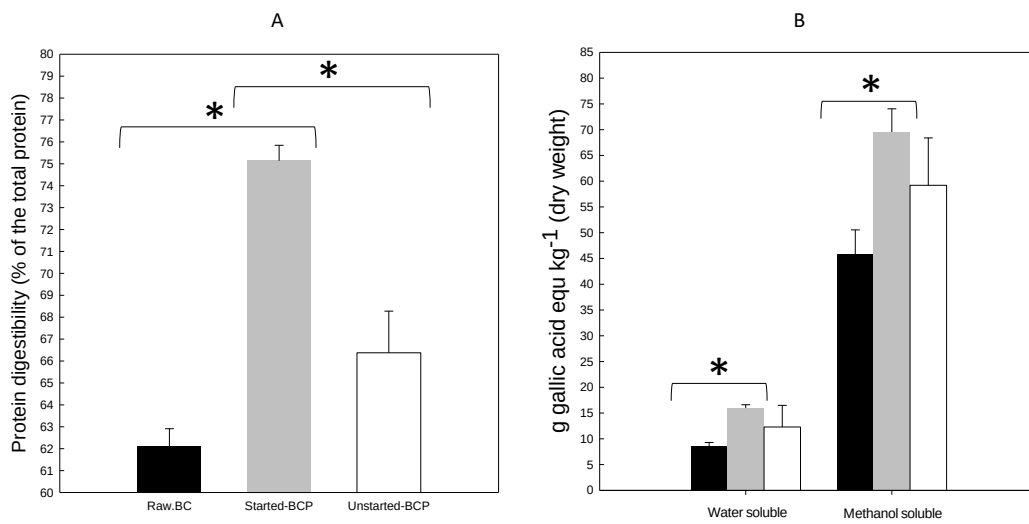


Figure 2 (A) *In vitro* protein digestibility of raw-BCP (black bar), started-BCP (grey bar) and unstirred-BCP (white bar) after fermentation at 30°C for 216 h. (B) Waters and methanol soluble free phenolics concentrations (g gallic acid equ/kg dry weight of bee collected pollen) in raw bee collected pollen (raw-BCP) (black bars), started-BCP (grey bars) and unstirred-BCP (white bars) after fermentation at 30 °C for 216 h. Data are the means ($\pm$ SD) of three independent experiments analyzed in triplicate. Data were subjected to Student's *t*-test. The asterisks indicate values statistically different ($P < 0.05$).

4. Selection of Fructophilic Lactic Acid Bacteria Strains Capable of Fully Reducing FODMAPs in Wheat-Derived Products.

4.1 Material and Methods

Twenty-five FLAB strains were used as fermentation starters of wheat dough. *L. plantarum* DC400 and *Lactobacillus sanfranciscensis* DD8, previously isolated from sourdough, were used as control strains. Strain selection was based on their fructose and mannitol consumption ability. A fructose-glucose based medium was singly inoculated with the strains and used to investigate the fructose consumption kinetics. Glucose and fructose concentrations were detected through high-performance liquid chromatography (HPLC) (Filannino et al., 2017). FLAB strains were selected also basing on their mannitol consumption ability as reported by Cantatore et al. (2018). Selected strains were subsequently used to set up different fermentation trials of wheat dough, both as single and mixed starters. Cells were inoculated into the dough at the initial cell density of ca. 7 Log CFU/g, as described above. Control dough was prepared just with durum wheat flour and sterile tap water, without bacterial inoculum. Doughs were incubated for 72 h at 30°C. Acidification and bacterial growth were monitored every 12 h for 72 h. Glucose, fructose and total fructans levels were monitored every 12 h during doughs fermentation. The water/salt-soluble extracts of doughs were prepared according to Weiss et al. (1993) and used to analyze the glucose and fructose levels by HPLC. Total fructans were measured using an AOAC-approved commercially available, enzymatically based assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland), as per manufacturer's instructions. The extraction process was carried out as described by Muir et al., (2009), with few changes. With the aim to reduce fermentation time, a final dough fermentation

protocol was set up adding commercial enzymes (data not shown). For each condition, samples obtained from three independent experiments were analysed in triplicate.

4.2 Results and Discussion

This study proposed an innovative protocol suitable to speed up the FODMAPs degradation, thanks to complementary solutions, such as a consortium of selected FLAB starters and the addition of commercial enzyme. Under dedicated conditions, targeted exogenous enzymes hydrolyzed wheat fructans with different linkages and range of polymerization, in synergy with the FLAB fructanase activity. Lastly, FLAB completed the consumption of fructose and mannitol. FLAB had both the capabilities to convert fructans into fructose, and to metabolize the resulting fructose. Extracellular fructanase activity is extremely rare in LAB (Loponen and Gänzle 2018), but FLAB have been shown to significantly contribute to the fructans hydrolysis during wheat dough fermentation. The hydrolysis of fructans released fructose that was easily metabolized through the 6-PG/PK pathway or converted to mannitol by FLAB. Since mannitol is a polyol included into the FODMAPs group, processing of low FODMAPs food necessitates mannitol-degrading organisms. Enzymes for mannitol conversion have been found in homofermentative lactobacilli of the *L. delbrueckii*, *L. casei*, *L. plantarum*, and *L. salivarius* groups (Loponen and Gänzle 2018). Previously, FLAB have been shown to metabolize mannitol when this latter is the only carbon source available (Endo and Okada 2008; Endo et al. 2009, 2011, 2012; Filannino et al 2016). Under the condition of this study, the restriction of carbohydrate sources allowed for mannitol fermentation by FLAB after fructans and fructose were completely consumed. Thus, fermentation by FLAB was a sustainable and valuable biotechnology for decreasing the FODMAP content below cutoff values (0.3 g per 100 g of bread) in cereal-based products.

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New Perspective on Taste: exploring Association Among Oral Perception, Tongue Physiology and Oral Microbiota Composition

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The aim of this PhD project is to evaluate taste perception in relation to genetic, physiological and microbiological variables, using a multidisciplinary approach. To achieve this aim different groups of subjects have been recruited: 1) subjects characterized by different taste responsiveness to the bitter compound 6-n-propylthiuracil, PROP (Supertasters vs Non-tasters); 2) subjects characterized by different nutritional status, (Obese vs Normal-weight children and adolescents); 3) subjects characterized by different genetic and cultural background (Asian vs Caucasian).

Nuovi punti di vista nello studio delle relazioni tra percezione gustativa, fisiologia della lingua e composizione del microbiota orale

L'obiettivo di questo progetto di dottorato è stato quello di valutare la relazione tra percezione gustativa, fattori genetici, fisiologici e microbiologici, applicando un approccio multidisciplinare. Per raggiungere tale obiettivo sono stati reclutati differenti gruppi di soggetti caratterizzati da: 1) differente sensibilità gustativa al composto amaro 6-n-propiltiuracile, PROP (Supertaster vs Non-taster); 2) differente stato nutrizionale, BMI (bambini e adolescenti obesi vs normopeso); 3) differente background culturale e genetico (Asiatici vs Caucasici).

Key words: Taste perception; oral microbiota; eating behaviour; obesity; cross-cultural differences;

1. Introduction

In accordance with the PhD thesis project previously described (Cattaneo, 2017), this oral communication presents the main results obtained during the three years of project and reported in the following papers: a) Cattaneo, Gargari, et al., (2019) – *published in Scientific Reports*; b) Cattaneo, Riso, et al., (2019) – *published in Nutrients*; c) Mameli, Cattaneo, et al. – *submitted to Plos One*; d) Cattaneo, Liu, et al. – *submitted to Food Quality and Preference*. The presentation of the results has been divided in three main activities regarding:

A1) Taste perception, oral microbiota composition and eating behaviours

There are many known drivers of food choice and habits, however, taste is considered one of the main predictors. It is well known that taste varies greatly among individuals and it has been shown that impairments in taste perception and hedonic experience of taste can even cause unhealthy eating behaviours. Interestingly, it has been suggested that also microbes in the gastrointestinal tract could have a potential direct role in shaping individuals' eating behaviours and food preferences (Alcock et al., 2014). However, fairly poor literature about this topic is available and the relationship between taste perception and oral microbiota has not been systematically investigated so far.

A2) Taste perception, food neophobia and oral microbiota composition in relation to children and adolescents' nutritional status

Obesity is considered a disease with a multifactorial aetiology, which seems to be genetically based, but requires environmental, psychological and social influences to exhibit. Recently, it has been suggested that differences exist in taste sensitivity between obese and non-obese adults (Proserpio et al., 2016) as well as children (Overberg et al., 2012). Besides individual variation in taste perception, food neophobia (literally the reluctance to eat novel foods) is another aspect to be considered as an important trait in shaping food habits and affecting subjects' nutritional status (Proserpio et al., 2018). Moreover, it has been suggested that oral bacteria could have a potential direct role in development of obesity (Ley, 2010).

A3) Cross-cultural differences in taste perception and oral processing behaviour

Cultural and genetic differences in consumer populations across the world are important determinants for food preferences. The variation in oral texture perception of foods across consumers is supposed to depend on individual differences in tactile acuity. There are some indications that Asian and Caucasian consumers have different oral chemosensory ability. Moreover, the way that individuals manipulate food in their mouths could also influence the food textures they prefer (Jeltema et al., 2015). Therefore, population-wide differences in texture sensations are still not well understood.

2. Materials and Methods

2.1 Taste perception, oral microbiota composition and eating behaviours

One hundred and five normal-weight young adults were involved and a screening procedure with PROP solution was performed. Participants were selected according to their thiourea taste sensitivity (PROP status) and they required to be Non-tasters (NT) or Supertasters (ST). Thus, 59 subjects (NT: n=30 and ST: n=29) were admitted to the main experiment. Taste perception was assessed by evaluating the taste thresholds for the four basic tastes (3-AFC, ISO/DIS 13301:2018) and the Fungiform Papillae (FP) density. The oral microbiota composition was assessed through 16 S rRNA gene profiling. A Food and Beverage Frequency Questionnaire (FB-FFQ, Porrini et al., 1995) was used to assess the frequency consumption of specific categories of foods and beverages over the previous month and subjects were asked to complete a seven-day food diary to assess their food and nutrient intake.

2.2 Taste perception, food neophobia and oral microbiota composition in relation to children and adolescents' nutritional status

34 children and adolescents affected by obesity and 33 controls were involved. Taste perception was assessed using the 'Taste Strips' method (Mueller et al., 2003) in which a Total Taste Score (TTS) of 16 was the possible maximum score achieved by subjects, obtained by calculating the sum of all four taste qualities presented in the four different concentrations. FP density was also evaluated. The oral microbiota composition was assessed through 16 S rRNA gene profiling. Moreover, Food Neophobia was measured using the Italian Children Food Neophobia Scale (ICFNS, Laureati et al., 2015).

2.3 Cross-cultural differences in taste perception and oral processing behaviour

152 consumers (75 Chinese and 77 Danish) were recruited. Oral processing behaviours were identified using a questionnaire. Lingual tactile acuity was assessed according to responses to stimulation with Von Frey filaments (RIF). PROP taster status and FP density were evaluated as well.

3. Results and Discussion

3.1 Taste perception, oral microbiota composition and food habits

An important inter-individual variability was found for all taste stimuli, as reported in Cattaneo, Gargari, et al., (2019), between the two groups, with NT subjects showing significant ($p < 0.01$) higher threshold values compared with ST. A significant difference was also found in FPD between the two groups of subjects ($p < 0.01$), with NTs subjects showing a significantly reduced FPD compared with ST. Present findings are in line with most of literature data, confirming that ST and NT subjects differ in their taste ability, with NT subjects showing a significantly lower sensitivity than ST subjects and presented anatomical differences in the tongue.

Significant correlations were found between different taste perception variables, especially between tastes that share many common features in the transduction mechanisms as reported in Table 1. Thus, these findings seem to confirm the presence of the well-known dichotomy in taste coding for perception of pleasant (e.g. sweet and savory compounds) vs noxious stimuli (e.g. sour and bitter compounds).

Table 1 Correlation coefficients of the relationship among gustatory functions.

	FPD	PROP	Sweet threshold	Salty threshold	Bitter threshold	Sour threshold
FPD	1	0.47***	-0.08	-0.30*	-0.06	-0.16
PROP		1	-0.35**	-0.32*	-0.34**	-0.33*
Sweet threshold			1	0.10	0.34**	0.07
Salty threshold				1	0.14	0.34**
Bitter threshold					1	0.31*
Sour threshold						1

Correlation coefficients determined by Pearson's correlation coefficient. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 59$.

The main results of the present study showed that interindividual differences in taste perception may influence habitual food consumption and intake, as reported in Cattaneo, Riso, et al., (2019). For instance, subjects who were orally hypersensitive to sodium chloride solution seem to significantly increase consumption of less healthy foods, like bakery ($p < 0.01$) and salty baked products ($p < 0.05$), saturated-fat-rich products ($p < 0.05$) and soft drinks ($p < 0.01$). Moreover, participants who have a higher threshold for sweet taste (hyposensitive) reported consuming significantly more frequently sweets and desserts than the hypersensitive group ($p < 0.05$). Nevertheless, we observed a relationship between bitter taste sensitivity and total energy and carbohydrates intakes, suggesting a potential shift towards less healthy dietary patterns in the hypersensitive group. Overall, These findings have implications for eating behaviour, as perceived sensory properties of foods and beverages clearly influence preferences and the type and amount of food consumed.

As reported in Cattaneo, Gargari, et al., (2019), we found that five bacterial genera were significantly higher in ST compared to NT subjects, as shown in Figure 1. In particular, the Gram-positive *Actinomyces* (belonging to the phylum Actinobacteria; $P = 0.012$), *Oribacterium* (Firmicutes; $P = 0.034$), *Solobacterium* (Firmicutes; $P = 0.040$) and *Catonella* (Firmicutes; $P = 0.009$), and the Gram-negative *Campylobacter*

(Proteobacteria; $P = 0.009$) were overrepresented in ST group.

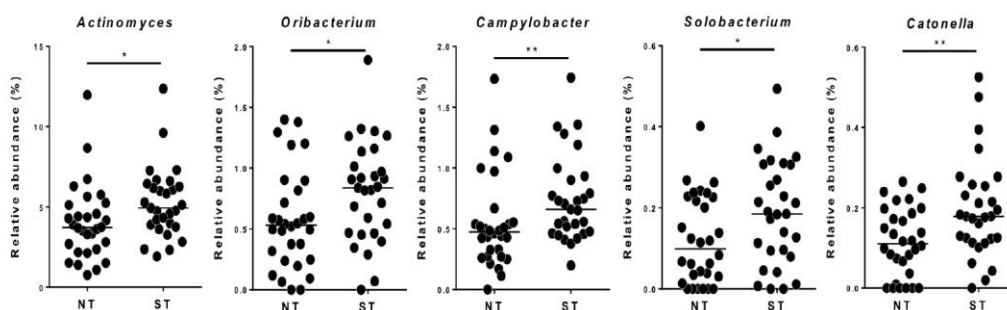


Figure 1 Scatter plots representing the relative abundance of bacterial genera that resulted significantly different between non-taster (NT) super-taster (ST) samples. Statistics according to Mann-Whitney test; ** $p < 0.01$; * $p < 0.05$.

Overall, these results suggest that the oral microbiota composition deserves to be considered as an influencing variable when investigating peri-receptor events involved in chemosensory processes, since oral bacterial community may influence and modulate taste perception.

Interesting correlations between the relative abundance of oral bacterial taxa and dietary intake were found, as shown in Figure 2.

		Microbial Taxa																											
Energy and macronutrients intake	Energy (kcal)							+																					
	Protein (% En)									++						+													
	Fat (% En)																												
	Carbohydrates (% En)																												
	Protein (g/die)								++		++																		
	Fat (g/die)								++		+																		
	Carbohydrates (g/die)	+	+																										
	Total Fibre (g/die)	+	+	++																									

Figure 2 Correlations between the relative abundance of bacterial taxa on tongue dorsum and nutritional variables. The heatmap represents the Spearman's correlation R -values. + and – represent positive and negative correlations, respectively. Asterisks relate to the Kendall rank correlation p values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Indeed, Clostridia class was positively associated with total energy, fat, and protein intakes (e.g., genera *Selenomonas*, *Ruminococcaceae*, *Johnsonella*, and *Veillonella*) but negatively associated with fiber intake (e.g., genera *Catonella* and *Peptostreptococcus*), whereas Proteobacteria phylum and *Prevotella* genus showed the opposite association.

Overall, our results are in line with the general assumption that some microbial taxa are positively associated with vegetable-rich (*Prevotella*) or protein/fat-rich diets (*Clostridia*) (Wu et al., 2011).

3.3 Taste perception, food neophobia and oral microbiota composition in relation to children and adolescents' nutritional status

Obese children and adolescents presented significantly ($p < 0.001$) more difficulties in correctly identifying the different taste qualities compared to controls, resulting in a lower Total Taste Score (TTS). Moreover, the density of FP is significantly lower in obese subjects ($p < 0.001$). Our findings, as reported in Mameli, Cattaneo, et al., (*Submitted*), are in line with the hypothesis that obese and non-obese children and adolescents presented differences in their taste sensitivity, with obese subjects showing a lower ability in correctly identifying taste qualities compared to the group of controls (Overberg et al., 2012). This impaired taste perception in obese children and adolescents supports the assumption that the taste system is affected in obese subjects.

Although various evidences suggest that body weight could be related to personality traits such as food neophobia, our results did not highlight any relationship between nutritional status and food neophobia. However, independently of nutritional status, children and adolescents who present an higher FP density are significantly more neophobic than less sensitive individuals, which suggests that higher sensitivity might lead to neophobic reactions.

In this study, we also focus on the link between oral bacterial community and obesity. The analysis of the salivary bacterial consortia revealed that people with obesity have significantly ($p < 0.05$) higher bacterial richness than normal weight controls, as shown in Figure 3a.

Even if the oral microbiota composition in saliva resulted very similar in cases and controls (Figure 3b), some difference was highlighted at phylum level for Proteobacteria (22% in controls vs. 17% in cases, $p < 0.05$) and at class level for Gammaproteobacteria and Negativicutes (9% in controls vs. 6% in cases for both classes, $p < 0.05$).

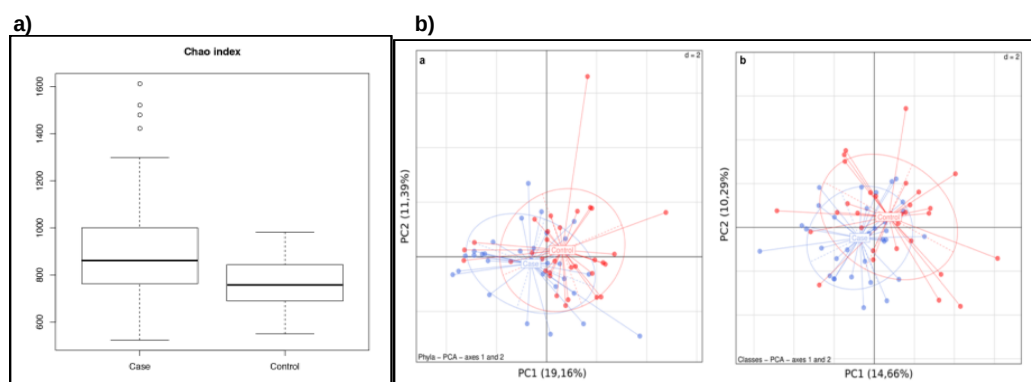


Figure 3 a) Box-plots representing the α -diversity (observed species) of the saliva microbiota composition in case and control groups through Chao index. b) Principal Component Analysis of the microbiota profile in both groups at phylum level (left) and at class level (right).

Cross-cultural differences in taste perception and oral processing behaviour

This study (Cattaneo, Liu et al., *Submitted*) was performed in collaboration with prof Wender Bredie of the Department of Food Science at Copenhagen University and

Arla Foods company. Latent Class Analysis (LCA) highlighted that Asian population (Chinese) deviated significantly from the Caucasians (Danish) for their oral processing behaviours. Chinese were characterized by a larger number of 'Soft processing likers' (77%), who preferred foods that could be held in the mouth for a longer time and manipulated between the tongue and roof of the mouth. On the contrary, Danish consumers mostly belonged to the 'Firm processing likers' group (73%), who preferred foods that require to use the incisors and/or molars to break down or deform the food in the mouth.

Regarding tactile acuity data, cross-population differences in lingual tactile acuity were not found, but interestingly, differences in taste sensitivity were found between the two population cohorts, as reported in Table 2.

Table 2 *Subjects' characteristics according to R-index values, FPD and PROP responsiveness in Chinese and Danish population cohorts.*

	Chinese	Danish	p-value
R-index (%)	84.6 ± 1.0	82.0 ± 1.0	n.s.
FPD (FP/cm ²)	62.9 ± 1.4	56.6 ± 1.3	***
PROP responsiveness	51.4 ± 2.0	38.3 ± 1.9	***

*Significant differences between Chinese and Danish cohorts, detected according to post-hoc test using the Bonferroni test adjusted for multiple comparisons, are indicated by *** for $p < 0.001$ and n.s. for $p > 0.05$, respectively.*

Chinese consumers generally presented significantly greater FPD ($p < 0.001$) and PROP responsiveness ($p < 0.001$) compared to the Danish subjects. Moreover, Chinese 'Soft processing likers' were characterized by a greater FPD (65.8 ± 1.4 FP/cm²) than 'Firm processing likers' (59.9 ± 2.5 FP/cm²), suggesting that Chinese preferred Mouth Behaviors might be related to FPD and, to a lesser extend to PROP status, which could somehow explain the greater percentage of 'Soft processing likers' in this Asian population.

4. Conclusions and Future Perspectives

In conclusion, in this PhD thesis several aspects involved and potentially affected by taste perception were evaluated. In general, these three years activities suggested that it is important to provide further insights into the complexities of human eating behaviour, understanding how and which host-related factors could affect people food choices and habits. Moreover, these outcomes could be used as a starting point to understand the driving force of food preferences and help food industries in developing food products that match different consumers' needs and could help develop further strategies of obesity prevention and therapy.

Additionally, the potentiality of this multidisciplinary approach opens new avenues of research by highlighting associations between sensory and consumer science, food technology and nutrition.

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Innovative Analytical Methods Merged with Chemometric for Food Integrity Assurance

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The topic of this PhD research project was the development of innovative “non-targeted” analytical methods that, merged with chemometric data analysis, were able to help in the detection of food frauds. Both rapid and confirmatory approaches were applied for the detection of specific fraudulent activities on different raw materials. This project was developed jointly by Barilla G. & R. Fratelli S.p.A. and the University of Parma, department of Food and Drug.

Metodi Analitici Innovativi Accoppiati ad Analisi Chemiometriche per l'Assicurazione dell'Autenticità Alimentare

L'obiettivo di questo progetto di dottorato è stato lo sviluppo di metodi analitici “non-targeted” innovativi che, accoppiati all'analisi chemiometrica, fossero in grado di identificare frodi alimentari. Sono stati applicati sia metodi rapidi che metodi di conferma per l'individuazione di specifiche attività fraudolenti su diverse materie prime. Il progetto è stato sviluppato in collaborazione tra Barilla G. & R. Fratelli S.p.A. e l'Università di Parma, dipartimento di Scienze degli Alimenti e del Farmaco.

Key words: Food Frauds; Non-targeted mass spectrometry; metabolomics; rapid methods.

1. Introduction

In accordance with the PhD thesis project previously described (Cavanna, 2017), this oral communication reports the main results of the following three activities directed to:

A1) the development of «non-targeted» confirmatory analytical methods for the detection of frauds in extra virgin olive oil;

A2) the development of «non-targeted» rapid and confirmatory analytical methods for the detection of frauds in eggproducts;

A3) the development of «non-targeted» confirmatory analytical methods for the detection of frauds in wheat

2. Extra Virgin Olive Oils

2.1 Overview

Extra Virgin Olive Oil (EVOO) is one of the most important and expensive edible oils, and therefore it is also one of the most adulterated food commodity over the global market. Typical frauds like the addition of other type of oils (i.e. seed oils) or mixtures with other refined oils can be easily detected with standards methods. For this reason, the fraudster are now focused on developing more sophisticated adulterations that would

3. Eggproducts

3.1 Overview

Eggs, mostly in the egg products form, are largely used for the creation of different industrial products and their freshness is a crucial characteristic for the production of safe and high quality commodities; however, rapid and confirmatory robust methods able to assess this criterion are still lacking and only the official parameter “Lactic Acid” is monitored up to now (Reg. CE 853/2004).

On the basis of what described above, the aim of this part of the project was the development of methods able to assess eggs freshness, for both rapid and confirmatory purposes. The rapid way explored the volatile components of the egg products using a HSGC-IMS (Head Space Gas Chromatograph coupled to an Ion Mobility Spectrometer) instrument, that allowed the creation of a predictive chemometric model. The confirmatory approach used the metabolomic LC-HRMS non targeted analyses as a tool for the identification of new chemical markers responsible of eggs freshness.

3.2 Experimental design

For the rapid method, the experimental design was applied to 52 batches collected directly from Barilla's production plant. They were analyzed immediately after the receipt in the plant, then were left at room temperature and analyzed again after 1 day (n=29), 2 days (n=21), 3 days (n=15), 4 days (n=11) and 5 days (n=9). With this robust amount of samples (n=137) a preliminary PCA and then a robust OPLS-DA classification model was created, comparing the group of “fresh” samples with the group of “not fresh” samples. The predictive abilities of the model were validated with the analysis of seven new batches not used for the model creation (*validation set*) and with the prediction of mixtures in which the predominant component was the fresh egg product and different aged eggs were added as adulterants.

The non targeted metabolomic study was executed on 29 batches of the samples set collected for the volatiles survey, with the same experimental design and analyzing the samples as fresh and after 1 day and after 2 days spent at room temperature (n= 79). The markers selected at the end of the process were searched in the validation set previously mentioned.

3.3 Results obtained

For the rapid method, a robust predictive OPLS-DA classification model was created ($R^2X = 0.824$; $R^2Y = 0.950$; $Q_2 = 0.920$), as presented in figure 2.

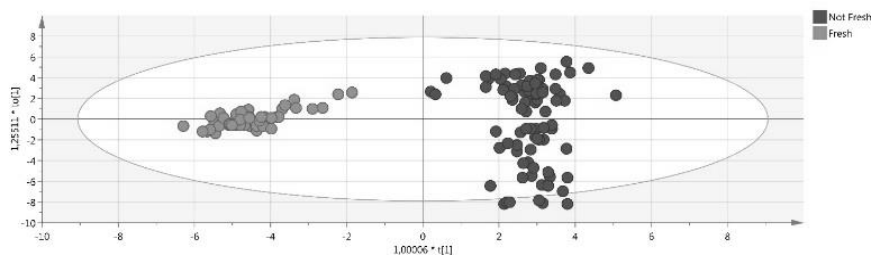


Figure 2 OPLS-DA scores plot of the egg products samples, colored according to the groups. Grey: “fresh samples”; black: “not fresh samples”.

In addition, 97% of the validation set was correctly predicted by the model and all the mixtures were classified at least as “suspect”, certifying the reliability of this approach also in the detection of more sharp fraudulent activities (Cavanna et al, 2019b).

Moving to the non-targeted method, at the end of the metabolomic study, 15 compounds responsible of freshness and 16 compounds responsible of not freshness of egg products were selected and identified. If available, the analytical reference standards of these molecules were injected in order to certify beyond any reasonable doubt their identity (Sumner, et al., 2007).

Furthermore, the same compounds were detected in seven new egg products batches not used for the model creation and treated with the same experimental design (n=21). As example, table 1 shows the comparison of the area values of Arginine (a freshness marker) across the different time points in the samples used for the markers selection and in the samples used for model validation.

Table 1 Comparison of the mean area values (+/- Standard Error) of Arginine between the markers selection and the markers validation. Time points: first bar, “t zero”; second bar, “1 day”; third bar, “2 days”.

COMPUND NAME	MARKERS SELECTION	MARKERS VALIDATION
Arginine		

The presence and the trends of the markers are the same between the two groups of samples and these results certify that these molecules can be considered real freshness or not freshness compounds and are not related only to some specific chemometric models (Cavanna et al., 2018).

4. Wheat

4.1 Overview

The declaration of durum wheat geographical origin, sometimes claimed on pasta labels, represent an added value to the commodity, especially if it is made only with wheat coming from a specific nation or region (i.e. “100% Italiano”).

The chemical composition of wheat, indeed, is partially related to the cultivation soil and to the climate conditions, that are different by geographical areas and that can affect the quality and subsequently the commercial value of this raw material (Zhao, et al., 2011).

For these reasons, the false origin declaration of this commodity or the creation of fraudulent mixtures of wheat coming from different countries could represent an illegal economical gain for wheat sellers and this is the reason why the attention of pasta producers in the development of methods able to assess the geographical origin of wheat largely increased in the last decade (Morin et al., 2018).

This part of the project was focused on the development of a non-targeted Liquid Chromatography –High Resolution Mass Spectrometry study for the detection of new chemical markers responsible of the geographical origin of wheat and able to discriminate if the raw material comes from Italy, Europe or Extra Europe.

4.2 Experimental design

Authentic samples coming from different Italian, European and Extra European regions were collected during two years of harvesting, 2016 and 2018: the 2016 campaign was used for the model creation and for markers selection, while the 2018 campaign was used for model and markers validation.

91 samples were collected during the 2016 campaign (56 from Italy, 21 from other European countries and 14 from Extra European countries) and 49 samples were collected during the 2018 campaign (11 from Italy, 21 from other European countries and 17 from Extra European countries)

A predictive OPLS-DA model was created and, subsequently, a new multivariate data elaboration study was executed in order to select markers responsible of the “Italian”, “European” or “Extra European” origin of wheat.

4.3 Results obtained

The predictive OPLS-DA model (figure 3) was able to correctly classify 100% of the samples used for its creation and approximately 80% of the samples of the test set.

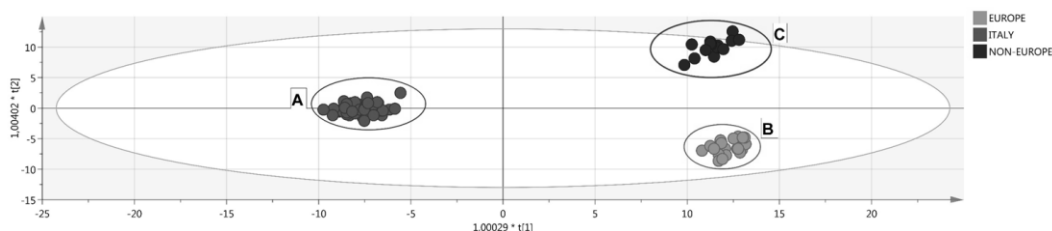


Figure 3 OPLS-DA scores plot of the 2016 wheat samples. Group “A”: Italian samples; group “B”: European samples; group “C”: Extra European samples;. R^2X (cum): 0.532 R^2Y (cum): 0.981 Q^2 (cum): 0.900.

25 chemical compounds were selected and classified as “Italian” or “Not Italian” depending on whether they were present only or mostly in the Italian or in the not Italian samples; in addition, two molecules were selected as discriminant markers for the “Extra European” geographical origin of wheat; subsequently, a group of these molecules was putatively identified.

All the selected features were searched in the 2018 wheat samples and their presence or absence, together with their trends across the different geographies, were evaluated and compared with the results obtained with the 2016 samples used for the model creation. As example, table 2 shows the comparison of the area values of Vanillylactic acid (an “Italian” marker”) across the different regions for the 2016 samples and for the 2018 ones.

Table 2 Comparison of the mean area values (+/- Standard Error) of Vanillylactic Acid in the three geographical areas between the 2016 and the 2018 campaigns. First bar, "European wheat"; second bar, "Extra-European wheat"; third bar, "Italian wheat".

COMPUND NAME	2016 CAMPAIGN	2018 CAMPAIGN
Vanillylactic acid		

All the target molecules were detected with almost the same trend through the different geographies, certifying that the compounds identified are robust markers for the assessment of the geographical origin of wheat.

Further improvements of this study should lead to increase the identification level of the markers and to the development of specific targeted methods.

5. Industrial Impact

The rapid HSGC-IMS method developed for the assessment of eggproducts freshness was tested in Barilla's quality control laboratories for three months and the results obtained were compared with the typical enzymatic approaches applied up to now; approximately 70 samples were analyzed with encouraging results: this more rapid, more sensitive and cheaper way to control the incoming raw materials will be introduced permanently in QC laboratories in the future.

The confirmatory methods presented in this document were used to analyze some suspect samples intercepted by the Quality Assurance department. Moreover, in the future, new tools for routine controls will be available: laboratories should only be able to detect the molecules responsible of each target fraud, avoiding the need of high-resolution mass spectrometry and chemometric softwares, that are much more expensive than a simple singlestage liquid chromatography–mass spectrometry instrument.

6. Conclusions and Future Perspectives

This PhD project provided new tools for the detection of specific food frauds of three different raw materials; in the future, quality control laboratories will have more possibilities to face these issues and prevent the entrance of counterfeit batches in their production plants.

For the markers selected with the metabolomic studies, the activities will continue in order to identify the molecules that are unknown up to now.

The analytical approaches presented in this document will be applied also for the detection of other frauds; the most critical points will be the clear identification of the studies aim and, subsequently, the collection of authentic samples.

In a parallel direction devoted not to food authenticity/safety issues and instead oriented to maximize added value of food products in front of consumers preferences, a similar analytical approach will be also conceived within the framework of Sensomics Science, trying to identify active sensory molecules within different products.

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Aspergillus from Grapes: Ochratoxin A Risk in Relation to Climate Change and New Strategies for Reducing Contamination

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With the present PhD project we studied the impact of combined climate change factors (i.e. increasing temperature, water stress and elevated CO₂ concentrations) on the ecophysiology, OTA production and the expression of key toxin genes by *Aspergillus carbonarius*, which is considered the main responsible of OTA contamination in grapes. Moreover, it has been evaluated the possibility to develop new strategies to reduce the risk of OTA contamination by exploiting the already reported ability of *Aspergillus* species to degrade the toxin (Bejaoui et al., 2005; Gallo et al., 2012). The ultimate aim, is to identify and characterize proteases responsible of OTA degradation.

Aspergillus isolati da uva: rischio ocratossigeno in relazione ai cambiamenti climatici e nuove strategie per ridurre la contaminazione

Con la presente tesi di dottorato, abbiamo studiato l'impatto di diversi fattori climatici (ad es. aumento di temperatura, attività dell'acqua ed aumento della concentrazione di CO₂) sull'ecofisiologia, produzione di OTA ed espressione di geni chiave in *Aspergillus carbonarius*, il quale è considerato il principale responsabile della contaminazione di OTA nell'uva. Inoltre, è stata valutata la possibilità di sviluppare nuove strategie per la riduzione del rischio di contaminazione da OTA sfruttando la già nota capacità di ceppi di *Aspergillus* di degradare la tossina (Bejaoui et al., 2005; Gallo et al., 2012). Lo scopo finale, è quello di identificare e caratterizzare proteasi responsabili della degradazione di OTA.

Keywords: *Aspergillus carbonarius*; ochratoxin A; climate change; decontamination; proteases.

1. Introduction

In accordance with the PhD thesis project previously described (Cervini, 2016), this oral communication reports the main results of the following activities:

A1) Effect of two-way interacting treatments (day/night temperatures x a_w) on growth, gene expression and OTA production;

A2) Effect of three-way interacting treatments (day/night temperatures x a_w x CO₂ concentrations) on growth, gene expression and OTA production;

A3) Evaluation of *A. carbonarius* capability of degrading OTA and identification of new putative proteases from *A. carbonarius* genome.

2. Ochratoxigenic risk in relation to climate change

Ochratoxin A (OTA) is a potent pentaketide nephrotoxin widely contaminating food and feed products (grains, legumes, coffee, dried fruits, meat derived products, beer and wine) (Bayman & Baker, 2006). OTA is the primary mycotoxin risk in wine and dried vine fruits, produced by fungi belonging to the genus *Aspergillus* section *Nigri* of which, *A. carbonarius* is considered the main producer (Visconti et al., 2008).

The contamination of grapes and wine is strongly related to climatic conditions during cultivation, phenological stadium of grapes, damage by insect (Visconti et al., 2008; Somma et al., 2012). Based on recent evidences on climate change, it is expected that in the next 10-25 years the environment in which crops will grow may drastically change with temperature expected to increase by +2-5°C and atmospheric CO₂ to double or triple (400 ppm vs 800/1200 ppm) (Botana et al., 2015).

In this regard, several authors already studied how the mycotoxigenic risk, may mutate under a possible climate change scenario (Vaughan et al., 2014; Medina et al., 2014; Akbar et al., 2016; Verheecke-Vaessen et al., 2019). Nevertheless, there is little information on the impact that climate change may have on fungal colonization and ochratoxin A contamination of grapes.

3. Strategies for OTA decontamination

It is reported that in high risk years, the legislative limit (2 µg/l) of OTA content in wine and grape derived products (Commission regulation no. 123/2005) could exceed in 20-30% of the final product. In this respect, various strategies for decontamination of wine from OTA have been studied in the last decade but no reliable technique/systems are available except the use of activated charcoal by reducing also wine quality (Varga, 2010). Recently, an innovative and environmentally friendly corrective measure to reduce OTA level by repassage of contaminated musts or wines over grapes pomaces was developed (Solfrizzo et al., 2010; Solfrizzo et al., 2016).

A better solution for OTA decontamination could be biodegradation. Enzymes may be a practical way to reduce the OTA levels. Some proteases from microbial origin (carboxypeptidase A, lipase, amidase) may be involved in the degradation of OTA. However, little information is available and very few enzymes have been purified and characterized. The most acknowledge pathway of OTA microbiological degradation involves the hydrolysis of the amide bond that links the L-β phenylalanine molecule to the OTα moiety (Abrunhosa et al., 2010). The degradation products OTα seems to not exert cytotoxic effects on cellular metabolism. Therefore, this mechanism can be considered a detoxification pathway.

4. Experimental Procedure

In this PhD thesis three main activities subdivided in sections were planned in order to study:

1) the effect of different 1.1) day/night alternating temperatures x water activity, 1.2) day/night alternating temperatures x water activity x CO₂ on growth and OTA production in *Aspergillus carbonarius* strains;

2) the expression of 2.1) biosynthetic and 2.2) regulatory genes related to OTA production;

3) new strategies for OTA reduction in grape and derived products 3.1) exploiting the capability of *A. carbonarius* to degrade OTA, 3.2) identify proteases potentially involved in OTA degradation, 3.3) characterize an aspartyl protease already identified in OTA biosynthetic cluster of *A. carbonarius*.

5. Materials and methods

A1/A2) Three *A. carbonarius* (ITEM 7444, ITEM 5010, B7) isolated from Italian wine grapes, were used for this study although, the strain ITEM 5010 will be considered in the description of our results. A Grape Juice Medium (GJM) was prepared at 0.99 and 0.93 a_w.

The two-way interacting effect (A1) of water stress (0.99 vs 0.93 a_w) and two day/night temperatures simulating nowadays conditions and climate change scenario of

South Apulia (18-31°C vs 20-37°C 10h/14h dark/light), during the ripening season, was studied.

The three-way interacting effect (A2) of water stress (0.99 vs 0.93 a_w), two day/night temperatures simulating a nowadays and climate change scenario of North Apulia (15-28°C vs 18-34°C 11.5h/12.5h dark/light), during the ripening season and existing (400 ppm) vs predicting (1000 ppm) CO₂ concentrations, was also investigated.

Lag phases prior to growth and growth rates calculated from the linear regression model, expression of key biosynthetic genes (*PKS*, *NRPS*, *HAL*, *p450*) (Gallo et al., 2012; Gallo et al., 2014; Ferrara et al., 2016) analysed by qRT-PCR and phenotypic OTA production determined by HPLC-FLD (Gallo et al., 2014) were investigated, either in A.1 and A.2 activities.

A3) From the *in silico* screening of the *A. carbonarius* genome (<http://jgi.doe.gov/carbonarius/>) and from the results of transcriptomic analysis carried out on the fungus grown in different OTA producing conditions, seven proteases (from *AcProA* to *AcProG*), expressed at increased level during OTA production were selected.

In order to perform activities described, the wild-type strain ITEM 5010 and three mutant strains Δ *AcOTApks*, Δ *AcOTANrps*, Δ *AcOTAHal*, previously obtained in our laboratory from ITEM 5010 and unable to produce OTA, were used in this study. The *A. carbonarius* strains were incubated in presence of exogenous OTA (3 μ M in 10 ml) under two different conditions:

- 1) incubation in BM (Basic medium) containing OTA, then culture liquid and mycelium of each strain were collected after 3 and 4 days of growth.;
- 2) exogenous OTA was added after 3 days of growth in BM; then culture liquid and mycelium of each strain were collected after 12h and 24h.

Ochratoxin A content by HPLC-FLD and expression levels of seven proteases (from *AcProA* to *AcProG*) encoding genes by qRT-PCR, were investigated.

6. Results and discussion

6.1 Effect of two-way interacting treatments (day/night temperatures x a_w) on growth, gene expression and OTA production.

In our study, we observed that 18-31°C, 10h/14h dark/light and high water availability (0.99 a_w) determined a higher growth rate (19.4 mm/day) and a shorter lag phase (26.39 hrs), compared to water stress (0.93 a_w). Increasing temperature at 20-37°C 10h/14h dark/light caused a reduction of *A. carbonarius* growth rate at 0.99 a_w (5.97 mm/day) which is lower than that observed at 0.93 a_w (11.49 mm/day) in the same condition (data not shown). This behavior in *A. carbonarius* resulted partially in agreement with previous reports (Belli et al. 2005b; Selouane et al., 2009). Moreover, a general upregulation of all the genes tested at 18-31°C vs 20-37°C 10h/14h dark/light varying from 0.73 (*Hal*) to 3.44 (*p450*) was observed (Table 1). These molecular evidences, mirrored the OTA production that was significantly higher at 18-31°C after 4 days (553.91 ng/g) than at 20-37°C (184.72 ng/g) (Figure 1).

	$\log_2$ (2 ^{-$\Delta\Delta$CT})
	18-31°C
<i>PKS</i>	↑ 0.74
<i>NRPS</i>	↑ 0.73
<i>Hal</i>	↑ 0.77
<i>p450</i>	↑ 3.44

Table 1 Summary of fold changes ($\log_2$ (2^{- $\Delta\Delta$ CT})) of OTA related genes (*PKS*, *NRPS*, *Hal*, *p450*) at 18-31°C, after 4 days. Numbers represent the fold changes that were calculated considering 20-37°C 10h/14h dark/light as the control condition.

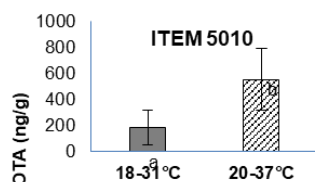


Figure 1 OTA production (ng/g) at 18-31°C and 20-37°C after 4 days by *A. carbonarius* ITEM 5010. Letters indicate significant differences ($p < 0.05$).

6.2 Effect of three-way interacting treatments (day/night temperatures x a_w x CO₂) on growth, gene expression and OTA production.

In both non-stressed (0.99 a_w) and drought stressed (0.93 a_w) conditions the effect of elevated CO₂ (1000 ppm) caused an increase of the growth rates at both temperature cycles (data not shown). In particular, at 18-34°C 11.5h/12.5h dark/light a significantly faster growth (18.38 mm/day) was observed compared to 15-28°C 11.5h/12.5h dark/light (16.55 mm/day). Moreover, water activity (0.99 vs 0.93 a_w) significantly influenced the lag phases while the CO₂ treatment (400 vs 1000 ppm) exerted a significant effect only at 0.93 a_w (data not shown). Gene expression analysis (Table 2) showed that elevated CO₂ concentration (1000 ppm) caused a positive stimulation of all OTA biosynthetic genes (*PKS*, *NRPS*, *Hal*, *p450*) both at 15-28°C and 18-34°C 11.5h/12.5h dark/light. These molecular data, were supported by the chemical analysis in which we found that high CO₂ concentration (1000 ppm) caused an overall increase of OTA production after 4 days, especially at 15-28°C, 11.5h/12.5h dark/light (3978.86 ng/g). Conversely, at current CO₂ concentration (400 ppm) no significant differences were detected between the two day/night alternating temperatures (Figure 2).

Table 2 Summary of fold changes $\log_2(2^{-\Delta\Delta CT})$ of OTA related genes (*PKS*, *NRPS*, *Hal*, *p450*), at 15-28°C and 18-34°C 11.5h/12.5h dark/light, 1000 ppm, after 4 days. Numbers represent the fold changes that were calculated considering, for each temperature, 400 ppm as the control condition.

	$\log_2(2^{-\Delta\Delta CT})$	
	15-28°C	18-34°C
<i>PKS</i>	↑ 3.62	↑ 4.89
<i>NRPS</i>	↑ 4.93	↑ 4.2
<i>Hal</i>	↑ 2.33	↑ 1.11
<i>p450</i>	↑ 2.13	↑ 0.13

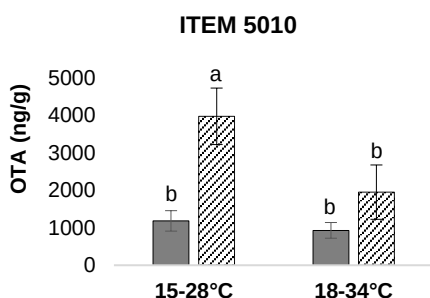


Figure 2 OTA production (ng/g) at 15-28°C and 18-34°C 11.5h/12.5h dark/light, at 400 () vs 1000 () ppm, after 4 days by *A. carbonarius* ITEM 5010. Letters indicate significant differences ($p < 0.05$).

6.3 Evaluation of *A. carbonarius* capability of degrading OTA and identification of new putative proteases from *A. carbonarius* genome.

Both when 30 nmol of OTA were added in the medium at time 0 and after 3 days (Table 3) of fungal growth, we observed a reduction of OTA from 65% to 80% in the different cultures of tested strains. Concomitantly, a slight increase of the degradation product OT α was registered. We also analyzed the OTA and OT α content in the mycelium (data not shown) of the strains in order to exclude that the most of the mycotoxin would have remained entrapped in the mycelium. Based on the results observed, we concluded that most of the decrease of OTA was due to the degradation activity by the fungus. However, the amount of OT α appeared lower than expected, even though we had already observed in a previous work (Gallo et al., 2012) that the accumulation of OT α as degradation product was less than 30% equivalent to the initial amount of OTA.

Increased levels of expression after the addition of OTA were observed for some proteases genes especially in Δ AcOTAnrps and Δ AcOTAhal, mutant strains. In particular, AcProA and AcProB showed an increase of activation after 12h in Δ AcOTAnrps (data not shown). All the genes considered reached a higher transcription level after 24h in Δ AcOTAhal with a maximum fold change detected for AcProD (Figure 3).

		3d	+12h	+24h
WT	OTA	0.02	0.13	0.24
WT+OTA	OTA	0.02 (+30)	10.2	5.8
	Ota		0.22	0.06
Δ AcOTApks +OTA	OTA	0 (+30)	10.9	10.3
	Ota		0.07	0.09
Δ AcOTAnrps +OTA	OTA	0 (+30)	10.7	9.5
	Ota		0.09	0.16
Δ AcOTAhal +OTA	OTA	0 (+30)	10.8	10
	Ota		0.12	0.08

Table 3 OTA and OT α production (nmol) of ITEM 5010 (wild type and mutant strains) after adding exogenous OTA (30 nmol) after 3 days of growth.

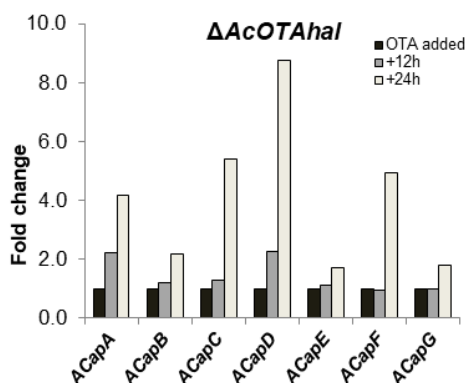


Figure 3 Expression levels of proteases (from AcProA to AcProG) in Δ AcOTAhal at 3d, +12h and +24h, after OTA addition.

7. Conclusion

In the first part of the present PhD project, we aimed to clarify the behaviour of *A. carbonarius* under a possible climate change scenario. Our *in vitro* study simulated a real

field condition in the Apulia region, South Italy, during the month preceding harvest which is considered the appropriate moment for the contamination by *A. carbonarius* in grapes. On the basis of our results, we can assume that increasing temperature to +37°C, a diurnal temperature that we tested, may not be associated to an increase of the OTA risk. On the other hand, the increase of CO₂ air concentration to 1000 ppm may lead to a shift in the distribution of ochratoxigenic risk areas.

By the way, it would be worthy to perform also experiments on grape berries, in order to get data even more reliable than on a grape medium.

This type of ecophysiological study can be useful to develop predictive models which can be utilized in evaluating the relative risk of mycotoxin contamination in the future.

Furthermore, during this PhD project we also focused on the identification of proteolytic enzymes as a possible strategy to degrade OTA into a less toxic compound. The expression profile of different *A. carbonarius* proteases was analysed to establish a correlation with the fungal OTA degrading activity. Some of these proteases were further investigated to characterize their specific enzymatic activity with the final aim to identify a protein able to reduce the ochratoxin A content in grape-derived products, such as wine.

8. Nomenclature

a_w: water activity; HPLC-FLD: High-performance liquid chromatography with fluorescent detector; qRT-PCR: quantitative real time polymerase chain reaction.

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Production of Natural Aroma Compounds by Biocatalysis in Water with Acyltransferase

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The present PhD thesis deals with the development of new methods for the production of natural aroma compounds using acyltransferase from *Mycobacterium smegmatis* (MsAcT). This is an interesting enzyme for industrial applications due to its hydrophobic active site, which allows the catalysis in aqueous environment, different from the other common hydrolases. The main purpose of this thesis work is the development of preparative biotransformation of alcohols in esters with flavour property using bioreactor system in continuous flow.

Produzione di composti aromatici naturali per biocatalisi in acqua tramite aciltransferasi

Lo scopo di questa tesi di dottorato è sviluppare nuovi metodi per la produzione di composti aromatici naturali utilizzando un'aciltransferasi isolata da *Mycobacterium smegmatis* (MsAcT). La MsAcT è un enzima interessante da utilizzare in campo industriale, poiché a differenza delle idrolasi più comuni, ha un sito catalitico idrofobico e per questa ragione è in grado di lavorare in ambiente acquoso. L'obiettivo principale di questo progetto di tesi è mettere a punto delle biotransformazioni in sistema di bioreattori di flusso continuo per la produzione di esteri con proprietà aromatiche a partire da alcoli.

Key words: flavor esters, aroma, enzymatic acylation, biocatalysis, acyltransferase

1. Introduction

Esters play a significant role in the food industry, they are among the most important and versatile components of natural flavors and fragrances in food, drinks and cosmetics (Burdock, 2010). Their preparation starting from natural substrates and using bioprocesses (e.g., fermentation or enzymatic reactions) is appealing, since the final product can be labelled and commercialized in EU and USA as natural (Berger, 2009). Therefore, new biotechnological approaches for obtaining flavors are highly demanded as long as they are efficient and sustainable (Hofmann and Fraatz, 2018). Many flavor/fragrance esters can be enzymatically obtained using lipases that catalyze esterification, transesterification or interesterification reactions in media (e.g., organic solvents) characterized by low water activity (Sà, 2017). The presence of water is critical for the equilibrium of lipase-catalyzed reactions in organic media, strongly limiting the overall yields; in fact water may compete as a nucleophile with the alcohol in the attack of the acyl-enzyme intermediate, thus favoring hydrolysis over transesterification (Klibanov, 2001), and for this reason water must be avoided or removed during the reaction for achieving high yields.

MsAcT is able to catalyze synthetic reactions in aqueous medium because of its highly hydrophobic active site, which enables efficient transesterification reactions even at 99% v/v buffer solution, differing from others hydrolases that may catalyze synthetic reactions only in non-aqueous media (Leeuw, 2017). This unique feature of MsAcT

among hydrolases may open new opportunities to conduct synthetic biocatalysis in aqueous media, conferring importance and practical interest at industrial level, once is not necessary to use organic solvents as media (Wiermans, 2013). The possibility of applying this enzyme for catalysis in flow reactor allows more sustainability of organic synthesis and advantages compared to the batch methods, including increased safety, better control of reaction parameters, possibility of automation and in-line purifications (Tamborini, 2018). In this work, we have explored the potential of MsAcT for flavor ester preparation.

In accordance with the PhD thesis project previously described (Chiarelli, 2018), this oral communication reports the main results of the following four activities directed to:

A1) purification, quantification and determination of the activity of the MsAcT by *p*-nitrophenyl acetate;

A2) determination of molar conversion several different flavor esters derived from transesterification of primary alcohols with ethyl esters bearing short-chain acyl groups;

2. Materials and Methods

In this project, a codon-optimized synthetic gene MsAcT from *Mycobacterium smegmatis* including a C-terminal his-tag was cloned into vector pET26b (+). The cloned vector was transformed in *E.coli* BL21 (DE3). A single colony was used for an overnight preculture in Terrific broth (TB) with kanamycin at 37°C. A shake flask with same conditions of broth and antibiotic was inoculated with agitation at 37°C until the OD₆₀₀ reached 0.6 and followed cold shock. Expression of the MsAcT gene was then induced by addition of IPTG and the culture was incubated at 28°C overnight. The cells were harvested by centrifugation, pellets were washed and centrifuged again, the supernatants were removed and the wet weight of the cells was determined. The pellets were resuspended in 50mM Tris HCl buffer, pH 8 with 6.6mM of imidazole, in a volume 5 times the wet weight. The cells was placed on ice, and sonicated. The crude extract was ultracentrifuged. The supernatant was filtered and loaded on Ni-NTA column, equilibrated in 6.6 mM of imidazole and eluted by linear gradient using 50 mM Tris HCl buffer, pH 8 with 6.6mM imidazole and 250 mM imidazole. Eluate containing the MsAcT was quantified by Bradford assay and identified by SDS-PAGE analysis, and dialyzed at 4°C with 100mM potassium phosphate buffer pH 8.0.

Activity was spectrophotometrically evaluated at 400 nm by determining the release of *p*-nitrophenol after hydrolysis of *p*-nitrophenylacetate at 25 °C in a half-microcuvette (volume 1 mL) for 2 min. One unit (U) of activity is defined as the amount of enzyme which catalyzes the consumption of 1 μmol of *p*-nitrophenylacetate per minute. Reference conditions were: 0.1 mg/mL *p*-nitrophenylacetate, 0.1% v/v EtOH, different amounts of MsAcT in 100 mM phosphate buffer, pH 8.0. The specific activity of the purified enzyme was 110 U/mg.

The kinetic of biotransformations were determined with different concentrations of substrate, 10% (v/v) of acyldonor was added, 1mg/mL of the enzyme purified in potassium phosphate buffer (100 mM; pH 8.0), the biphasic reaction mixture was left under magnetic stirring at 25 °C. The reactions were stopped by extraction with ethyl acetate (1:1). The organic phase was dried over Na₂SO₄, and measured by gas chromatography (GC).

3. Results and Discussion

The reaction between geraniol and ethyl acetate (EtOAc) using the acyltransferase from *Mycobacterium smegmatis* (MsAcT) as biocatalyst was studied in a two-liquid phase system composed by water and EtOAc; the product (geranyl acetate) presents a green type flavor and a floral type odour (Burdock, 2010). Different parameters (pH, temperature and enzyme concentration) were optimized, while keeping the amount of geraniol (0.065 mM) and the phase ratio between water and EtOAc (9:1) fixed; a Multisimplex optimization design was employed (Romano, 2011) using initial rate and molar conversions after 30 min as response variables. The best results (corresponding to a conversion >98% after 30 minutes) were obtained using 1.0 mg/mL of MsAcT at pH 8.0 and 25 °C; under these conditions the production of the ester was studied using different initial geraniol concentrations (Table 1).

Table 1 Acetylation of geraniol at different initial concentrations using MsAcT (1.0 mg/mL, 110 U/mL) in the presence of EtOAc (10% v/v) in phosphate buffer (0.1 M, pH 8.0) at 25 °C.



Geraniol (mM)	Initial rate ($\mu\text{mol}/\text{mg min}$)	Maximum conversion ^a (%)	Maximum product yield (mg/mL)	Time ^b (h)
100	2.25	> 98	19.6	2
200	4.56	> 98	39.2	24
250	6.36	98	48.1	24
300	6.57	95	55.9	24
400	7.68	79	62.0	48
500	7.65	68	66.7	48

^a As determined by gas chromatography. ^b Time refers to maximum conversion observed.

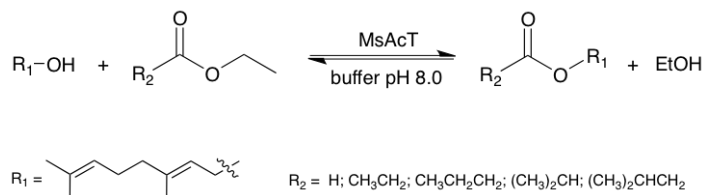
The highest rates of geranyl acetate synthesis were achieved between 400-500 mM geraniol concentration, while almost quantitative yields were obtained only at alcohol concentrations lower than 300 mM. Possible product inhibition was evaluated by assessing the activity of MsAcT in the presence of increasing concentrations of EtOH and geranyl acetate (0-500 M); the activity of the enzyme was assayed (with *p*-nitrophenyl acetate) in the presence of concentrations of EtOH between 250 and 500 mM showed that the enzyme was 40-50% inhibited, while no significant effects were observed with geranyl acetate.

The best compromise between rates and conversion was observed starting from 250 mM geraniol, allowing for 98% conversion (48.1 mg/mL analytical yield) after 24 hours. Notably, good conversion (68%) after 48 hours was achieved even when the biotransformation was carried out starting from 500 mM geraniol, allowing for the accumulation of 66.7 mg/mL of geranyl acetate.

Acetylation of geraniol was performed on semipreparative scale (10 mL) starting from 250 mM alcohol concentration; the reactions were stopped and worked-up when conversions reached the maximum yield, allowing for the recovery of 442 mg of geranyl acetate (90% recovered yield).

Acylation of geraniol were then studied with other acyl donors, which varied in acyl chain length; the results of the biotransformations carried out under optimized conditions (alcohol 250 mM, acyl donor 10% v/v, enzyme 1.0 mg/mL, buffer pH 8.0, 25 °C) are summarized in Table 2.

Table 2 Acylation of geraniol (250 mM) using MsAcT (1.0 mg/mL, 110 U/mL) in the presence of different ethyl esters (10% v/v) in phosphate buffer (0.1 M, pH 8.0) at 25 °C.



Acyl donor	Product flavor property ¹	Initial rate (μmol/mg min)	Maximum conversion ^a (%)	Time ^b (h)
Ethyl formate	rose	7.69	> 98	3
Ethyl propionate	fruity	1.51	86	48
Ethyl butyrate	fruity/apricot	1.15	62	48
Ethyl isobutyrate	rose/apricot	1.42	74	48
Ethyl isovalerate	rose/sweet apple	0.88	38	48

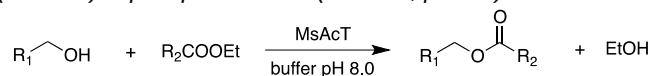
^a As determined by gas chromatography. ^b Time refers to maximum conversion observed. ¹ (Burdock, 2010).

The ability of acting as acyl donor decreased by increasing the acyl chain length; geranyl formate was obtained in quantitative yield after 1-3 h, whereas, with geranyl propionate and butyrate, the maximum yields (ranging between 65-98%) were reached only after 1-2 days.

Enzymatic acylation was then studied with different primary alcohols and different acyl donors; all the reactions studied were aimed at preparing esters used as flavor or fragrance components (Table 3). Biotransformations were carried out starting from 100, 250, and 500 mM alcohol concentrations.

Formylation and acetylation of all the tested primary alcohols allowed for quantitative or almost quantitative conversions with high rates ($\geq 90\%$ within 30-60 min) when the initial concentration of alcohols was kept between 100 and 250 mM. When the biotransformation was carried out with higher alcohol concentration (500 mM), still high molar conversions of the flavor-esters were observed (from 80 up to $>98\%$), albeit with longer reaction times. Slower reactions were generally found with acyl donors of increased chain length (propionyl- and butyryl-). The only biotransformation occurring with relatively low yields (30-70% depending on the initial concentration) was the propionylation of *n*-hexanol to give *n*-hexyl propionate; interestingly, in this case hydrolysis of the formed ester was predominant after reaching its maximum accumulation, as noticeable from an increase of the alcohol concentration (data not shown).

Table 3 *Acylation of different primary alcohols (starting 100-500 mM concentrations) with short chain ethyl esters using MsAcT (1.0 mg/mL, 110 U/mL) in the presence of different ethyl esters (10% v/v) in phosphate buffer (100 mM, pH 8.0) at 25 °C.*



Alcohol R1	Initial alcohol (mM)	Acyl donor R2	Product flavor property ^a	Maximum conversion ^b (%)	Time ^a (h)
Isoamyl alcohol	100	Ethyl formate	black currant/currant	93	0.5
	250			93	0.5
	500			86	1
	100	Ethyl acetate	banana/pear	94	0.5
	250			95	0.5
	500			88	0.5
	100	Ethyl propionate	Pineapple/apricot	97	0.5
	250			97	1
	500			95	1
Hexanol	100	Ethyl butyrate	fruity	94	24
	250			93	24
	500			92	24
	100	Ethyl formate	apple	97	0.5
	250			97	1
	500			88	3
	100	Ethyl acetate	fruity/pear	97	0.5
	250			95	1
	500			90	3
Benzyl alcohol	100	Ethyl propionate	earthy/metallic-fruity	72	1
	250			50	1
	500			35	2
	100	Ethyl butyrate	apricot/pineapple	94	2
	250			96	6
	500			95	24
	100	Ethyl formate	floral-fruity/apricot	92	0.5
	250			84	1
	500			86	3
Phenethyl alcohol	100	Ethyl acetate	jasmine	92	0.5
	250			90	1
	500			87	3
	100	Ethyl propionate	floral-fruity/peach	96	0.5
	250			95	1
	500			89	3
	100	Ethyl butyrate	plum/pear	95	8
	250			92	8
	500			95	24
Cinnamyl alcohol	100	Ethyl formate	hyacinth	95	0.5
	250			88	2
	500			86	3
	100	Ethyl acetate	rose/raspberry	96	0.5
	250			91	0.5
	500			82	0.5
	100	Ethyl propionate	red rose/strawberry	97	0.5
	250			97	0.5
	500			96	0.5
	100	Ethyl butyrate	rose/honey	97	4
	250			97	8
	500			90	24
	100	Ethyl formate	fruity/apple	>98	0.5
	250			>98	4
	500			82	24
	100	Ethyl acetate	floral/pineapple	>98	1
	250			>98	4
	500			96	24
	100	Ethyl propionate	fruity/woody	>98	1
	250			>98	5
	500			97	24
	100	Ethyl butyrate	fruity/honey	>98	16
	250			92	24
	500			80	48

^aAs determined by gas chromatography. ^b Time refers to maximum conversion observed. ¹ (Burdock, 2010).

4. Conclusions and Future Perspectives

In conclusion, a highly efficient preparation of different flavor-esters was achieved using the acyltransferase from *Mycobacterium smegmatis* (MsAcT), which catalyzes the acylation of different primary alcohols in aqueous systems. The results collected so far indicate that the biotransformation can be applied not only to the production of acetate esters, but can be expanded to a range of other acyl donors (e.g., ethyl formate, propionate, butyrate), useful for the formation of different flavor/fragrance esters, making this process strongly versatile. Notably, all the primary alcohols tested were accepted as substrates, even at higher concentrations (up to 500 mM); preparation of geranyl acetate was carried out on semipreparative scale (10 mL) starting from 250 mM alcohol concentration, allowing for the recovery of 44.2 mg/mL (geranyl acetate) of the product. Biocatalysis is sometimes perceived to be inefficient compared with conventional chemical processes because of the low productivity, mostly due to substrate/product inhibition of the enzymatic activity which often occurs at somewhat low concentrations. To become an industrially attractive technology. A biocatalytic process must be engineered to improve space time yields (Sheldon, 2017): in this work we have described that the use of relatively low amount of MsAcT allowed the conversion of remarkably high substrate concentrations with good yields. The proposed enzymatic method may pave the way for an efficient and environmentally sustainable preparation of natural esters; immobilizing the enzyme may further intensify the biotransformation and carry out continuous processes in suited reactors (Tamborini, 2018), thus making it appealing at industrial level.

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Cradle-to-Grave Environmental Profile of Dry Pasta: Assessment and Mitigation Measures

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This PhD thesis research project was firstly aimed at assessing the cradle-to-grave carbon footprint of organic durum wheat semolina dry pasta. Since pasta cooking was the most impacting life cycle stage, a minimum energy consumption cooking system was developed and tested to keep the cooked pasta quality unchanged. The overall environmental profile of dry pasta was assessed in compliance with the IMPACT 2002+ methodology to provide a basis for a more sustainable production.

Profilo ambientale complessivo della pasta secca: stima ed azioni di mitigazione

Questa tesi di dottorato è stata inizialmente finalizzata a valutare l'impronta di carbonio dalla culla alla tomba di pasta secca biologica. La cottura della pasta è risultata la fase più impattante del ciclo di vita e si è messo a punto un sistema di cottura di minimo consumo energetico atto a garantire la qualità della pasta cotta. L'impatto ambientale complessivo della pasta secca è stato valutato in base alla metodologia IMPACT 2002+ al fine di prefigurarne una produzione più eco-sostenibile.

Key words: Carbon footprint; cooking water-to-dried pasta ratio; energy consumption; IMPACT 2000+; Overall Weighted Damage Score; PAS 2050.

1. Introduction

In accordance with the PhD thesis project previously described (Cibelli, 2017), this oral communication described the main results of the following three activities directed to:

A1) Assess the cradle-to-grave carbon footprint of 1 kg of organic durum wheat semolina dry pasta (CF_{CG}) packed in 0.5-kg polypropylene (PP) bags using the *Publicly Available Specification* (PAS 2050) method (BSI, 2008), as well as the contribution of each product life cycle stage.

A2) Develop a novel sustainable pasta cooking system to minimize the energy and water needs and assure an appropriate instrumental and sensorial quality of cooked pasta.

A3) Characterize the environmental profile of organic dry pasta using the IMPACT 2002+ methodology (Jolliet et al., 2003) to suggest a more sustainable dried pasta production.

2. State-of-the-art

Since the current food system is regarded as ecologically unsustainable, the food and drink industry is looking for improving its environmental performance. Dry pasta is a basic food mostly produced and consumed worldwide. The cradle-to-grave carbon footprint (CF_{CG}) of 1 kg of dried pasta was estimated as equal to 1.93 or 3.03 kg carbon dioxide equivalents (CO_{2e}), depending on the use of a gas or electric stove, respectively (Barilla, 2017). Durum wheat cultivation represented 32 or 20 % of CF_{CG} , while pasta cooking 31 or 56 % of CF_{CG} . Such a great contribution was due to the great volume of water (i.e., 10-12 L) that has to be boiled to cook 1 kg of dried pasta. Thus, the

greenhouse gas (GHG) emissions associated with the cooking of pasta should be primarily reduced.

The environmental performance of food and drink production may be assessed by various standard methods (Cimini and Moresi, 2018). Some of them (i.e., PAS 2050; Bilan Carbone®; GHG Protocol) make use of only the impact category of climate change (CC) and give no hint about the overall environmental impact of the products, even if the emissions from direct land-use changes over the previous 20 years are generally included. Other standard methods evaluate seven (i.e., *Life Cycle Assessment*, LCA, and *Environmental Product Declaration*, EPD®), 14 (*Product Environmental Footprint*, PEF) or 15 (IMPACT 2002+) mid-point impact categories to measure the potential impacts on the human health (HH), ecosystem quality (EQ), and depletion of natural resources (RD). Any generic impact category (IC_i) is estimated by summing up the release to air, water or soil (Ψ_i , expressed in mass, energy, mass-km basis) associated to the system boundaries times its corresponding characterization factor ($F_{i,j}$). There is, thus, a strong need for reliable databases to assess a product life cycle environmental performance, as observed by the food and drink companies involved in several PEF pilot tests (FoodDrinkEurope, 2017).

3. Materials and methods

3.1 Assessment of the environmental impact of dried pasta

The life-cycle analysis was sequentially performed in compliance with the PAS 2050 (BSI, 2008) and IMPACT 2002+ (Joliet et al., 2003) standard methods. All the four LCA canonical stages complied with the recommendations of the ISO 14040 series, and were referred to a functional unit consisting of 1 kg of dried pasta, produced in a medium-sized pasta factory located in the South of Italy in the years 2016 and 2017, and packed in 0.5-kg PP bags. The system boundaries for this study included the upstream, core and downstream processes (Fig. 1). The former implied the organic durum wheat cultivation, and fertilizer, electricity, fuel, and packaging material production. In the core processes wheat milling, and pasta making and packaging were considered. The downstream ones included the distribution, use and post-consumer waste management phases. Primary data were collected from the above pasta factory, while secondary ones from several technical reports and databases embedded in the professional tool SimaPro 8.5.2 (PRé Consultants, Amersfoort, NL), as detailed by Cimini et al. (2019d). The CF_{CC} of the functional unit chosen was estimated by accounting for the 100-year time horizon global warming potentials (IPCC, 1996). The others impact categories of the IMPACT 2002+ method were calculated using the specific characterization factors $F_{i,j}$.

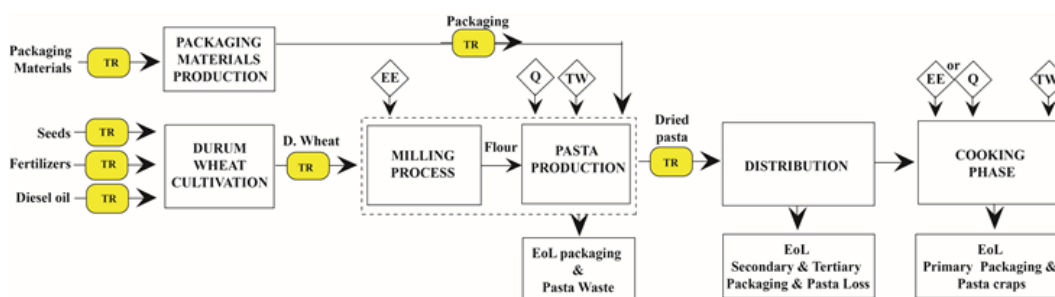


Figure 1 Dry pasta system boundaries, as adapted from Cimini et al. (2019d): EE, electric energy; Q, thermal energy; TR, transport; TW, process water.

3.2 Assessment of cooked pasta quality

Several commercial brands of durum wheat semolina dried pasta of the *Spaghetti* and *Penne rigate* types were used. Their labelled raw protein content and recommended cooking time ranged from 108 to 139 g kg⁻¹, and 9 to 10-12 min, respectively. Their composition was previously reported (Cimini et al., 2019abc), while the pasta cooking system and procedure used, as well as the cooking water and energy balances during pasta cooking, were described by Cimini and Moresi (2017) and Cimini et al. (2019c). Several cooking tests were performed by varying the water-to-pasta ratio (WPR) from 2 to 12 L kg⁻¹. To limit pasta adhesion during cooking, a mechanical stirrer was kept rotating at 50 rev min⁻¹ for 30 s, but resting for the subsequent 90, 60 or 30 s at WPR equal to 6, 4, or 3 L kg⁻¹, respectively. Cooked pasta properties were characterized by assessing the relative water uptake (WU) and cooking loss, starch gelatinization degree, hardness at 90 (F₉₀) or 98 (F₉₈) % deformation using a Universal Testing Machine UTM mod. 3342 (Instron Int. Ltd., High Wycombe, UK) equipped with a 1000-N load cell, and a few sensory attributes (i.e., Firmness, Stickiness, Bulkiness, Overall Cooking Quality) by a panel of six trained judges, as detailed by Cimini et al. (2019abc). The carbon footprint of pasta cooking (CF_{PC}) was evaluated by multiplying the overall electric energy consumed by its corresponding emission factor of 323.6 g CO_{2e} kWh⁻¹ (ISPRA, 2018). All data were shown as average ± standard deviation, and analyzed by Tukey test at a probability level (α) of 0.05.

4. Results and discussion

4.1 The cradle-to-grave carbon footprint of organic dry pasta

As shown in Fig. 2, the CF_{CG} of 1 kg of organic dry pasta amounted to ~1.8 kg CO_{2e} kg⁻¹, while the contribution of all the life cycle phases was ranked as follows: field phase (666 g CO_{2e} kg⁻¹), home pasta cooking (651 g CO_{2e} kg⁻¹), pasta production and packaging (201 g CO_{2e} kg⁻¹), transportation (148 g CO_{2e} kg⁻¹), packaging material manufacture (107 g CO_{2e} kg⁻¹), durum wheat milling (52 g CO_{2e} kg⁻¹), end of life of packaging materials (33 g CO_{2e} kg⁻¹) and pasta losses (19 g CO_{2e} kg⁻¹). Wheat milling by-products, and pasta making and packaging wastes were used as cattle feed and gave rise to a CO_{2e} credit of 69 g CO_{2e} kg⁻¹ (Cimini et al., 2019d). Thus, the sustainability of dry pasta might be improved by reducing the contribution of the consumer, field, and transportation life cycle phases.

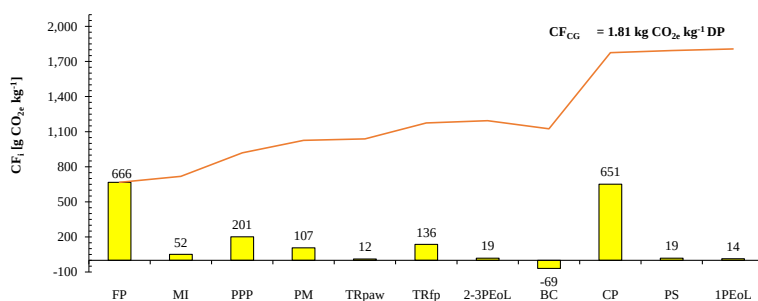


Figure 2 Contribution of the different life cycle stages to the CF_{CG} of 1 kg of dried organic pasta, as extracted from Cimini et al. (2019d), and its cumulative score (see continuous line): 1PEoL, primary packaging end of life; 2-3PEoL, secondary and tertiary packaging end of life; BC, byproduct credit; CP, consumer phase; FP, field phase; MI, milling phase; PM, packaging material manufacture; PPP, pasta production and packaging; PS, pasta scraps; TRfp, transport of final product; TRpaw, transport of packaging and auxiliary materials, and wastes.

4.2 Eco-sustainable pasta cooking system

The effect of WPR on the cooking quality of long and short pasta types was assessed using an induction hob (Cimini et al., 2019abc) or a gas-fired one (Cimini et al., 2019e).

The induction hob was set at its maximum power rating ($P_H=2$ kW) to heat water up to the boiling point, and at a power rating (P_C) of just 0.4 kW during pasta cooking (Cimini and Moresi, 2017; Cimini et al., 2019abc). The main cooking quality parameters (WU , F_{90} or F_{98}) of two commercial pasta types, both with a raw protein content of about 135 g kg^{-1} , as well as the specific pasta cooking energy consumption (e_{PA}) and carbon footprint (CF_{PC}) were compared at two WPR levels, as shown in Fig. 3. As WPR was reduced from 10 to 3 L kg^{-1} , the water uptake and cooking loss (data not shown for brevity) by cooked penne was practically constant, while those for cooked spaghetti reduced by circa 17% (Fig. 3a). On the contrary, cooked pasta hardness (F_{90} or F_{98}) was not statistically different at $\alpha=0.05$ (Fig. 3b). In all cases tested, the energy efficiency of the induction hob (η_C) was practically constant ($67\pm 3\%$), while both e_{PA} and CF_{PC} decreased from 1.55 ± 0.04 to $0.55\pm 0.01 \text{ Wh g}^{-1}$ (Fig. 2c), and from 0.55 to $0.19 \text{ kg CO}_{2e} \text{ kg}^{-1}$ (Fig. 2d), respectively. At $WPR=3 \text{ L kg}^{-1}$, the mixing energy consumed to avoid pasta sticking ($\sim 0.0011 \text{ Wh g}^{-1}$) was definitively negligible with respect to e_{PA} . Thus, instead of cooking 1 kg of dried pasta in 10 L of water, as commonly suggested by pasta manufacturers, the cooking procedure tested with $WPR=3 \text{ L kg}^{-1}$ would about halve the climate change impact of the current Italian consumption pasta of dried pasta ($\sim 1.61 \times 10^6 \text{ Mg yr}^{-1}$).

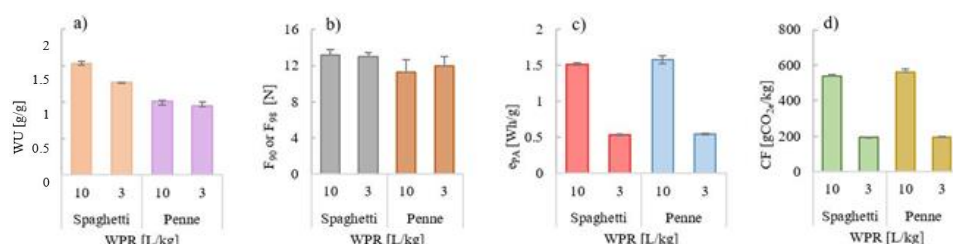


Figure 3 Effect of WPR on the **a)** relative water uptake (WU); **b)** hardness at 90 or 98% deformation (F_{90} or F_{98}), **c)** specific pasta cooking energy consumption (e_{PA}), and **d)** carbon footprint (CF_{PC}) for the long and short pasta formats examined.

Table 1 Environmental profile of 1 kg of dried organic pasta as estimated using the IMPACT 2002+ method: percentage contribution of the different life cycle stages (symbols as in Fig. 2), and overall score of each mid-point impact category (IC_i).

Impact category (IC_i)	Life Cycle Phase Contribution (%)					IC_i Score	Unit
	FP	MI+PPP	PM	TR	CP		
Carcinogens	8.8	46.3	35.5	1.7	7.8	1.02×10^{-2}	kg $C_2H_3Cl_e$
Non-carcinogens	40.5	16.7	15.0	19.6	8.2	1.11×10^{-2}	kg $C_2H_3Cl_e$
Respiratory inorganics	76.1	7.4	3.1	4.2	9.2	1.85×10^{-3}	kg $PM_{2.5e}$
Respiratory organics	47.8	13.3	10.9	6.4	21.6	3.16×10^{-4}	kg C_2H_{4e}
Ionizing radiation	63.8	11.4	3.9	7.5	13.3	9.34	Bq $^{14}C_e$
Ozone layer depletion	36.2	24.3	5.8	16.4	17.2	1.23×10^{-7}	kg CFC-11 _e
Aquatic ecotoxicity	23.0	14.3	36.7	14.5	11.5	58.4	kg TEG water
Terrestrial ecotoxicity	25.2	15.7	17.8	36.0	5.3	28.1	kg TEG soil
Terrestrial acidification/nutrication	91.0	3.0	1.0	1.1	3.9	0.154	kg SO_{2e}
Aquatic acidification	85.6	4.7	1.7	1.5	6.5	2.1×10^{-2}	kg SO_{2e}
Aquatic eutrophication	43.8	14.5	11.6	7.0	23.1	1.13×10^{-4}	kg PO_4^{3-}
Land occupation	99.6	0.0	0.3	0.0	0.1	3.8	m ² org. arable
Global warming	28.5	16.1	6.3	7.5	41.5	1.45	kg CO_{2e}
Non-renewable energy	28.2	16.4	6.9	6.9	41.7	24.3	MJ primary
Mineral extraction	38.3	0.8	4.6	0.3	56.0	0.003	MJ surplus

4.3 The environmental profile of organic dry pasta

Table 1 shows the environmental profile of 1 kg of dry pasta as resulting from the framework provided by the Impact 2002+ V2.14 life cycle impact assessment method embedded in SimaPro 8.5.2. The environmental impact of the great majority of the mid-point ICs firstly arised from the field phase (i.e., non-carcinogens, respiratory inorganics and organics, ionizing radiation, ozone layer depletion, terrestrial and aquatic acidification and nitrification), and secondly from the cooking one (i.e., global warming, non-renewable energy and mineral extraction), as pointed out by the underscore and italics characters in Table 1, respectively. Even if this method referred to a time horizon of 500 years, the global warming effects were still dominated by the cooking phase. The 15 ICs shown in Table 1 were then grouped into four damage categories (DCs) to underpoint the environmental compartments damaged by dry pasta in its life cycle. The effect on human health (HH) derived from different ICs (i.e., carcinogens, non-carcinogens, respiratory inorganics and organics, ionizing radiation, ozone layer depletion) and was expressed in disability-adjusted life years (DALY), these indicating the amount of years (of life) lost due to premature mortality and/or disability, after an exposure to toxic chemicals. The impact on the Ecosystem Quality (EQ) resulted from several ICs (i.e., aquatic and terrestrial ecotoxicity, acidification, eutrophication, land use) and was measured in potentially disappeared fraction (PDF) of biological species that have a high probability of not surviving in the geographical area where dry pasta is produced. Since the impact of CC on EQ and HH is not accurate enough to derive reliable damage characterization factors, the global warming was considered as a stand-alone endpoint category by referring to a 500-yr time horizon to account for both short- and long-term effects (Jolliet et al., 2003). Finally, the environmental impact resulting from the depletion of non-renewable resources (RD) was measured by the additional primary energy required to extract a unit of mineral and of total non-renewable primary energy for energy carriers. As shown by the underscore and italics characters in Table 2, the damage impact on HH and EQ mainly derived from the field phase, while that on CC and RD from the cooking one, respectively. The assessment was extended to the endpoint approach via the phases of *normalization* and *weighing*. The HH, EQ, CC and RD normalization factors of 0.0071 DALY, 13,700 PDF m²yr, 9,900 kg CO_{2e}, and 152,000 MJ per person by year were obtained as the ratio between the overall results for all pollutants, potentially damaged fraction of species, total GHG emissions and non-renewable energy consumption in Western Europe, and Western European population, respectively. Then, a default weighting factor of one was used to aggregate such damage categories (Table 2). EQ was the damage category mostly affected (39.9% of the overall weighed score), followed by HH (23.1%) and RD (19.3%), while CC was that suffering the lowest environmental damage (17.7%). The dry pasta life cycle phase exerting the greatest percentage contributions to the overall weighted damage score (829 μ pt) was the cultivation one (65.7 %), followed by the cooking phase (17.7 %), grain milling and pasta making (8.3 %), transportation (4.4 %), and packaging material manufacture (3.9 %).

Table 2 IMPACT 2002+ damage assessment related to the production of 1 kg of dried organic pasta packed in 0.5-kg PP bags: percentage contribution of the different life cycle stages (symbols as in Fig. 2), single (SS_i) and weighted scores (WS_i) of each damage category (DC_i), and overall weighted score (OWS).

Damage category (DC _i)	Life Cycle Stages Contribution (%)					SS _i	Unit	WS _i (μ pt)
	FP	MI+PPP	PM	TR	CP			
Human Health (HH)	<u>73.8</u>	8.5	4.1	4.5	9.2	1.36x10 ⁻⁶	DALY	191.4
Ecosystem Quality (EQ)	<u>95.6</u>	0.9	1.2	1.8	0.5	4.53	PDF m ² yr	330.7
Climate Change (CC)	28.5	16.1	6.3	7.5	<u>41.5</u>	1.45	kg CO _{2e}	146.5
Resource Depletion (RD)	28.2	16.4	6.9	6.9	<u>41.7</u>	24.3	MJ primary	160.1
Overall Weighted Score (OWS)	65.7	8.3	3.9	4.4	17.7			828.8

4.4 Effect of some mitigating options on dry pasta environmental profile

To improve the sustainability of dry organic pasta, the only GHG emissions of the most critical hot spots were reduced as follows. Fig. 4a shows that the eco-sustainable pasta cooking procedure (ESCP) mentioned above ($WPR=2 \text{ L kg}^{-1}$ and $P_C=0.4 \text{ kW}$) allowed the CF_{CG} to be reduced by 29% with respect to the reference case (RC). Use of organic crop rotation (OCR) with a crop yield of 7.5 Mg ha^{-1} and durum wheat carbon footprint of $0.36 \text{ kg CO}_{2e} \text{ kg}^{-1}$, as tested in Emilia-Romagna by Ruini et al. (2013), enabled the CF_{CG} to be decreased by another 13 %. Both these mitigating options allowed the CF_{CG} to be reduced by 42 % (Fig. 4a). Further options were tested by Cimini et al. (2019d). Secondly, the ESCP yielded ca. 31 % reduction in the DCs of CC and RD, but exerted a limited lessening in those of HH (2.0 %) and EQ (0.1 %). The overall weighted damage score decreased from 829 to 731 μpt (Fig. 4b). Use of the above OCR too yielded a reduction of 46 % in the DC of EQ, and of just 5.0-6.5% in those of RD, CC and HH. Altogether, both these mitigating actions allowed the OWS to be decreased to circa 550 μpt (Fig. 4b).

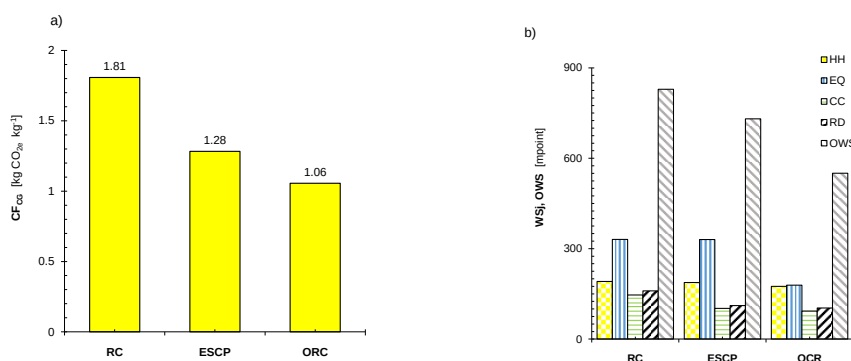


Figure 4 Marginal abatement curves of the (a) CF_{CG} and (b) weighted scores (WS_i) of the damage categories of HH, EQ, CC, and RD, and overall weighted damage score (OWS) of 1 kg of dry organic pasta as resulting from the mitigation strategies (ESCP, and OCR) described in the text.

5. Conclusions and Future Perspectives

The business-to-consumer carbon footprint (CF_{CG}) of 1 kg of dry pasta made of decorticated organic durum wheat semolina was estimated and primarily and secondarily controlled by the use and field phases. Since the cooking energy requirements accounted for a significant share of the energy consumed along the product life cycle, a more eco-sustainable cooking practice was developed to limit energy and water usage. The environmental profile of the product was assessed in compliance with the IMPACT 2002+ methodology and the ecosystem quality resulted to be the damage category mostly affected. Among the product life cycle phases, the cultivation and cooking ones exerted the highest contributions. By resorting to the eco-sustainable pasta cooking procedure developed here and to the most effective organic crop rotation tested so far, it was possible to cut CF_{CG} and OWS by 42 and 34 %, respectively. Since the characterization of the environmental profile of a single product is quite expensive, and the climate change impact category is by far more reliable than all the other ones accounted for in this PhD thesis, the carbon footprint assessment seems to be the cheaper tool to identify the major hotspots of the product life cycle stages. Thus, it is probably the best method to start improving the sustainability of the 99 % of the food and beverage small- and medium-sized enterprises. Future research activities should, on one side, be addressed to develop

novel energy-saving pasta cookers capable of replacing the domestic appliances and cookware sets used nowadays, and, on the other one, drive the pasta manufacturers to evaluate if the production of novel pre-gelatinized pasta products might be regarded as a feasible option to reduce the energy consumption and improve the environmental performance of dry pasta.

Acknowledgements

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Influence of Psychological Traits on the Acceptability of Healthy Foods

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The aim of the PhD thesis project is to investigate the influences of psychological traits and taste responsiveness on the acceptability of healthy foods characterized by “warning” sensations such as bitterness and astringency, using a multidisciplinary and multidimensional approach to food choices and preferences.

Influenza dei tratti psicologici e dell’acuità percettiva sull’accettabilità di prodotti salutari

Lo scopo del progetto di tesi di dottorato è quello di indagare l’influenza dei tratti psicologici e dell’acuità percettiva sull’accettabilità di cibi salutari caratterizzati da proprietà sensoriali “allarmanti” come l’amaro e l’astringente, utilizzando un approccio multidisciplinare e multidimensionale allo studio delle scelte e delle preferenze alimentari.

Key words: healthy foods, psychological traits, intensity, liking, bitterness, astringency

1. Introduction

In accordance with the PhD thesis project, this oral communication reports the main results of the following three activities directed to investigate the effect of psychological traits and taste responsiveness on:

- A1) on intensity and emotional responses to tastes in water solutions;
- A2) the familiarity with and the choice for phenol-rich foods and beverages;
- A3) the sensory and hedonic evaluation of real phenol-rich foods.

Food choice is influenced by many interacting factors in humans and the selection of a given food depends on the interplay of its intrinsic and extrinsic characteristics with person-related dimensions that are biological, physiological, psychological, and socio-cultural (Monteleone et al., 2017). This multicomplex process appears particularly relevant in the acceptability of healthy foods that are characterized at the same time by health benefits and warning sensations such as bitterness and astringency.

Bitterness is notoriously a sensory property for which humans have an innate dislike and aversion, as it represents potential sources of toxic compounds. Astringency also elicits negative consumer reactions when perceived at high intensities, probably because tannins may have anti-nutritional effects in animals and humans by reducing the digestibility of dietary proteins. Recent studies investigated the effect of basic tastes on emotions and on autonomic nervous system responses. Bitterness induced the strongest electrodermal, thermovascular and cardiac responses (Robin, 2012), evoked negative emotions (Robin et al., 2003) and elicited aggressive affective and behavioral responses (Sagioglou & Greitemeyer, 2014). However, several studies showed that responses to bitterness can be modulated by many conditions, as exposure to mild stressors, after which bitter substances were rated as more intense (Platte et al., 2013), highlighting the potential for a shift in the hedonic properties of food with change in affect. To our knowledge, the effect of astringency on arousal and emotions has not

been studied yet and the study of the effect of psychological traits on bitterness in water solutions need to be implemented.

Individuals substantially differ in chemosensory perception and the inherited capacity to perceive the bitterness of propylthiouracil (PROP) is considered a reliable broad marker for individual differences in taste responsiveness that may influence food preferences. PROP supertasters showed a lower preference of bitter vegetables and gave higher bitterness and astringency ratings for coffee, even if these did not significantly affect liking (Masi et al., 2015) or consumption. In general, results are inconsistent and the associations between taste abilities and food choice and perception need to be further explored.

Psychological traits play an important role in food preferences and choices. One key personality variable is food neophobia, originally defined as the reluctance to try or eat unfamiliar foods. Food neophobia could be considered an efficient adaptive strategy to avoid the risk of ingesting novel and potentially poisonous foods, but in the modern society it is considered a maladaptive behavior as it decreases diet variety, thus having potentially important nutritional consequences. High levels of food neophobia have been associated with reduced liking, preference and intake for fruits and vegetables (Knaapila et al., 2011). Neophobics (subjects high in food neophobia) perceived pungency and astringency in food products as more intense and liked the most pungent and astringent samples less than neophilics (subjects low in food neophobia) (Laureati et al., 2018; Spinelli et al., 2018). Differences were also detected in the autonomic nervous system activation: neophobics exhibited greater physiological arousal (pulse, skin conductance, respiration) when food stimuli were presented, while no significant differences were detected during the presentation of non-food stimuli compared to neophilics (Raudenbush & Capiola, 2012). Moreover, they exhibited reduced salivary responses regardless if the food was novel or familiar. As food neophobia is a conservative behavior, which keeps the organism's feeding behavior 'locked in on a safe track', it can be reasonably hypothesized that higher arousal in food neophobics could increase perceptual sensitivity via increased alertness when approaching food and that arousal could be unpleasant, therefore producing dislike of stimulus. These mechanisms need to be deeply investigated.

The aim of this PhD thesis project is to overcome the limitations of previous studies, as the sample size and the approach based on a limited perspective, and to take into account at the same time taste sensitivity, psychological and sensory and hedonic responses to foods based on evaluations of samples and not only of names or tastant in solution. The combination of sensory evaluations, implicit and physiological measurements would help to better understand the underlying mechanisms of taste perception and liking, and the role played by personality traits and PROP status in the acceptability of bitterness and astringency in water solutions and in foods.

2. Materials and Methods

2.1 Measuring psychological traits

Food neophobia, sensitivity to reward, to punishment and to disgust, sensation seeking (the need for varied, novel and complex sensations), alexithymia (the inability of individuals to identify and name their emotional states), private body consciousness (awareness of internal sensations), state and trait anxiety were measured using self-report questionnaires. Based on the percentile limits, participants were grouped into Low (1st quartile), Medium (interquartile) and High (3rd quartile) level of expression of each trait.

2.2 Measuring PROP taster status

PROP taster status was assessed using a 3.2mM PROP solution, prepared by dissolving 0.545 g/L of 6-n-propyl-2-thiouracil into deionized water (for example, see Prescott et al., 2004).

2.3 Measurement of intensity and emotional responses to tastes in water solutions (ws)

The effect of psychological traits and taste responsiveness on the perception of tastes in ws was investigated in two studies. In the first study (N=101), the perceived intensity of two series of bitterness (caffeine 0.00, 0.75, 1.50, 3.00, 6.00 g/L) and astringency (K Aluminum Sulfate 0.00, 0.40, 0.80, 1.60, 3.20 g/L) were evaluated using the generalized Labelled Magnitude Scale (gLMS; Bartoshuk, 2000). These concentrations were chosen to induce a perceived intensity from weak to strong on gLMS. Solutions were arranged in a subset for each taste and were presented in a randomized order. In the second study (N=109), the perceived intensities and the emotional responses in terms of valence (trustworthiness) and arousal (skin conductance) to bitterness, astringency and sweetness as a function of food neophobia were measured. Participants were selected based on PROP status (only Medium-Tasters) and food neophobia (31% Low, 41% Medium, 28% High). Two concentrations for each taste were prepared in order to create two equintense levels perceived as weak/moderate and moderate/strong on gLMS (caffeine 1.50 and 3.00 g/L; K Aluminum Sulfate 0.80 and 1.60 g/L; sucrose 71.9 and 200.0 g/L). All samples were presented in blind conditions, in a randomized order and in triplicate in two sessions. During the first one, subjects were asked to taste a sample; then a neutral face was briefly presented on the screen and they were asked to indicate if they trusted the face (yes/no) and how much they trusted it (9-point Likert Scale). Skin conductance was simultaneously and continuously recorded. During the second session, participants were asked to rate the perceived intensities of solutions.

2.4 Measuring familiarity with and choice for phenol-rich foods and beverages

The choice of phenol-rich vegetables and coffee/tea between pairs of two food items characterized by different levels of bitterness and astringency was assessed with the V-IT-FCQ and C-IT-FCQ (Tab. 1). For more details, see De Toffoli and colleagues (2019a).

Table 1 Pairs of food items included in the vegetable choice questionnaire (V-IT-FCQ) and coffee/tea choice questionnaire (C-IT-FCQ).

Vegetable Choice Questionnaire (V-IT-FCQ)	
Options lower in bitterness and astringency	Options higher in bitterness and astringency
Pumpkin risotto	Risotto with radicchio
Lettuce and valerian salad	Radicchio and rocket salad
Green salad	Bean sprout salad
Chard	Chicory
Zucchini	Asparagus
Carrots	Cauliflower
Cucumber	Radish
Coffee/Tea Choice Questionnaire (C-IT-FCQ)	
Macchiato	Coffee
Coffee with sugar	Coffee without sugar
Cappuccino	Coffee
Tea with sugar	Tea without sugar

Vegetable and coffee/tea pairs in the choice questionnaires were selected so that the options in each pair significantly differed for bitterness and astringency. For each pair, participants were asked to indicate which food they would ideally choose, pointing out that the answer would describe not what they usually choose but rather what they would like to choose in a situation of absence of restrictions (e.g., due to health or weight concerns). Options within the pairs were coded as “0” for the lowest level of bitterness and astringency and “1” for the highest level of bitterness and astringency. For each subject, a choice index was calculated for vegetables (CV) and coffee/tea (CC) as a mean of the choices of the more bitter/astringent option (range 0-1).

Familiarity with vegetables and coffee/tea items was assessed by a five-point labelled scale (1= I do not recognize it; 2= I recognize it, but I have never tasted it; 3= I have tasted it, but I don't eat it; 4= I occasionally eat it; 5= I regularly eat it) developed by Tuorila and colleagues (2001). Two indices of familiarity with vegetables and coffee/tea higher in bitterness and astringency (+) were obtained by the sum of ratings of familiarity with the items that, within each pair, were higher in these sensations. Two indices of familiarity with vegetables and coffee/tea lower in bitterness and astringency were obtained by the sum of ratings of familiarity with the items that, within each pair, showed a lower level of bitterness and astringency (-).

2.5 Measuring sensation intensity and liking for real phenol-rich foods

The effect of psychological traits and taste responsiveness on sensory and hedonic evaluation of real phenol-rich foods was investigated in two studies. In the first study (N=1200), four samples of chocolate pudding varying in sucrose concentration were produced by adding different amounts of sucrose (C1= 38, C2= 83, C3= 119, C4= 233 g/Kg) to the base. In the second study (N=797), four samples of bean purée added with different levels of phenols from Olive Mill Waste Water (OMWW; 0.00, 0.44, 1.00, 2.25 g/Kg) were evaluated. For the concentrations selection see De Toffoli and colleagues (2019b).

In both studies, during the first session, respondents were asked to rate their liking using the Labeled Affective Magnitude Scale (LAM; Schutz & Cardello, 2001). During the second session, respondents evaluated the intensity of the target sensations using the gLMS.

3. Results

3.1 Effect of personality traits and PROP status on intensity and emotional responses to tastes in water solutions

Intensity ratings to tastes - The first two dimensions on the Principal Component Analysis computed on psychological traits scores explained 49.2% of the total variability. Two clusters were identified: Cluster 1 was composed with subjects high in food neophobia and sensitivity to disgust, whereas Cluster 2 was composed with subjects high in sensitivity to reward and sensation seeking. Clusters did not differ for gender and PROP status. Concentration and cluster affected the perceived intensity in the bitterness ($p<0.0001$ and $p<0.001$) and astringency ($p<0.0001$ and $p=0.037$) series. Intensity ratings increased with the increasing of concentration in both tastants, ranging from weak to very strong on gLMS. Cluster 1 perceived the highest concentrations of caffeine (3.0 and 6.0 g/L) as more intense and the second sample of astringency (0.40 g/L) as less intense than Cluster 2. The same group tasted the functionalized bean purée series.

Emotional responses to tastes – ANOVA models showed that concentration significantly affected the perceived intensity of tastes ($p<0.0001$) and the trustworthiness to neutral faces ($p<0.0001$). Low concentrations (-) were perceived as weak/moderate and high concentrations (+) were perceived as moderate/strong on gLMS (fig. 1a). Sweetness- evoked the highest trustworthiness to neutral faces, while bitterness+ evoked

the lowest one (fig. 1b). Skin conductance was affected by taste and bitterness+ evoked the strongest response. High levels of food neophobia were associated with reduced trustworthiness, lower intensity ratings of sweetness- and higher intensity ratings of bitterness-.

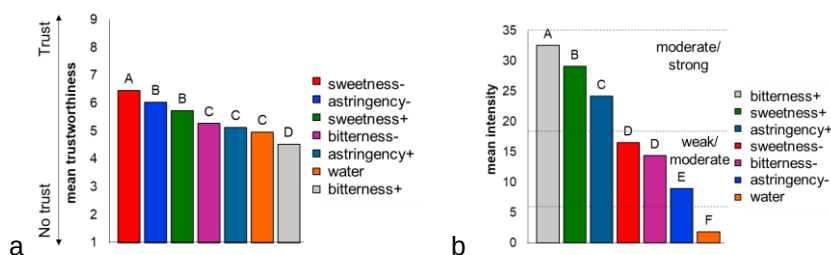


Figure 1 Effect of taste on perceived intensity (a) and on trustworthiness (b). Different letters represent significant different values ($p \leq 0.05$).

3.2 Effect of personality traits and PROP status on familiarity with and choice for phenol-rich foods and beverages

ANOVA models showed that the effect of food neophobia, sensitivity to punishment and to disgust was significant on both Vegetable Choice Index and Coffee/Tea Choice Index indices (Fig. 2a-b). Individuals who scored higher in food neophobia, sensitivity to punishment or to disgust reported significantly lower choice indices than individuals low in these traits, meaning that they systematically opted for the least bitter/astringent option within the pairs. No effect of PROP responsiveness was found on either choice index.

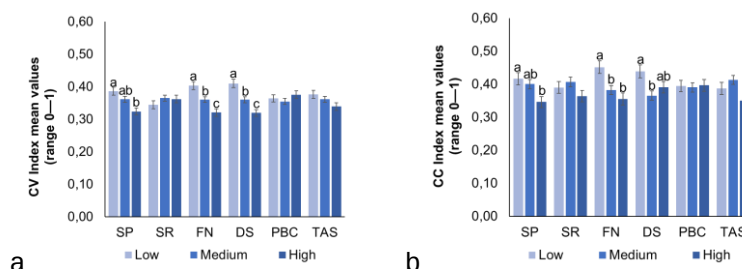


Figure 2 Effect of psychological traits (sensitivity to punishment, SP; sensitivity to reward, SR; food neophobia, FN; sensitivity to disgust, DS; private body consciousness, PBC and alexithymia, TAS) on the Choice Index for vegetables (a-CV Index) and the Choice Index for Coffee/Tea (b-CC Index). Different letters represent significant different values ($p \leq 0.03$).

Subjects with high sensitivity to disgust, food neophobia and alexithymia had a lower familiarity index with vegetables higher in bitterness and astringency ($p < 0.0001$) and were less familiar with the without sugar coffee/tea group of products ($p = 0.03$). No significant effect of PROP was found on either index of familiarity.

3.3 Effect of personality traits and PROP status on sensory and hedonic evaluation of real phenol-rich foods

Chocolate pudding – ANOVA models showed that sweetness increased with sugar concentration, while bitterness and astringency decreased. Food neophobia and sensitivity to disgust affected both liking ($p < 0.0001$) and the perception of bitterness ($p = 0.05$) and astringency ($p < 0.0001$). Alexithymia and sensitivity to reward affected the perception of bitterness and astringency ($p < 0.0001$) but not the liking. Sensitivity to punishment affected only the perception of bitterness ($p < 0.0001$). Subjects high in food

neophobia and sensitivity to disgust liked less the most bitter samples (C1 and C2) and, as subjects high in alexithymia and sensitivity to reward and punishment, perceived C1 as more bitter and more astringent. No differences between groups in liking and perceived intensities were detected in the least bitter and astringent sample (C4), except subjects highly sensitive to reward who liked more the sweetest sample.

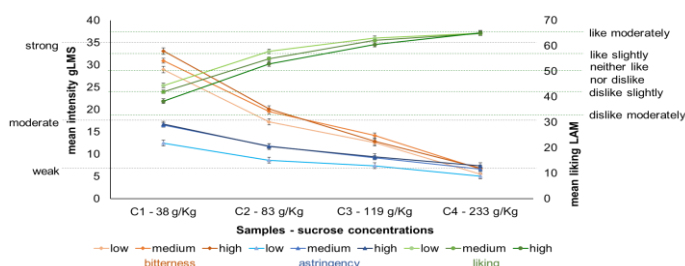


Figure 3 Mean intensity ratings for bitterness (orange lines), astringency (blue lines) and mean liking ratings (green lines) by sample (C1 less sweet sample, C4 sweetest sample) and by FN level (low neophobics, medium neophobics and high neophobics). Error bars are standard errors.

Bean purée added with OMWW - Phenol concentration of OMWW significantly affected sensory and hedonic ratings ($p=0.001$). The increase of phenol concentration had the strongest effect on bitterness and, to a lesser extent, on astringency and negatively impacted the liking. Food neophobia, alexithymia and sensation seeking significantly affected bitterness, astringency and liking ($p=0.04$). Sensitivity to reward affected the perception of both target sensations ($p=0.03$) and sensitivity to punishment significantly affected only the astringency ratings ($p=0.04$). Subjects high in food neophobia, alexithymia and sensitivity to reward provided intensity ratings which were systematically higher than almost one of the other groups. Sensation seekers showed the opposite pattern, with low sensation seekers who perceived bitterness and astringency as more intense than almost one of the other groups. Liking tended to decrease with increasing of perception of bitterness and astringency among groups.

PROP affected sensory properties ($p=0.0001$) but not hedonic ratings, with Super-Tasters who evaluated the samples as more bitter and astringent.

4. Conclusion

The approach proposed in this PhD project allowed the investigation of the role of individual differences in chemosensory perception and psychological traits as modulators of acceptability of healthy foods. Results related to PROP status are inconsistent and, in line with previous studies, showed a lack of association with bitter vegetable preference and choice (Laureati et al., 2018). Evidences from recent studies highlighted that a complex network of both genetic and environmental factors appear to influence responsiveness to PROP and required to be deeply investigated in future studies.

Taken together, these findings confirm the hypothesis that psychological variables may act to facilitate or inhibit preference and intake of vegetables and beverages characterised by unpleasant sensations, consistent with previous results. Individual differences in psychological traits resulted to be associated with a hypersensitivity to the alarm sensations, evaluated with implicit and explicit measurements, both in water solutions and in real foods. The intercorrelation between these traits seems to play a key role in the modulation of sensory and hedonic responses making the alarm sensations been perceived as more intense and less acceptable. With the increasing challenges of

food diversity and global health, the identification of possible barriers to the consumption of healthy foods could allow the planning of targeted preventive programmes for children and adults and the development of personalised food products.

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New Insights into Pasta Cooking: The Continuous Cooking Procedure in Professional Appliances

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The aim of the PhD project is to propose a new approach for food scientists in the food service sector to fulfil the gap between engineers and kitchen operator perspectives. As study case, the continuous cooking procedure in professional pasta cookers and its critical issues were investigated. The procedure was simulated on a laboratory scale for understanding the underlying phenomena. Continuous cooking affected pasta cooking quality and water properties requiring technological strategies to retain fresh water properties and the final pasta quality. Finally, the implementation of these strategies in the professional appliance was evaluated in terms of technical feasibility.

Nuove prospettive sulla cottura della pasta: la cottura continua nella ristorazione professionale

Questo progetto di dottorato ha mirato a proporre un nuovo approccio per la ricerca alimentare nella ristorazione professionale per colmare il divario tra le prospettive di ingegneri e cuochi. Come caso studio, è stata studiata la procedura di cottura continua della pasta nei cuocitori professionali e sono state evidenziate le criticità. Per comprenderne i meccanismi alla base, la procedura è stata dunque sviluppata in laboratorio. I cambiamenti della qualità in cottura della pasta e delle proprietà dell'acqua di cottura hanno richiesto strategie tecnologiche per limitarli. Infine, è stata valutata la fattibilità tecnica dell'implementazione nell'apparecchiatura professionale delle strategie di ottimizzazione.

Key words: pasta; cooking quality; continuous cooking; food service; unconventional technologies.

1. Introduction

In accordance with the PhD thesis project previously described (Diamante, 2018), this oral communication abstract reports the main results of the following three activities directed to:

A1) assess the cooking water properties and determine the most critical changes occurring throughout the service;

A2) determine the cooking behaviour of pasta during the continuous cooking procedure and its final quality during a service shift;

A3) propose technological interventions for cooking water properties management to be applied in the professional appliance.

2. The food service sector requests

In the food service sector (Fig. 1), both the user of the professional appliance, i.e. the kitchen operator, and the customer of the establishment, i.e. the food consumer, drive the appliance choice in terms of model, category and brand. Organization, efficiency, time

management, productivity, personalization, and safety are just examples for needs of kitchen operators. Food quality, service environment and service performance are the three main criteria used by food consumers to evaluate the establishment. Professional appliances play a critical role in almost all of them and result from a collaboration between engineers and kitchen operators. So far indeed, in most cases, the research and development in the manufacturing of such appliances has been entrusted to engineers having a technical background. The performance of the food process is validated on a culinary base by kitchen operators, instead. This dialogue has different languages and lacks a multidisciplinary and scientific knowledge of food and food processing. Food technologists are asked to fill this gap, who can give a boost to the food processing optimization, pleasing both the kitchen operators and the food consumers. The food technologist answer to the food service sector requires a new experimental approach and basic scientific research, since almost all studies in the literature are based and focused on domestic or industrial procedures (Fusi *et al.*, 2016).

Based on these considerations, this PhD thesis aimed at contributing to fulfil the gap by considering the continuous cooking of pasta in the food service as a study case. In fact, the pasta cooker represents an emblematic case of food service requests to the manufacturer. Kitchen operators ask to the appliance for stress inputs minimization, safety, high efficiency and productivity. End-consumers more and more rise the expectations in terms of the quality of food, which has to be promptly served always fresh-like.



Figure 1 *Food service sector schematical representation.*

3. Continuous cooking procedure of spaghetti in the food service

As mentioned above, as study case of professional appliances, this PhD project considers the pasta cooker and its optimization for the cooking of spaghetti. First, the use and issues of the appliance were investigated. The pasta cooker is filled with fresh water and when the boiling point is reached, pasta is manually added and removed when it is cooked. The same water is used many times (up to 13). During this continuous cooking process, working conditions for kitchen operators worsen. Cooking water properties change, affecting in turn the final product quality. Also, a foam layer may form upon cooking, causing water to flow into the overflow drain and representing a safety issue for kitchen operators. Nowadays, the solution is to replace this volume with fresh water that has to be heated, with consequent high time and energy consumption and thus operating costs. Second, the industrial procedure was simulated and investigated on a laboratory scale. Third, a deep investigation on the cooking behaviour of pasta and the role of cooking water characteristics was carried out. Fourth, high pressure homogenization and ultrasound treatment were used, for their mechanochemical and thermal action, on cooking water (Che *et al.*, 2009; Martini *et al.*, 2010). Finally, the technical feasibility of the implementation of the proposed technologies was evaluated on industrial scale.

4. Materials and Methods

Durum wheat spaghetti (5 mm) were cooked in a laboratory-made colander in 300 mL of tap boiling water with no salt added (Diamante *et al.*, 2019). Pasta was strained and rinsed with tap boiling water above the beaker. The procedure was repeated 13 times. Pasta was poured into a Büchner funnel over a beaker while collecting cooking water and rinsed with 100 mL of cold distilled water. Samples of cooked pasta, freeze-dried and ground, were analysed for cooking loss and water absorption. Cooking loss (CL, %) was the percentage ratio between the dm material leached into the cooking water and the sum of dm weight of dry pasta. Water absorption (WA) was calculated with the following equation 1:

$$WA \left(\frac{g}{g \text{ dm}} \right) = \frac{CP (g) - [DP (g) - CL' (g, dm)]}{[DP (g, dm) - CL' (g, dm)]} \quad 1)$$

CP, cooked spaghetti; DP, dry spaghetti (weight as is or on dm basis); CL', cooking loss

Pasta firmness and stickiness were determined by the texture profile analysis on TA-XT Plus Texture Analyzer (Stable Micro Systems Ltd., Godalming, UK). After cooking, draining and rinsing, samples were resting in a covered container (25 °C, 10 min). Firmness was measured using a light knife blade (A/LKB) (speed 0.17 mm s⁻¹). Pasta stickiness was evaluated using a stickiness rig (HDP/PFS) (compression speed 0.5 mm s⁻¹, compression force 1 kg for 2 s). No rinsing was performed.

Freeze-dried and ground cooking water samples were analyzed for solid and starch content. Solid content (SC, %) was the dm of the freeze-dried sample and starch content was determined by gas-liquid chromatography and calculated as 0.9 times the glucose content (Courtin *et al.*, 2000). Cooking water was analyzed fresh for pH, rheological and foaming properties, the latter with a standardized stirring test (Wouters *et al.*, 2016). The rheological measurements were carried out using an RS6000 rheometer (Thermo Scientific RheoStress, Haake, Germany), equipped with a coaxial cylinder geometry (CCB25 Din). Steady shear measurements (flow curves) were performed over a shear rate from 3 to 100 s⁻¹. The power law model was used to fit the flow data ($\sigma = K \cdot \dot{\gamma}^n$).

The high pressure homogenization (HPH) was carried out at 20 and 100 MPa, one pass, in a high pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Italy) with samples conditioned at 20 °C before processing.

5. Results and Discussion

5.1 Cooking water properties

The simulated continuous cooking procedure on a laboratory scale supported the empirical evidences reported by kitchen operators. As expected, the solid content in the cooking water increased reaching up to 4% of solids, which were partly dispersed as suspended pasta fragments and partly solubilized in the boiling tap water (Table 1). During continuous cooking, starch content increased in cooking water up to 2.8% (wb), which means that it represented up to 69% of the dry matter material in cooking water.

The continuous cooking procedure led to a pH drop of cooking water from alkalinity in batch 1 to acidity in batch 13 (Table 1). This drop was related to the solid concentration increase and tap water (pH 8.2±0.2) addition in between each cooking

batch, because of water evaporation and water uptake by pasta. This result highlights the importance of assessing and choosing water characteristics for pasta cooking, as previously reported by other authors (Alary *et al.*, 1980; Feillet, 1984). pH might not only affect leaching by changing starch-protein interactions, but also the foam layer formation ability during cooking.

Table 1 pH, solid and starch content of cooking water samples at different batches.

Batch (n)	pH	Solid content (%)	Starch content (% wb)
1	8.3 a	0.59 c	0.36 c
7	6.8 b	2.91 b	1.89 b
13	6.5 c	4.04 a	2.77 a

^aValues followed by the same letter in the same column are not significantly different ($p < 0.05$)

Solid content and pH represented two factors that could affect the rheological and foaming properties of cooking water. Fig. 2 shows the flow properties of cooking water of different batches at room temperature (25 °C). Samples showed a shear thinning behaviour under steady shear flow. The decrease in viscosity with the increase in shear rate can be attributed to the disentanglement of polymer chains under shear flow and breaking of possible structure in solution. The latter are exposed to mechanical and thermal stresses during the subsequent cooking batches (e.g. pasta fragments from batch 1 are cooked for about 3 more hours) which progressively dissolve parts of these fragments and favour the interaction. This flow behaviour was described by a power law model and its suitability was confirmed by the coefficients of determination (R^2) (Table 2). Flow index values (n) of 0.83-0.98 were consistent with a shear thinning behaviour particularly for higher batch numbers. K values increased from 1.4 to 31 mPa·s ^{n} increasing the batch number. This resulted from the increase in solid concentration which restricted the motion between polymer chains. These rheological properties have different negative aspects on industrial scale. They are related to foaming capacity during continuous cooking, they are perceived when tasting the cooked pasta and lead to more clumped strands in the subsequent cooking batch if stirring is not more frequently performed. In particular, batch number 7 had been indicated as acceptability threshold for working conditions.

Foamability represented a negative property of cooking water leading to cost consuming issues on industrial level. Batch 13 produced significantly ($p < 0.05$) more foam (96 ml) than batch 7 and 1 (80 and 48 ml, respectively) (Fig. 3). Foams from batch 7 and 13 were quite stable over resting time, with remaining foam volumes being 69 and 70 ml, respectively, after 60 min. Batch 1 foam was relatively unstable, resulting in a low foam volume of 15 ml after 60 min. This increase in foaming capacity is related to more proteins adsorbing to the air-water interfaces and stabilizing the resulting foam. On industrial scale, this phenomenon is observed in terms of foam layer formation during the vigorous boiling. In addition, the increase in stability over time gives an additional drawback, since the formed foam layer might be more stable in the appliance tank. Moreover, the decrease in pH might minimize the electrostatic repulsion between proteins leading to a rapid protein adsorption to the interface and improving the foaming properties (Allen Foegeding *et al.*, 2006).

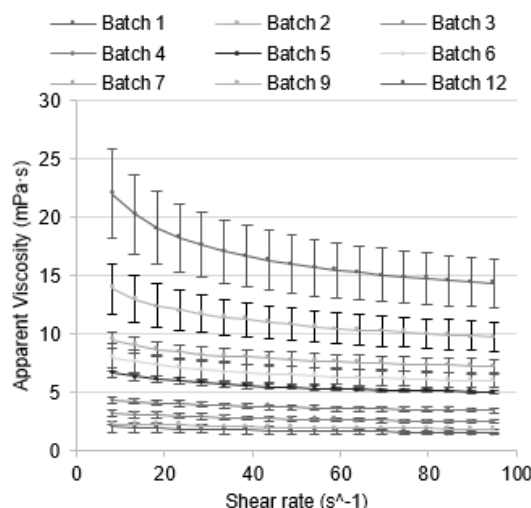


Figure 2 Apparent viscosity as a function of shear rate for different batch number of the cooking water (25°C).

Table 2 Power law parameters for cooking water at different batch number.

Batch (n)	n (-)	K (mPa·s ⁿ)	R ²
1	0.986 ± 0.016	1.4 ± 0.4	0.997
2	0.914 ± 0.012	2.8 ± 0.3 · 10 ⁻²	0.998
3	0.899 ± 0.008	4.0 ± 0.3 · 10 ⁻²	0.999
4	0.896 ± 0.002	5.3 ± 0.3 · 10 ⁻²	0.999
5	0.885 ± 0.005	8.3 ± 0.9 · 10 ⁻²	0.999
6	0.888 ± 0.004	9.6 ± 3.1 · 10 ⁻²	0.999
7	0.894 ± 0.001	11.9 ± 0.7 · 10 ⁻²	1.000
9	0.864 ± 0.005	18.9 ± 0.2	0.999
12	0.834 ± 0.005	30.7 ± 2.2	1.000

Power law parameters: K, consistency coefficient; n, flow behaviour index; means ± standard deviation.

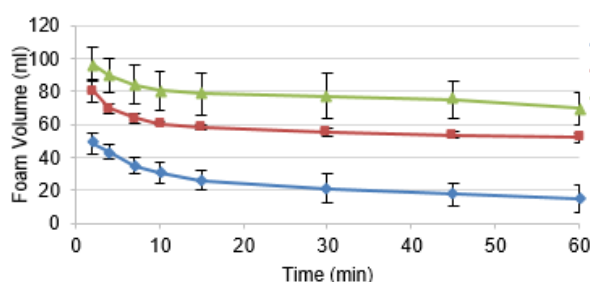


Figure 3 Foam volume over time of whipped cooking water samples.

5.2 Spaghetti cooking quality

In light of the observed changes of cooking water properties, continuous cooking procedure might affect also the cooked pasta quality. During continuous cooking, cooking loss decreased from 7.4 to 3.8%, while water absorption decreased from 1.9 to 1.7% increasing the batch number (Table 3). This suggests that the increase of solid content and the pH drop in cooking water leads to a reduction in water imbibition through the spaghetti strand determining a lower hydration and consequent lower solid leaching at the optimal cooking time. Furthermore, at acidic pH, protein molecules are positively charged, and starch molecules are negatively charged. Under these conditions, electrostatic interactions between proteins and gelatinised starch readily occur, enhancing starch-protein interactions preventing the leaching of molecules into the cooking medium (Delcour *et al.*, 2000).

Table 3 *Effect of batch number on technological properties and texture of cooked pasta.*

Batch (n)	CL (%)	WA (g/g dm)	Firmness (N)	Stickiness (N)
1	7.41 a	1.90 a	2.72 b	1.90 a
7	5.17 b	1.76 b	2.88 ab	2.07 a
13	3.77 c	1.74 c	3.04 a	2.04 a

a Values followed by the same letter in the same column are not significantly different ($P < 0.05$)

Textural values of spaghetti showed that continuous cooking would make the pasta significantly firmer ($P < 0.05$) (Table 3). This has been related to the solid retention inside the starch–protein network and the lower water absorption when cooking water is more concentrated and acidic. Stickiness remained surprisingly unaffected within the range of batch number tested. This finding supports the hypothesis that total solids lost to cooking water might not necessarily be related to stickiness and the mouthfeel stickiness is associated to the rheological properties of the cooking water.

5.3 Technological intervention on cooking water

Based on the previous results, the need for technological interventions to minimize foam formation by modifying physical properties of cooking water are needed. Cooking water was thus subjected at different pressures. Fig. 4 shows the apparent viscosity of cooking water samples treated by HPH as a function of shear rate. The flow pattern of samples was changed by high pressure homogenization more evidently with lower pressure. The apparent viscosities of cooking water after HPH were constantly lower than untreated one over the entire shear rate, suggesting that HPH changed the rheological properties of cooking water, reducing its viscosity. In particular, at lower applied pressure corresponded a lower flow curve. The breakup of the macromolecule aggregates homogeneously into their individual macromolecules occurs at the highest pressure. The initial structure can be related to inter-particle and intermolecular interaction, favouring a network with a higher initial resistance to flow. When the product is sheared this structure collapses, reducing the product resistance. For the purpose of this PhD project, a high pressure treatment of cooking water at the lower pressure of 20 MPa is suggested to retain rheological properties as close to Newtonian behaviour and initial cooking batches as possible.

6. Conclusion and future perspectives

The aim of this doctoral thesis was to propose a new approach for food scientists in the food service sector able to fulfil the gap between engineers and kitchen operator perspectives. As study case, the continuous cooking procedure of pasta in professional pasta cookers allowed to give new insights into pasta cooking and its issue was tackled with an unconventional technology. Future work is needed to successfully implement the technology in the professional appliance. Moreover, this study is a springboard for future pasta cooking studies with different cooking water characteristics and processing parameters. Overall, this research proves the possibility to implement a multidisciplinary and scientific approach to the food service sector, typically entrusted to engineers and chefs.

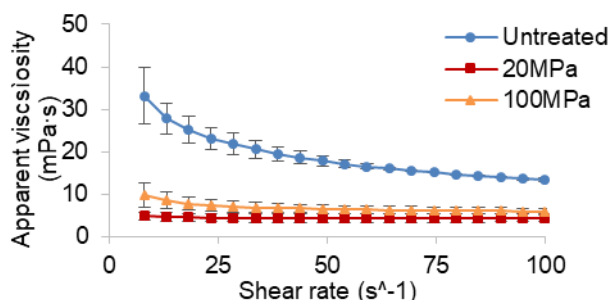


Figure 4 Apparent viscosity as a function of shear rate of the cooking water at batch 13 treated at different pressures (MPa).

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Overall Assessment of a Model Probiotic Bacterium: from Gut Colonization to Clinical Efficacy

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This Ph.D. thesis research project was aimed to study the impact of a specific probiotic bacterium on the intestinal microbial ecosystem (IME) focusing attention on its recovery and colonization at intestinal level. Furthermore, the Ph.D. project was also aimed to study the efficacy of this probiotic bacterium in a IBS population. In order to reach this goal, particularly sensitive methodological strategies were developed and appropriate designs of intervention and clinical trials were adopted.

Valutazione complessiva di un batterio probiotico modello: dalla colonizzazione intestinale all'efficacia clinica

Questo progetto di tesi di dottorato ha avuto lo scopo di studiare approfonditamente l'impatto di uno specifico batterio probiotico sull'ecosistema microbico intestinale puntando l'attenzione soprattutto su recovery e colonizzazione a livello intestinale. Inoltre, il progetto di tesi di dottorato ha avuto anche lo scopo di studiare l'efficacia del batterio probiotico in una popolazione con IBS. A questo scopo, sono state sviluppate strategie metodologiche particolarmente sensibili e sono stati adottati appropriati design di studio di intervento e studi clinici.

Key words: gut colonization; probiotic; *Lactobacillus paracasei* DG; recovery study; IBS.

1. Introduction

The gut is inhabited by microbial communities in so massive way that the prokaryotic cells probably overpasses of one order of magnitude the eukaryotic cells present in the human organism forming an intimate beneficial association with the host (the concept of "holobiont"). For this reason, the intestinal microbiota has been proposed as an additional organ of the human body that is deputed to numerous functions ranging from vitamin production and immunomodulation to nutrient bioavailability and competitive exclusion against potential detrimental microorganisms (Bäckhed et al., 2015). Consequently, the modification of the gut microbiota composition plausibly induces functional changes affecting the host physiology (Bäckhed et al., 2015). In this context, the ability to modulate the intestinal microbial ecosystem is clearly an important element in supporting a healthy life. Notably, it appears possible to modify the relative abundance of specific bacterial groups by means of dietary interventions and, consequently, modulate the concentrations of health-affecting microbial metabolites in the gut such as butyrate, a bacterial fermentation product that plays a regulatory role on intestinal motility, epithelial barrier, and mucosal inflammation (Ferrario et al., 2014). Among these interventions, probiotics play a very central role.

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). This definition is inclusive of a broad range of microbes and applications, whilst capturing the essence of probiotics (microbial, viable and beneficial to health; Hill et al., 2014).

The Italian Ministry of Health has regulated the use of probiotic bacteria in the food sector over the past 12 years and, in 2013, confirmed the use of the word probiotic for food and food supplements under certain conditions, including a minimum number of viable cells (1×10^9 CFU) administered per day, a full genetic characterization of the probiotic strain and a demonstrable history of safe use in the Italian market (Hill et al. 2014).

The identification of the actual impact on IME of a probiotic, prebiotic, pharmacologic or dietary intervention in general is surely of importance, but it is a difficult task, since the effects may be hindered by the complexity and the profound variability of the microbiota compositions among subjects. For this reason, the IME can be studied only by adopting proper trial design associated to next-generation analysis supported by solid statistical approach (Veiga et al. 2014).

In this project, we have selected a specific strain, *L. casei* DG® (*Lactobacillus paracasei* CNCM11572), that is contained within the probiotic product Enterolactis®, commercially available from more than 10 years. This strain has already demonstrated its efficacy in healthy subjects and in many diseases, such as diverticular disease, *Helicobacter pylori* infection, Small Intestinal Bacterial Overgrowth (SIBO), Ulcerative colitis and Irritable Bowel Syndrome (IBS).

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following three activities directed to:

A1) evaluate the ability of the strain to cross alive the gastrointestinal tract and colonize it in children and adults (recovery studies);

A2) investigate on mice the preferential site of colonization of the probiotic bacterium (animal study);

A3) design the efficacy study on IBS patients, to define the endpoints and the centers to involve, followed by a microbiological part, based on 16S rRNA gene profiling (by MiSeq Illumina) of the metagenomic DNA isolated from fecal samples.

In order to reach these goals, some intervention and clinical trials have been conducted. Fecal samples have been collected in order to be analyzed according to microbiomic protocols in order to determine the overall structure of the microbial communities by 16S rRNA gene profiling. The same fecal samples have been also used for the quantification of the short chain fatty acids (SCFAs) by liquid chromatography coupled to mass spectrometry. Finally, biopsies and blood sample have been collected in studies conducted with mice and patients with IBS respectively, in order to investigate the preferential intestinal site of colonization of the probiotic bacterium (mice) and the blood microbiota and the gut permeability.

2. Material and methods

2.1 Recovery studies

To conduct these two recovery studies, it was necessary to develop a novel method for discriminating and enumerating culturable *L. paracasei* DG cells based on the unique sticky, filamentous phenotype of this strain on MRS agar containing vancomycin and kanamycin (as shown in Fig. 1). The identity of DG was also confirmed with strain-specific primers by colony PCR.

First of all, to develop culture conditions for selective and discriminative growth of *L. paracasei* DG it was implemented the cultivation protocol suggested by the Italian

Higher Institute of Health (ISS) for the enumeration of heterofermentative lactobacilli in probiotic products (ISSN 1123-3117 ISTISAN 08/36). The ISSN protocol suggested the use of 1 mg/ml vancomycin for the selective counting of heterofermentative lactobacilli; however, during the preliminary experiment, the use of such a medium allowed the growth of many non-DG colonies. For this reason, based on the antibiotic resistance profile of *L. paracasei* DG it was added 10 mg/ml kanamycin to the medium (vk-MRS medium), which resulted in an evident decrease in the background without affecting the growth of the DG strain compared to the growth of this strain in normal MRS medium or MRS supplemented with the only vancomycin.

Furthermore, it was observed that the DG colonies on vk-MRS agar had a peculiar sticky, filamentous phenotype, allowing the discrimination of this strain from the colonies of closely related lactobacilli, which typically have a creamy consistency (see Fig. 1).

Finally, to unambiguously confirm that the colonies isolated on the vk-MRS plates belonged to the DG strain, we performed end-point colony PCR analysis with strain-specific primers on randomly selected colonies.

This method allowed us to precisely distinguish the DG colonies from the other fecal microorganisms and to selectively count colonies of the probiotic strain under study.

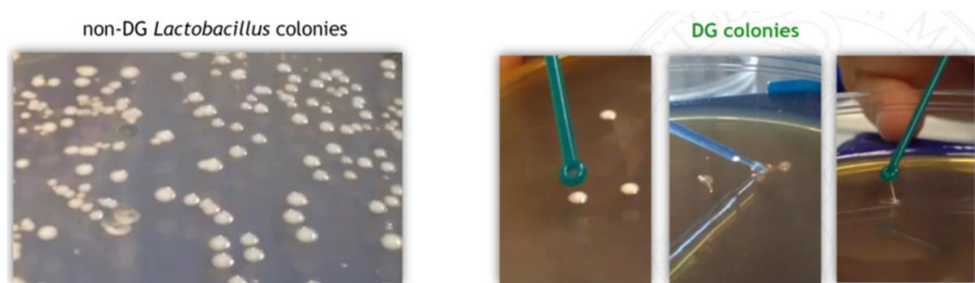


Figure 1 Sticky, filamentous consistency of the colonies of *Lactobacillus paracasei* DG grown on vk-MRS agar plates, compared to the non-DG colonies.

The above method was used for two recovery studies aimed to quantify viable cells of *L. paracasei* DG in the fecal samples of healthy subjects during a 1-week probiotic consumption period and a 1-week follow-up in the following populations:

- 20 adults, aged 18-55 years, enrolled at Department DEFENS, Milan
- 20 children, aged 3-12 years, enrolled at CROSS Research, Arzo, Switzerland

Both the trials were open-label pilot microbiological studies, that wanted to demonstrate the capability of the strain *Lactobacillus paracasei* DG, administered in a drinkable formulation containing 1×10^9 UFC, to really be able to cross alive the gastrointestinal tract and potentially colonize the intestine in humans according to the study design shown in Fig. 2.

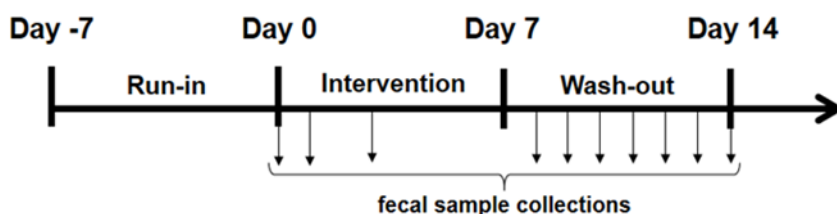


Figure 2 Design of the study. Vertical arrows indicate the day of collection of the fecal sample (when available) from the volunteer

2.2 Animal study

Two-month old female C57BL/6 mice were housed in a traditional, specific pathogen-free, nonsterile environment in the animal facility of the Department of Agricultural and Environmental Sciences of Università degli Studi di Milano. After one-week adaptation, mice were separated in cages in 2 groups of 5 mice each. Mice were treated with bacteria or the vehicle via oral gavage once a day for 5 days. Finally, mice were euthanized 4 hours after the gavage. Following sacrifice, biopsies of distal ileum, caecum and proximal colon portions were collected from each mouse and DNA was then extracted.

In the specific case, DNA was obtained from mouse biopsies by means of a PowerFecal® DNA Isolation Kit and it was employed to quantify bacterial cell number through qPCR. The total number of bacteria was quantified by using 16S rRNA-targeting primers 357F-907R. Bacterial DNA isolation was carried out with the same kit employed to extract DNA from biopsies.

2.3 IBS study

Taking into account carefully the trial previously designed and published (Cremon et al. 2018) in patients with IBS, in collaboration with the Principal Investigator (PI), we have designed a multicenter, randomized, double-blind, parallel-group, placebo controlled trial to assess the effect of *L. casei* DG® (formulated in capsules containing 24×10^9 UFC) on abdominal symptoms and the gut microbiota metabolism/composition in nonconstipated patients with IBS. It was also decided that at the end of the study as an exploratory objective we will evaluate the possibility to assess also the blood microbiota.

The microbiota of fecal samples will be characterized by 16S rRNA gene profiling on the Illumina MiSeq platform. Briefly, metagenomic DNA extraction from feces will be performed by means of a DNeasy PowerSoil Kit and a DNA fragment encompassing one or two variable regions of the 16S rRNA gene will be amplified from metagenomic DNA with validated primers. Proper bioinformatic pipelines (QIIME and MOTHUR) will be used to define bacterial richness and evenness (alpha-diversity through Chao1, Shannon and invSimpson indexes), intersample relationships (beta-diversity through Unifrac algorithms), and the relative abundance of bacterial OTUs; afterwards, both conventional parametric/non-parametric statistics and the DESeq negative binomial distribution method will be used to identify significant variations in microbiomic data.

Short chains fatty acids (SCFAs) will be also analyzed in fecal sample according to previous published study (Cremon et al., 2018).

3. Results and Discussion

3.1 Recovery studies

The conventional process of selecting novel potential probiotic strains includes in vitro assessment of the ability of the strains to survive at low pH, in simulated gastric juice or in the presence of bile salts. However, this evaluation is only useful for a first screening, but does not testify to the actual survival to gastrointestinal transit in humans. The fecal recovery study is the only demonstration that the strain is really able to cross alive the gastrointestinal tract and potentially colonize the intestine in humans. Nevertheless, the above study has been carried out so far for only a limited number of well-known commercial probiotics.

This may be due to the fact that in vivo assessment of probiotic viability is a more challenging task, possibly due to the difficulties associated with setting up intervention trials with human volunteers and because of technical limitations. To solve these problems, culture-based methods have been combined with molecular approaches to obtain adequate sensitivity and specificity.

The two recovery studies, carried out by means viable count experiments, carried out by combining ad hoc culture-based discriminative conditions and strain-specific molecular biological protocols, unambiguously demonstrated that *L. paracasei* DG can survive gastrointestinal transit in healthy adults and children.

3.1.1 Adults subjects

L. paracasei DG was isolated from at least one fecal sample from all the volunteers. The highest concentration of viable DG cells [ranging from 3.6 to 6.7 log₁₀ colony-forming unit (CFU) per gram of feces] in the feces was observed between 4 and 8 days from the beginning of Enterolactis intake and for up to 5 days after cessation of intake (see Fig. 3).

As expected, the total DG count determined by real-time quantitative PCR (qPCR) was mostly higher than the viable DG cells recovered.

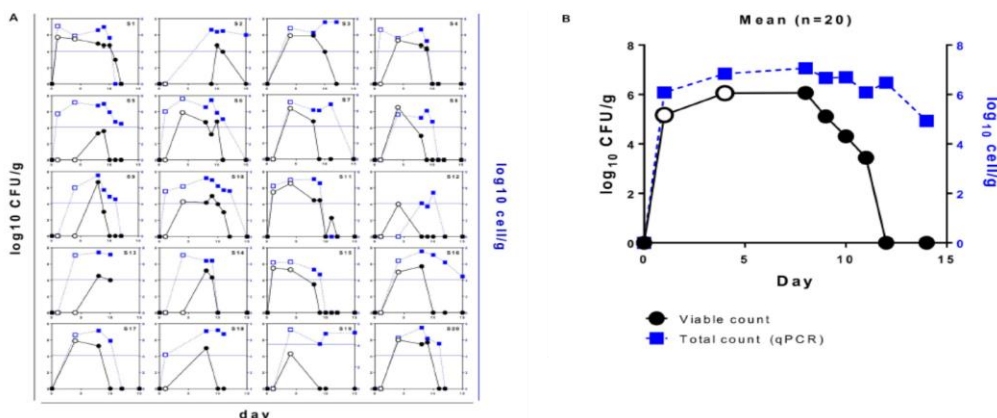


Figure 3 Viable (black lines and circles) and total (blue lines and squares) counts of *Lactobacillus paracasei* DG in the fecal samples of healthy adult volunteers who ingested *Enterolactis*® in drinkable vials once daily. (A) Data per subject; (B) mean data (n = 20). Open symbols refer to fecal samples collected during the week of probiotic intervention.

3.1.2 Pediatric subjects

During the administration period most subjects showed variable counts of live *L. casei* DG[®] CFU in their faeces. Nineteen (95%) of the 20 enrolled children presented viable *L. casei* DG[®] cells in their faeces at least once during the study (see Table 1), with a maximum count (mean $4.3 \log_{10}$ CFU/g ± 2.3) reached between day 4 and 6 from the beginning of consumption. Notably, for 11 (57.9%) of the 19 children with viable cells, *L. casei* DG[®] survived in faecal samples up to 3 days after treatment end.

Table 1 Percentage of children with viable *L. casei* DG[®] cells in faecal samples collected at baseline (day—2 [+ 1]), during treatment (Day 1 [+ 2], Day 4 [+ 2]) and at follow-up (Day 8 [+ 2] and days 11, 14, 17 and 20 [+ 2]).

Assessments	Subject number	Subjects, n (%) with viable <i>L. casei</i> DG in fecal samples				
		Baseline	1-week treatment		Follow-up	
		Day -2(+ 1)	Day 1(+ 2)	Day 4(+ 2)	Day 8(+ 2)	Day 11, 14, 17, 20 (+2)
Daily assessment	20	0(0.0%)	3(15.0%)	16(80.0%)	11(55.0%)	0(0.0%)
Overall	20	0(0.0%)	19(95.0%)			0(0.0%)

3.2 Animal study

L. paracasei DG colonized more the portion of caecum (media of total bacterial cell log: 11.43 ± 0.13) and colon (media of total bacterial cell log 10.99 ± 0.34) compared to ileum (media of total bacterial cell log: 9.22 ± 0.61) with a quantification of, respectively, 9.25 ± 0.08 (caecum), 9.38 ± 0.39 (colon) and 6.47 ± 0.83 (ileum) of bacterial strain cells (Fig. 4).

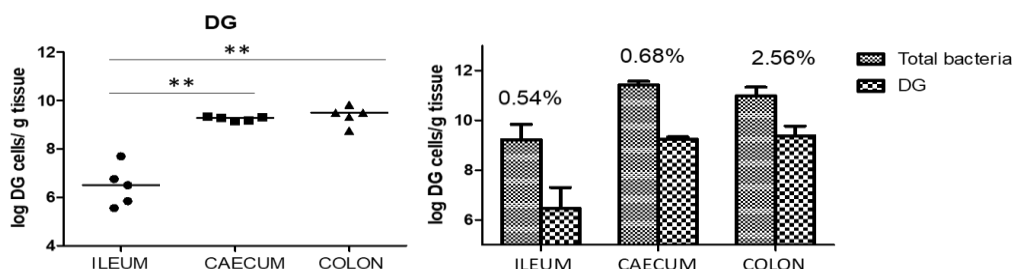


Figure 4 Quantification of *L. paracasei* DG cells expressed as media of total bacterial cell log (Mann-Whitney test (** = P value < 0.01)).

3.3 IBS study

The study, designed as above described, was approved by the Ethical Committee and was registered in clinicaltrials.gov (NCT03449628). Twenty centers have been involved in Italy in order to enroll 300 patients with IBS. The enrolment is still ongoing.

Based on previous pilot study, we suppose that overall abdominal pain and IBS symptoms will improve, *L. paracasei* DG cells will be present in the stools of exposed subjects, gut microbiota metabolism/composition will be significantly modulated, potentially conferring health benefit on the host following assumption of the strain.

4. Conclusions and Future Perspectives

Survival of gastrointestinal transit has been listed among the criteria that a microorganism should fulfill to qualify as a probiotic. Studies of probiotic viability in humans after ingestion mostly rely on quantification in feces and are also referred to as “persistence” or “recovery” studies. The viable recovery of probiotics from feces is technically challenging because feces are microbiologically very complex, hosting thousands of different microbial species. The ability to survive and persist in the gastrointestinal tract has been shown only for a limited number of probiotics available on market and is mostly limited to the well-known probiotic strains.

My Ph.D. project has shown that the strain selected from the market, *L. paracasei* DG, is really able to survive the gastrointestinal transit and to persist up to 5 and 3 days after the cessation of intake in adults and children, respectively. The capability to survive and reach alive the gut represents the first step for a potential colonization and modulation of the gut microbiota.

In addition, the animal study answered another important question in the world of probiotics: which is the preferential site of colonization of the probiotic bacterium? The obtained results may help us to identify a specific use of probiotic for in case of pathological situations with a localization at colon level, such as diverticular disease and IBD that in their ethiopathogenesis include the dysbiosis. So, by considering that the effects of probiotics are strain-specific, this set of tests could be useful to identify a high quality probiotic and to select a specific probiotic for a specific dysbiosis, according to the site of colonization. The *in vivo* studies in humans will then give us more information regarding also the clinical efficacy of the probiotic strain.

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Development, Characterization and Application in Food Systems of Innovative Functional Ingredients Obtained from Olive By-Products Phenolic Extracts

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The main aim of the PhD project is the valorization and exploitation of food by-products, in particular those originated from olive oil production chain which represent an effective source of phenolic compounds. Among olive by-products, the attention was mainly focused on olive leaves and the experimental design was structured on a three-steps plan: 1. evaluation of the technological properties of olive leaf phenolic extracts (OLE); 2. development and characterization of OLE-based functional ingredients by means of microencapsulation; 3. Exploitation of OLE-beads for the enrichment of real food matrices.

Ingredienti funzionali innovativi a base di estratti fenolici ottenuti da sottoprodotti olivicoli: sviluppo, caratterizzazione ed applicazione in sistemi alimentari

L'obiettivo principale del progetto di dottorato riguarda la valorizzazione e l'utilizzo di sottoprodotti alimentari, in particolare quelli originati dalla filiera dell'olio d'oliva e che rappresentano un'efficace risorsa di composti fenolici. Tra i sottoprodotti olivicoli, l'attenzione è stata rivolta principalmente verso le foglie di olivo ed il piano sperimentale è stato strutturato in tre step: 1. valutazione delle proprietà tecnologiche di estratti fenolici di foglie di olivo (OLE); 2. sviluppo e caratterizzazione di ingredienti funzionali contenuti OLE tramite microincapsulamento; 3. utilizzo di incapsulati per l'arricchimento di matrici alimentari reali.

Key words: Olive by-product, olive leaves, phenolic compounds, technological functionality, microencapsulation, functional ingredient.

1. Introduction

In the last few years there has been a growing interest on exploring the chemical composition of plant by-products in order to recover high value bioactive compounds for both food and pharmaceutical applications. Olive by-products, in particular olive leaves (OL), are rich in biophenols whose content and composition depends on biotic and abiotic factors as well as on processing technology (Talhaoui et al. 2015). Polyphenols are a class of natural substances with high biological value generally attributed to their antioxidant activity and are often associated with other beneficial effects such as anti-inflammatory, anti-carcinogenic and anti-atherosclerotic properties and gut microbial health. However, they are sensitive to several chemical, physical and environmental factors, which lead to instability with main effect also on their bioavailability.

Encapsulation technology is one of the most applied strategy to protect bioactive compounds towards degrading factors during utilization and storage. Enrichment and encapsulation of phenolic extracts into numerous food products have attracted the attention of scientists although literature data are quite limited until now in particular for

what concerns olive by-products. Gel-forming polymers from natural sources represent an important class of biomaterials for encapsulation because of their renewability, biodegradability, biocompatibility, and nontoxicity. Among this class of polymers, alginate has received much attention, likely because it can form hydrogels primarily by ionotropic gelation with divalent ions (i.e., Ca^{2+}) at room temperature, thereby allowing immobilization performed under mild and safe conditions. The most widely used encapsulation technique when using alginates is the extrusion method, which consists of dropping an aqueous solution of polymer and active principle into a gelling bath of CaCl_2 . Another less common method is the emulsification/gelation technique, which involves the dispersion of alginate/phenol compound solution into a vegetable oil. Thus, when the ionic induced gelation occurs, alginate forms the polymeric matrix trapping the active component inside. This is a promising method and it is useful for producing small diameter alginate beads, generally with a mean size ranging from 1 to 1000 μm .

In accordance with the PhD thesis project previously described (Flamminii, 2017; 2018), this paper summarises the main results of the three following activities:

A1) evaluation of technological properties of olive leaves phenolic extract (OLE) by means of surface tension, emulsifying activity, WHC/OHC, chemical stability, flow behavior;

A2) development and optimization of alginate-based microparticles through emulsion internal gelation method in combination with different biopolymers (pectin, whey protein, sodium caseinate). Encapsulation efficiency, particle size, morphology, swelling behavior, release kinetic were evaluated;

A3) production of enriched mayonnaise with OLE and OLE's microparticles. Physico-chemical and sensory properties of the experimental samples were evaluated; shelf-life tests were also carried out at different temperature conditions (ongoing).

2. Materials and Method

2.1 OLEs Technological Properties

The OLE were kindly provided by University of Bari, Department of Soil, Plant and Food Sciences (DiSSPA) and produced within the AGER 2 project, grant no. 2016-0105. The extraction from air-dried leaves was performed according to Difonzo et al., 2017. Three different extracts were obtained by using water and ethanol at different concentration (Eth0, Eth30 and Eth70). Extracts were analysed for their chemical composition (protein, sugar, mineral and fiber contents together with total phenol compounds, phenolic pattern) and antioxidant activity. The surface tension was assessed at the air/water interface by means of a tensiometer and a dose-dependent curve was obtained for each phenolic extract. The emulsifying activity was evaluated by measuring the particle size and distribution of oil-in-water model emulsions obtained with 0.3% (w/v) of each extract added to the water phase. Different percentages of oil dispersed phase were considered (5, 10 and 20% w/v) while keeping constant the amount of OLE added to the continuous phase. The flow behavior was investigated by means of a rheometer equipped with concentric cylinders; water and oil holding capacity (WHC/OHC) were determined by centrifugation as reported by Fuentes-Alventosa et al. (2009) with slight modifications. Furthermore, the chemical stability of the extracts was assessed during storage and results were expressed as decrease (in %) of oleuropein and its degradation was modelled by a two parameter Weibull distribution. The functional properties were evaluated at two different pH values (4.5, 7).

2.2 Microencapsulation of OLE

The microbeads were produced by emulsification/internal ionotropic gelation of sodium alginate following the method reported by Poncelet, Babak, Dulieu & Picot (1999), with some modifications. Encapsulant solutions were composed of sodium alginate (Alg), in combination either with pectin (Alg-Pec), or whey proteins (Alg-WPI) or sodium caseinate (Alg-Cas). Each ingredient was used at a concentration of 2% (w/v). The phenolic extracts were added to the encapsulant solution at 0.5 or 2% (w/v) concentration and to the washing media (CaCl_2 and ethanol 70%v), when an enriched procedure was used. The Encapsulation Efficiency (EE%), i.e. the phenolic compounds entrapped in the beads, was evaluated by the total content of phenolic compounds (Folin-Ciocalteu assay). Microparticles were analyzed for their size and morphology; moreover, phenolic-structurant interactions were evaluated by thermic (DSC) and spectroscopy (FT-IR) analyses. Swelling and release behavior were also investigated and release was modelled with the Peppas-Sahlin equation.

2.3 Production of enriched mayonnaise

In order to evaluate the exploitability of OLE-beads as functional ingredient, olive leaf phenolic extract and freeze dried microparticles were added as ingredient to a mayonnaise at the concentration of 200 mg of total phenolic compounds per kg of product. A standardized recipe was used for the mayonnaise as follows: 500 g of sunflower oil, 5 fresh egg yolk, 37.5 g of lemon juice, 10 g of vinegar and 5 g salt. Three kinds of samples were produced: a control sample, a sample enriched with the extract not encapsulated (mayo-OLE) and a sample enriched with OLE-beads (mayo-AlPec). Just after preparation (time zero), the evaluations carried out on the samples were: particle size distribution, microstructure, color, sensory analysis, viscosity and tribology. Then, ten grams of each sample were stored in 20 ml vials at 10°C, 20°C and 35°C (three independent replicates for each sample and temperature and time) and at 7, 14, 21, 28, 35, 50, 65, 80 and 95 days of storage the following determinations were carried out: color, hydroperoxides and TBARs for primary and secondary oxidation products, respectively.

3. Results and Discussion

3.1 OLEs Technological Properties

Most of olive by-products, such as olive leaves, are still undervalued despite their high potential as a source of functional compounds. However, to incorporate such compounds as active ingredients in complex food systems, it is of primary importance the knowledge of their technological functionality which represented the first objective of the PhD work. Results from the chemical characterization showed an influence of the extraction solvent both on the mineral and pigments contents and on the antiradical activity, while no significant differences were reported for sugars, fibre and total phenolic compounds with values ranging from 178.78 ± 8.43 , 163.98 ± 3.43 , 183.83 ± 9.43 mg GAE g^{-1} for Eth0, Eth30 and Eth70, respectively. The phenolic extract tested for surface activity showed a typical dose-dependent behavior; in particular, the extracts Eth30 at pH 4.5 and Eth70 at pH 7 resulted to be the most surface active.

These results were confirmed by the emulsifying capacity, in particular the model emulsions enriched with Eth30 at pH 4.5 showed a monomodal particle size distribution (PSD) with a mean diameter centered on 2 μm and a dimensional range within 0.93-6.30 μm while at pH 7 a polymodal distribution with different droplets populations was noticed. At increasing oil content and constant OLE level, the particle size distribution changed from monomodal to polymodal, with a shift towards bigger particles and an increase in

particle size. The results obtained for higher oil contents were due to the low extract-to-oil ratio which was not able to surround the newly formed oil/water interfaces and stabilize the droplets which rapidly underwent coalescence. Mean particle diameter ($D_{4,3}$) of the oil-in water model emulsions are reported in Table 1.

Table 1 Mean particle diameter ($D_{4,3}$) of oil-in-water model emulsions formulated with different oil ratio (5, 10 and 20%) added with OLE differently extracted and at constant added OLE

	$D_{4,3}$ (μm) 5% oil		$D_{4,3}$ (μm) 10% oil		$D_{4,3}$ (μm) 20% oil	
	pH 4.5	pH 7	pH 4.5	pH 7	pH 4.5	pH 7
Eth0	2.9±0.1 ^c	24.2±9.1 ^a	3.8±0.2 ^b	14.7±5.1 ^a	9.9±2.0 ^d	42.0±3.1 ^b
Eth30	2.8±0.1 ^c	5.1±1.5 ^{bc}	5.07±0.3 ^b	10.1±5.2 ^a	6.3±0.6 ^d	40.3±7.4 ^b
Eth70	3.4±1.2 ^{bc}	11.9±1.2 ^b	2.3±0.4 ^b	18.5±3.2 ^a	34.9±3.1 ^c	72.9±13.1 ^a

Values are means $\pm$ SD. Different letters for sample at different oil ratio (5, 10 and 20%) are significantly different by Tukey HSD

Surface properties and emulsifying capacity of the OLEs were affected mainly by pH of the medium and not by the phenolic compounds; considering the rather complex composition of the extracts (Difonzo et al., 2017) it is not to be excluded the contribution of proteins and other amphiphilic compounds as well as the formation of complexes with enhanced surface properties and

emulsifying activity.

Table 2 Water Holding Capacity (WHC) and Oil Holding Capacity (OHC) of OLE Eth0, Eth30 and Eth70.

	WHC g water/g OLE	OHC g oil/g OLE
Eth0	0.07±0.02 ^b	0.45±0.05 ^b
Eth30	0.05±0.03 ^b	0.33±0.05 ^b
Eth70	0.40±0.03 ^a	0.60±0.03 ^a

Values are means $\pm$ SD. Different letters for sample for WHC and OHC respectively, are significantly different by Tukey HSD test.

Water Holding Capacity and Oil Holding Capacity (OHC) results (Table 2), showed very low values in respect to those reported in literature for extracts recovered from food waste and by-products (i.e citrus peel) and can be considered in agreement with the chemical composition and in particular with the negligible content of TDF (data not shown). The OHC results show slightly higher values than those obtained for WHC and indicate a higher ability of OLE to bind oil rather than water. On the basis of the results obtained, OLEs could represent a useful ingredient in lipid-containing foods

thanks to their higher ability to bind oil rather than water.

3.2 Microencapsulation of OLE

In the first part of the second activity (A2), aimed at encapsulating OLE in alginate beads by emulsification and internal gelation, the importance of formulation and process-related factors were investigated and optimized (i.e. calcium source, concentration of emulsifier, gelation time and recovery procedures) to obtain the lowest particle polydispersity. Results (data not shown) indicated that both calcium citrate and carbonate were able to produce small alginate particles with limited polydispersity; an increase up to 2% (w/w) in the concentration of the emulsifier significantly decreased the mean particle diameter as obtained also by the increase in the gelation time. Based on these results four delivery systems made of plain alginate (Alg), alginate and pectine (Alg-Pec), alginate and whey proteins (Alg-WPI) and alginate and caseinates (Alg-Cas) were produced using the optimized procedure (not enriched procedure). In addition, beads

were also obtained by adding OLE in the recovery media at the same concentration than in the initial solution (enriched procedure).

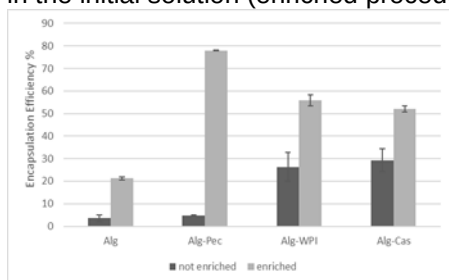


Figure 1 Encapsulation efficiency of the microparticles obtained with both procedures.

alginate and WPI/sodium caseinate and/or between WPI/sodium caseinate and OLE polyphenols, as reported also by Belscak-Cvitanovic et al. (2015), where the use similar proteins as the ones used in the present study, remarkably enhanced microspheres encapsulation efficiency. The production of the microspheres through the enriched procedure considerably increased the beads encapsulation efficiencies for all the tested systems and in particular for the Alg-Pec with values up to 77%. Delivery systems obtained by the enriched procedure were further characterized for the swelling behavior, Fig. 2 (a,b) and phenolic compounds release kinetics (data not reported).

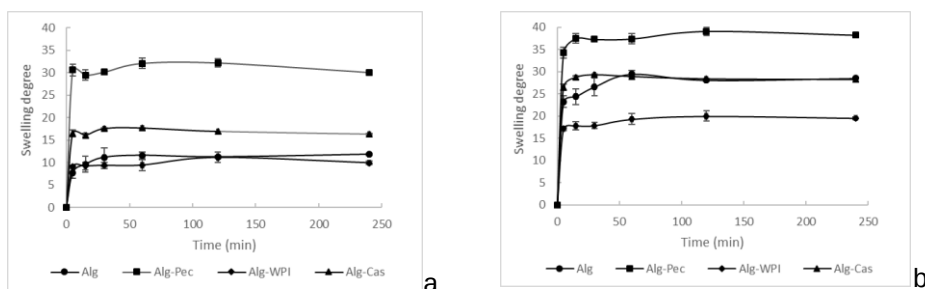


Figure 2 Swelling degree of (•) Alg, (▪) Alg-Pec, (♦) Alg-WPI, (▲) Alg-Cas microspheres at pH 4.5 (a) and pH 6.0 (b).

The swelling behaviour is an important index since it directly impacts on bioactives release and profile. All sample showed a similar swelling behavior, higher when in contact with phosphate buffer in respect to acetate buffer due to higher extent of protonization of carboxylate groups of calcium alginate which resulted in reduced electrostatic repulsion and favoured shrinkage. Swelling mainly took place within the first 5-60 minutes, quickly reaching a plateau. Our results confirmed what previously observed in by Rayment et al., (2009) about the swelling behaviour of alginate beads into gastric and intestinal fluids with the tendency to shrink in gastric environment (pH 2) and to swell in intestinal one (pH approaching 8).

Release data, fitted with Peppas-Sahlin equation, provided interesting information on the main mechanisms involved in the bioactive compounds release. The first one is due to Fickian diffusion, while the second is affected to relaxation/dissolution of the polymeric chains. All the systems exhibited m values lower than 0.43, which highlight a

pseudo-Fickian diffusional mechanism controlling the release of polyphenols with a negligible effect of any possible relaxation phenomena of the matrix biopolymers in case of spherical geometry. Similar results, with $m < 0.43$, $k_1 > k_2$ and $k_2 < 0$ were underlined by previous studies dealing with polyphenols encapsulation into alginate beads (Lupo et al., 2015, 2014). FT-IR spectra (4000–400 cm^{-1}) evidenced the presence of new intermolecular interactions which weakened the covalent bonds shifting their absorption to lower energy; this behaviour was confirmed by the DSC results that highlight endothermic peaks at around 75, 77, 79 and 85°C for Alg-WPI, Alg-Cas, Alg and Alg-Pec microbeads respectively and indicate possible chemical interactions between polymers and extract as well as a good thermal stability, as reported by Córdoba, Deladino & Martino. (2013).

3.3 Production of enriched mayonnaise

Taking into account the main results previously reported (i.e. good emulsifying capacity of OLE at pH 4.5 and better EE% of Alg-Pec system, controlled release properties at pH 4.5), the third activity of the project was focused on the fortification of mayonnaise (pH ca. 3.8) with OLE and Alg-Pec. This research activity is still ongoing.

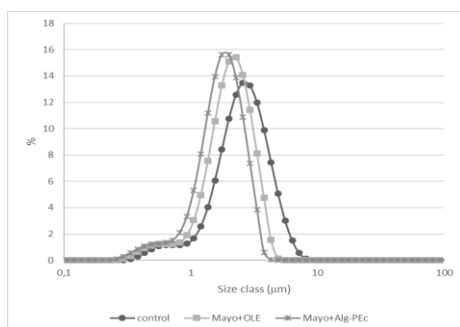


Figure 3 Particle size distribution of mayonnaise sample.

Table 3 Particle size parameters of mayonnaise sample.

	D [4;3]	D [3;2]	Specific Surface Area
Control	3.2±0.02 ^a	2.1±0.03 ^a	2981.7±59.1 ^a
Mayo+OLE	2.1±0.01 ^b	1.7±0.02 ^b	3663.4±43.2 ^b
Mayo+ Alg-Pec	1.8±0.01 ^c	1.5±0.01 ^c	4272.9±9.7 ^c

Values are means ± SD. Different letters for samples, are significantly different by Tukey HSD test.

Preliminary results obtained from the production day showed significant differences ($p < 0.05$) in all the chromatic parameters (L^* , a^* , b^*) of Mayo+Alg-Pec respect to the control and Mayo-OLE. Particle size distribution of mayonnaise samples (PSD), reported in Fig. 3, showed two populations and a shift towards lower size for the Mayo+Alg-Pec, as also confirmed by the particle size (Table 3). Results about flow behaviour highlight significant differences in terms of apparent viscosity ($p < 0.05$) between the system Mayo-Alg+Pec with respect to both Control and Mayo-OLE probably due to the interactions between lipid droplets and alginate/pectin microparticles which could exert a thickening effect. With respect to lipid oxidation, the enrichment with OLE and OLE-beads affected the production of both primary and secondary oxidation products with an efficacy influenced by storage temperature.

4. Conclusions and Future Perspectives

The proposed research could represent a valid contribution to the entire olive oil sustainability system since focused on the recovery of high added value compounds obtained from olive by-products and their use as functional ingredient in real food systems. Thanks to OLE surface properties, emulsifying capacity and oil binding ability results, OLE can be considered a promising functional ingredient in acidic oil-in-water emulsions. The encapsulation of OLE with emulsion/internal gelation technique in

alginate-polymer matrix improved both the retention, stabilization and controlled release of OLE respect to the compounds not encapsulated. From an industrial perspective, these results could open interesting opportunities in the exploitation of olive processing by-products and contribute to enhance the exploitability of olive leaves phenolic extracts as a multifunctional ingredient that can help in the production and stabilization of complex food products like emulsions.

5. Acknowledgement

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Effective Replacement in Food Packaging Laminates of the Conventional Oil Based Oxygen-Barrier Polymers with Bio-Composite Laminates Containing Cellulose Nanocrystals (CNCs) Extracted from Waste and Biomasses

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The main purpose of this doctoral thesis was to implement an innovative packaging material for extending the shelf life of oxidation-sensitive food products by using biocomposite multi-layers' laminates containing a biodegradable gas barrier such as cellulose nanocrystals coatings in replacement of the synthetic barrier such as ethylene vinyl alcohol copolymer still used today.

Uso e impiego effettivo dei bio-laminati ad alta barriera all'ossigeno a base di nanocristalli di cellulosa ottenuti da biomasse di scarto e sottoprodotti in sostituzione dei multistrati di laminati sintetici.

Lo scopo principale di questa tesi di dottorato è stato quello di implementare un packaging innovativo per l'estensione della shelf-life dei prodotti alimentari sensibili all'ossidazione utilizzando multistrati di laminati biocompositi contenenti materiali biodegradabili come i nanocristalli di cellulosa che esibiscono straordinarie proprietà barriera ai gas in sostituzione della barriera sintetica come il copolimero etilenvinil alcool tutt'oggi usato.

Key words: cellulose nanocrystals; food shelf-life extension; gas barrier properties; biocomposite multi-layers' laminates.

1. Introduction

The nanocellulose is obtained through the “top-down” process of the vegetal cellulosic sources (biomass, by-products) (Nurain et al., 2012) and it is divided into two categories: the cellulose nanocrystals (CNCs) and cellulose nanofibrils (CNFs) extracted chemically by acid hydrolysis and mechanically respectively. CNCs can be obtained either by acid hydrolysis (HCl, H₂SO₄) (Ping & Hsieh, 2001) or by oxidative hydrolysis with ammonium persulfate (Mascheroni et al., 2016) and TEMPO (Akira et al., 2011). In this work CNCs used derived from the acid hydrolysis of wood pulp by sulfuric acid (Ping & Hsieh, 2001). Polymeric films coated with very thin layer of cellulose Nanocrystals (<1μm) show very low values of oxygen permeability (0.06 cm³ * 24h⁻¹ * m⁻² * bar⁻¹) (Li et al., 2013) and outstanding mechanical properties in dry ambient. Therefore, CNCs might be used to replace synthetic barrier resins currently used in the food packaging sector (Mascheroni et al., 2016), with the aim of extending the shelf-life of oxygen-sensitive products with more sustainable solutions thus promoting a circular economy. However, the high hygroscopicity of the CNCs poses strong limitations of use because, nanocrystals coatings tend to lose their O₂ barrier when exposed to humidity. In fact, X-ray diffraction measurements explained the behavior of the CNCs in terms of hydration at the crystalline structure at a relative humidity of 100% and, in particular, the modifications of the short and long-distance interactions which allow the CNCs to contain a large amount of absorbed water. Furthermore, the gradually conformational modification of the crystalline domain by the water absorbed subsequently allows the gas to pass through the CNCs coatings easily (Fotie et al., 2017). Recently, the possibility of using cellulose nanocrystal coatings for modified atmosphere packaging (MAP) has been

investigated and the report concluded that it was feasible, but still, that moisture sensitivity was a significant obstacle to its implementation (Fotie et al., 2018). In this regard, the search of the best and most sustainable solution was carried out for an attempt of solving the current limit of CNCs before setting up any development of novel food packaging based on those nanocrystals. As major advantages, the use of cellulose nanocrystals will reduce the dependence on petroleum-based materials in food packaging, contribute to implement eco-compatible applications for more sustainable solutions and finally reduce food waste, which is one of the biggest environmental pollution factors (Poritosh et al., 2009).

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following four activities directed to:

A1) Characterization of the cellulose nanocrystals (CNCs): Z-potential, charges density and particles size assessment of CNCs suspensions;

A2) Coating CNCs onto polymers (CNCs-polymer) and gas permeability evaluation;

A3) Lamination of CNCs-polymer with solvent-based adhesives to obtain Biocomposite multi-layers' laminates (BMLCNCs);

A4) Gas permeability measurements of CNCs-polymer and BMLCNCs.

2. Pitfalls and potential solutions for cellulose nanocrystals (CNCs) implementation in packaging.

As mentioned above, the cellulose nanocrystals being very hydrophilic by nature, they are very sensitive to humidity. The structure of the CNCs contains hydrophilic compounds such as primary and secondary hydroxyl groups, which bind to water by hydrogen bonds (Oliver et al., 2001). Generally, the CNCs dispersion is prepared in water, applied onto plastic polymers and then thoroughly dried. CNCs coated polymeric films provide a very high barrier to gas permeation in the absence of water, but when placed in a relatively humid atmosphere, coatings are compromised and the gas permeability begins to increase abruptly (fig 1b). After a very thorough investigation on CNCs coated PET films, it was revealed that the water integrated by the CNCs coating acts as a plasticizer progressively and disrupts the crystal lattice; subsequently, CNCs conformation was modified allowing the gas to pass through the coated material with less hindrance (Fotie et al., 2017).

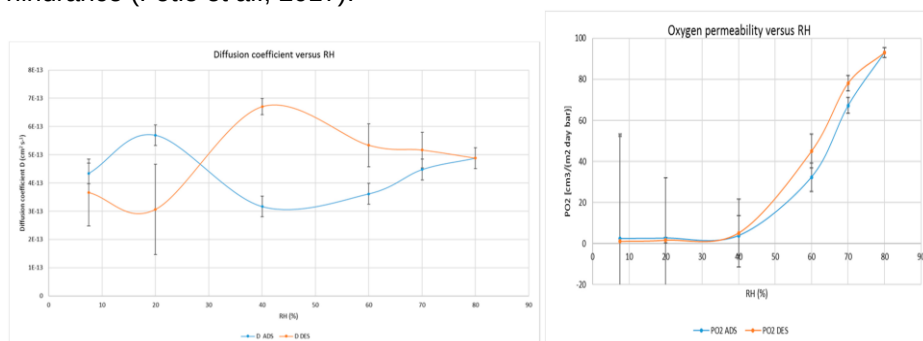


Figure 1 a and b Apparent diffusion coefficient (a-left) and oxygen permeability (b-right) at 25 °C under different relative humidity (RH) values, both in adsorption and desorption, through the CNCs coated PET film.

Furthermore, fig 1b reveals that the loss of the barrier in high relative humidity is not irreversible, but that is recovered gradually with the decrease in moisture. Finally, fig 1a and fig 1b show analogous thresholds in the desorption between 40-60% RH; thereby, it was concluded that the freezable water does not play a decisive role in the increase of oxygen permeability (Fotie et al., 2017). As a consequence, one of the solutions considered plausible to alleviate the effect of humidity on CNCs coatings was the lamination of the layer of CNCs between sealable and/or hydrophobic polymers that prevent their direct contact within the humid environment.

4. Experimental Procedure

My experimental PhD work was set up in 4 stages: 1) Characterization of cellulosenanocrystals 2) Coating process of the CNCs onto plastic films (CNCs-polymer); 3) Implementation of the Biocompositemuti-layers based on the cellulose nanocrystals (BMLCNCs) through lamination 4) Gas permeability measurements of CNCs-polymers and BMLCNCs.

5. Materials and Methods

Plastic films such as 12 µm PET (polyethylene-terephthalate), 75µm PE (polyethelene), 40µm PP (polypropylene), 15µm OPA (oriented polyamide), 30µm OPP (oriented polypropylene) and POLURFLEX 2644 / 58-01: two-component polyurethane solvent based adhesive system [mixing ratio 100:10:83 (OH: NCO: Solvent)] and 13 sec viscosity 25°C were all provided by Sapici spa, Cernusco sul Naviglio, Italy. Cellulose nanocrystals (CNCs) obtained by acid hydrolysis of the wood pulp were bought from CelluForce 609, Rang 12C.P.1010 Windsor (Quebec).

5.1 Cellulose nanocrystals characterization

Apparent hydrodynamic diameter and Z-potential were measured on suspended CNCs at 0.2 wt% and pH 8.2 by electrophoretic light scattering (ELS) and dynamic light scattering (DLS) respectively, using the PALS technology (mod. Litesizer 500, Anton Paar, Graz, Austria). Measures were replicated 5 times, at 25.0 ± 0.1 °C, by means of a 35 mW diode laser ($\lambda = 658$ nm) and at 15°, 90° detection angle. The charges density was obtained on suspended CNCs in water at 1 wt% through the conductimetric titration with NaOH 0.01M.

5.2 Coating process of CNCs onto plastic films (CNCs-polymers)

Plastic films were previously treated by corona treatment to enhance their surface energy and promote their adhesion with the water dispersed-CNCs at 6 wt%. The CNCs-polymer was then obtained (fig 2). The thickness of the coating applied onto the film was assessed by a gravimetric method. Four samples (10×10 cm²) were weighed (m_1 , g), then the coating was removed by conveying hot water (~70 °C) and the resulting uncoated film was dried and weighed (m_2 , g).

The coating thickness (L , cm) was estimated by Equation (1):

$$L = (m_1 - m_2) / (\rho \times 100) \quad (1)$$



Figure 2

CNCs-polymer after the coating process.

5.3 Biocomposite multi-lamination of the cellulose nanocrystals (BMLCNCs)

At room temperature, the solventless polyurethane adhesive was used to set up the BMLCNCs (fig 3), afterwards, the laminated plastic films were then stored at 50°C for 7 days to reinforce the bonded joint adhesives-polymers and for the delamination test, multi-coated films were conditioned at 23°C and 50% relative humidity for 40 h prior to test in accordance with Procedure A of Practice D618 in agreement with ASTM. The adhesive thickness was calculated as the remaining from the overall laminate knowing the thickness of polymers and CNCs layers involved in the BMLCNCs.



Figure 3 *BMLCNCs obtained after the lamination*

5.4 Gas permeability measurements of CNCs-polymers and BMLCNCs

All the oxygen permeability measurements were performed by an isostatic permeabilimeter (mod. Multiperm, PERMTECH S.r.l., Pieve Fosciana, Italy) according to ASTM standard methods (D-3985 and F-1249 respectively). Uncertainty was evaluated based on at least three replicates and were under 5%.

6. Results and Discussion

6.1 Particle size and z-potential measurements

Zeta potential and apparent hydrodynamic diameter, performed on suspended CNCs, gave -40 ± 0.2 mV and 115 ± 4 nm respectively. For the surface charges density, the conductimetric titration allowed to obtain 0.28 mmol/kg for strong acid ($-\text{SO}_3\text{H}$) and 0.35 mmol/kg for weak acid ($-\text{OH}$). The very high Z-potential demonstrates the stability of the colloidal suspension of CNCs dispersed in water; in addition, the presence of hydroxyl and sulphate half ester groups is a further confirmation of the hydrophilic character of the CNCs and the high functionality of their surface for a possible chemical interaction with other structures (polymers, papers...).

6.2. Oxygen permeability of standard polymers

The figure 4 shows the very high levels of oxygen permeability of standard polymers therefore, they are inadequate for oxygen-sensitive food products. As expected, a PET and OPA which are semi-crystalline and oriented polymer show a lower oxygen permeability but not still enough good for being used as gas barriers.

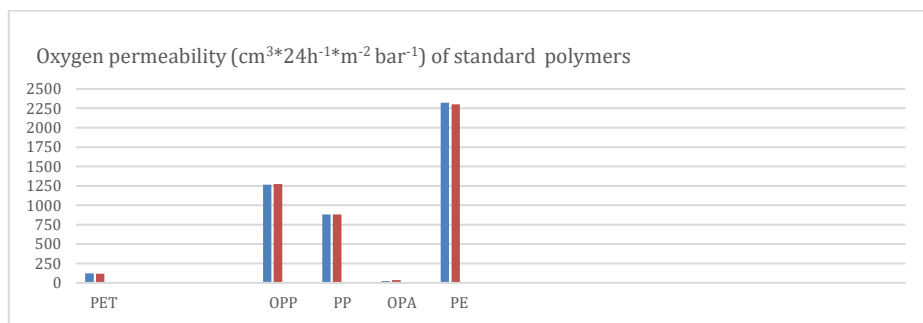


Figure 4 Oxygen permeability ($\text{cm}^3 \cdot 24\text{h}^{-1} \cdot \text{m}^{-2} \cdot \text{bar}^{-1}$) of uncoated polymers measured at 50% (blue) and 80% (red) of RH, at 23 °C

6.3 Oxygen permeability of CNCs-polymer

After the coating, the CNCs coated polymers showed a significant decrease of the oxygen permeability at 0% RH but slightly high at 80% RH (fig 5). First, it shows that all the coated polymers have a much lower O_2 permeability in particular PET, OPA, OPP and PP films whose values are close to 0 ($< 0.06 \text{ cm}^3 \cdot 24\text{h}^{-1} \cdot \text{m}^{-2} \cdot \text{bar}^{-1}$); therefore, this is an additional proof that CNCs coatings must be protected from the water to remain intact and give the same oxygen barrier. Second, fig 6 also reveals that the CNCs coatings are not affine at the same level with polymers; coated PE does not adhere perfectly with the CNCs dispersion, which was expected, based on the higher hydrophobicity of PE polymer.

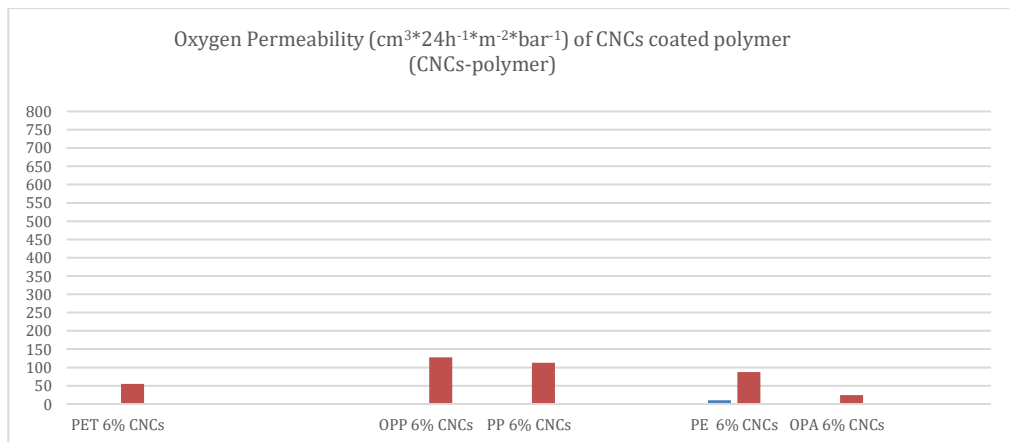


Figure 5 Oxygen permeability of CNCs-polymers measured at 0% (blue) and 80% (red) of RH, at 23 °C

6.4 Delamination Test of biocomposite multi-layers' laminates (BMLCNCs)

The delamination test of BMLCNCs proved that adhesion between the CNCs and the adhesive occurred perfectly because the stiffness, the breaking elongation and the percentage deformation at break are sufficiently high (table 1) for making it possible the use of the BMLCNCs in food packaging. The adhesive thickness calculated gave $1.2 \pm 0.2 \mu\text{m}$.

Table 1 Delamination Test of biocomposite multi-layers' laminates (BMLCNCs).

BMLCNCs	Maximum load (N)	Breaking elongation (mm)	Elastic Modulus (N/m)	Elongation At the end (mm)	Percentage deformation at break (%)	Breaking Strength (N/m)	Stress at yield (N/m)	Stiffness (N/m)
OP6%CNCs //OPP	1.14	83.17	35462	126.72	109.27	6.24	1.04	6986
PET6%CNCs //PP	0.23	84.39	5209	120.50	110.91	1.25	0.19	987
PET6%CNCs//PE6%CNCs	0.11	0.33	7246	105.97	0.41	9.68	0.11	1289
PET6%CNCs//OPP	0.22	0.1	9639	125	0.13	20.7		1883
PE6%CNCs//PE6%CNCs	0.72	61.78	2218	126.72	76.74	6.5	0.5	631

6.5 Oxygen permeability of BMLCNCs

After the lamination of the cellulose, the oxygen barrier improvement was evident and significant, multi-layers' laminates such as PET-CNCs//OPP, PET-CNCs//PP and PET-CNCs//PE were the best in terms of the oxygen barrier even in much higher relative humidity (fig 7). In particular, PET-CNCs//PP laminates give the highest oxygen barrier with permeability null at 50 and 80% RH (fig 6).

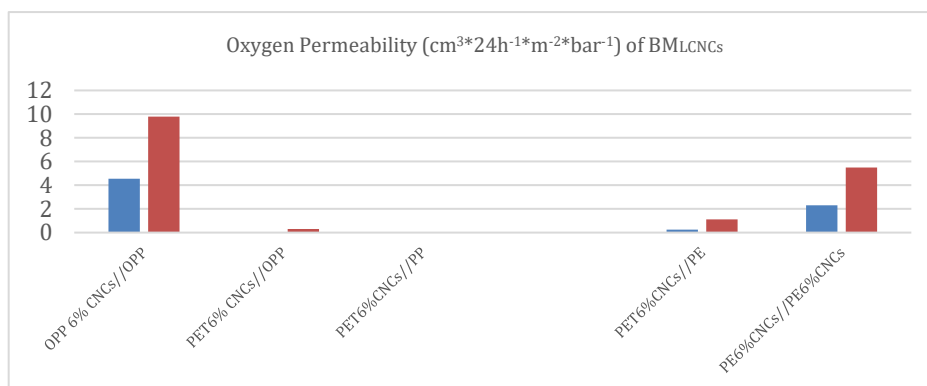


Figure 6 Oxygen permeability of laminated multi-layers (BMLCNCs) measured at 50% (blue) and 80% (red) of RH.

7. Conclusions and Future Perspectives

The problem to be sorted out was the sensibility of the cellulose nanocrystals to moisture that hampered their incorporation into food packaging. i) When comparing the oxygen permeability of CNCs-polymers and standard polymers, it can be seen that the contribution of cellulose nanocrystals in improving the oxygen barrier is really effective in absence of humidity but compromised in humid environment ii) After the lamination, the biocomposite multi-layers' laminates (BMLCNCs) were subjected to the delamination test and from the results, it can be observed that the stiffness, elastic modulus and elongation at the end are relevant for any test delamination of polymers iii) Oxygen permeability of

the BMLCNCs showed very low values even in higher relative humidity, which is the confirmation that the lamination has definitely isolated the CNCs coatings from the moisture and can be used for food shelf-life extension. This opens up a new gate for effective development of advanced materials suitable for food shelf-life extension, taking care of environmental protection and circular economy principles.

8. References

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Effects of Different Storage Technologies and Temperatures on the Quality and Nutritional Value of Yellow-Fleshed Kiwifruit

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The PhD thesis is aimed at comparing kiwifruit quality as a result of two storage methods – air storage and ozone treatment - different temperatures, different ozone concentrations of yellow flesh kiwifruit ‘Soreli’, in order to define the best storage method. Moreover, due to the emerging problem of allergenicity, the detection of the allergens on kiwifruit-containing food products, after ozone postharvest treatment, has been studied; in order to declare the potential allergenic ingredients on the products’ label.

Effetto di differenti tecnologie di conservazione e temperature sulla qualità ed il contenuto nutritivo del kiwi a polpa gialla

L'obiettivo della ricerca di dottorato è quello di confrontare gli effetti di diversi metodi di conservazione – aria ed ozono gassoso - a diverse concentrazioni di ozono ed a diverse temperature, del kiwi a polpa gialla ‘Soreli’, così da delineare i parametri per un’ottimale metodo di conservazione di tale prodotto ortofrutticolo preservandone le caratteristiche nutrizionali e qualitative. Inoltre, a causa dell’aumento dei consumatori affetti da allergie, diventa oggetto di studio la rintracciabilità degli allergeni in prodotti ottenuti dalla trasformazione di kiwi conservati secondo i trattamenti tecnologici post raccolta descritti precedentemente.

Keywords: cold storage, ozone, *Actinidia chinensis*, *Actinidia deliciosa*, postharvest quality, bioactive compounds

1. Introduction

In according with the PhD thesis project previously described, this oral communication reports the main results of the following activities directed to, in the first trial (1A), evaluate the effects of the synergic relation between 0 °C and the low concentration of gaseous ozone (150/180 ppb) during postharvest cold storage and shelf-life. Two cultivars were studied: green flesh kiwifruit ‘Hayward’ and yellow flesh kiwifruit ‘Soreli’. ‘Hayward’ has been taken as reference due to its worldwide importance in the kiwifruit industry. The storage at 0 °C was the first studied temperature due it is the recommended temperature to prolong postharvest storability of ‘Hayward’ kiwifruit. Whereas, the concentration of ozone applied during cold storage was lower compared to the concentration used in others works, in order to study the effectiveness of a minimum level of gaseous ozone.

During the second year (2A) the aim was to study the effects of gaseous ozone at higher concentrations (300 ppb) – compared to 150ppb of the first year - on qualitative and antioxidant-related properties of ‘Hayward’ and ‘Soreli’ kiwifruits when stored at 4 °C and during shelf-life conditions (18°C).

The purpose of the third trial (3A) was to maximise postharvest performance of ‘Soreli’, studying its behaviour when stored at 2 °C and 4 °C under continuous flow of gaseous ozone at 300 ppb and under air storage – untreated samples – for 60 days. The

used temperatures were higher than 0 °C due to Soreli's susceptibility to low temperatures.

Moreover, due to the allergenicity emerging problem, the detection of the allergens in kiwifruit-containing food products is under study.

The main focus was on ozone postharvest treatment, cold storage, a specific yellow flesh kiwifruit 'Soreli' and on the allergenic mechanism and traceability of allergens.

'Soreli' is a yellow flesh kiwifruit. It is a selection of tetraploid *Actinidia chinensis* cultivar originated from the controlled cross A137 x A134.41, carried out in 1997, and developed by Università di Udine, Italy. Soreli kiwifruits are characterized by mean fruit weight of 85 to 147 g, ovoid-long and regular shape, brilliant brown skin and intense yellow flesh. Harvest date, as determined by the fruit reaching 8.0 °Brix, varied by up to 25 days, with the extreme dates of 23 September and 18 October. Soluble solid content averaged from 12.5 to 15.7 °Brix. Fruits storage under normal atmosphere is limited to 90-120 days. A good key point of 'Soreli' is the marked citrus and tropical fruits flavour.

The ozone, used in aqueous or gaseous phase, is a strong oxidant. In 2001, it was approved by the US Food and Drug Administration as an antimicrobial additive for direct contact with foods. Ozone can be used as antimicrobial agent that causes the progressive oxidation of some cellular components (Barboni et al., 2010). Furthermore, Minas and coworkers (2014) demonstrated that ethylene biosynthesis and cell walls turnover were specifically targeted by ozone (0.3 µL L⁻¹ at 0 °C for 2 or 4 months) in 'Hayward'; generating the reduction of flesh softening and cell wall disassembly. Also, antioxidant capacity is affected by ozone treatment, due its oxidizing activity. Plants can develop some defense mechanism when under the oxidative stress. The reaction between ozone and antioxidants results in the formation of reactive oxygen species (ROS) and thereby protects cell structures from oxidative damage (Brodowska et al., 2017).

Allergy to kiwifruit belongs to the novel food-induced allergies. Kiwifruit is nowadays among the top ten food allergy sources according to recent studies. Up to now, the official IUIS Allergens Nomenclature database reports 13 allergens from green kiwifruits: the papain-like cysteine protease and actinidin Act d 1 (30kDa), the major allergens (Graziano 2017).

2. Experimental design

Kiwifruits were monitored during post-harvest cold storage in the absence (control) or presence of gaseous ozone.

1A) During the first trial a range of 150/180 ppb of continuous gaseous ozone flow was applied during storage at 0 °C on 'Hayward' and 'Soreli' kiwifruit over a period of 6 weeks, followed of 10 days of shelf-life at 18 °C. The untreated samples were stored at the same storage conditions but on air.

2A) During the second trial 300 ppb of continuous gaseous ozone flow was applied during storage at 4 °C on 'Hayward' and 'Soreli' kiwifruit over a period of 60 days, followed of 8 days of shelf-life at 18 °C. The untreated samples were stored at the same storage conditions but on air.

3A) During the third trial three main activities took place. First activity involved the use of 300 ppb of continuous gaseous ozone flow during storage at 4 °C and 2 °C over a period of 60 days on 'Soreli' kiwifruit. The untreated samples were stored at the same storage conditions but on air. The second activity consisted of the application use of 300 ppb of continuous gaseous ozone flow during storage at 2 °C over a period of 60 days on 'Soreli', after curing (10 days during which 24h fruits are stocked at ambient temperature,

and after it, during 7 days the temperature is gradually reduced up to 2 °C). The third activity was the transformation of the kiwifruits, stored according to the second activity, into juice, jam and biscuits in order to evaluate the traceability of the allergens.

The analysis carried out were:

1A) Tissue firmness (Instron Universal Testing Machine, penetrometer and NIR), soluble solids content, titratable acidity, color traits, ascorbic acid, carotenoids and total microbial count.

2A) The same analysis of the first trial and in addition to these: respiration rate, weight loss, dry matter, antioxidant capacity (DPPH), total phenols, flavonoids, MDA content and enzyme activities, including superoxide dismutase (SOD), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and polyphenol oxidase (PPO).

3A) The same analysis of the second trial and in addition to these the determination of lipoxygenase activities (LOX), catalase (CAT) and H₂O₂ content.

A principal component analysis (PCA) was applied to describe the relationship between physical-chemical attributes and treatments during postharvest storage. All analyses were carried out using the SPSS software package version 20.0 (SPSS. Inc., Chicago, IL, USA).

3. Results and Discussion

1A) The evidences from the first trial suggested that ozone post-harvest treatment is a suitable choice for the preservation of 'Hayward' and 'Soreli' kiwifruits. During cold storage and shelf life, it is mainly effective in preserving color (ΔE) (Fig.1a), flesh firmness (F) (Fig.1b) and total soluble solids concentration (TSS) maintenance. They are important quality attributes indicating the eating quality and postharvest storability of kiwifruits. Whereas, the microbiological data suggested that the low concentration of ozone is not so effective in preventing microbiological decay. Moreover, no meaningful changes in bioactive compounds were observed between treated and untreated kiwifruits at that storage conditions.

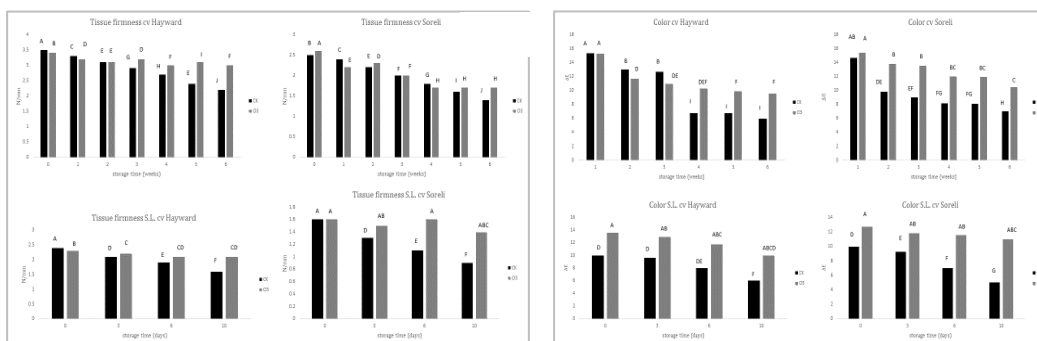


Figure 1a Color difference of 'Hayward' and 'Soreli' untreated (CK) and treated (O₃) kiwifruits during cold storage and shelf life.

Figure 1b Tissue firmness (N/mm) of 'Hayward' and 'Soreli' untreated (CK) and treated (O₃) kiwifruits during cold storage and shelf life.

2A) The findings from the second trial suggested that ozone post-harvest treatment at 300 ppb combined with 4 °C acted as strong oxidizing agent, inducing

oxidative stress. Various physiological responses have been observed, such as synthesis of antioxidants and secondary metabolites (ascorbic acid and phenols). On the hand, the chosen match wanted alleviate the low temperature breakdown, on the other hand, it accelerated the flesh softening and the oxidative stress, leading to senescence rapidly. During the storage 'Hayward' and 'Soreli' kiwifruits underwent senescence processes. Analysing 'Soreli' behaviour, 'Soreli' PCA biplot (Fig.2a) suggested that there was an induction of softening and oxidative stress due not to suitable match between ozone exposure concentration (300ppb) and temperature (4 °C). The spatial position of the clusters was focus on the storage time and during the storage the differences between the thesis decreased. MDA content (Fig.2b) suggested that ozone enriched-atmosphere could control the oxidative stress; the values of MDA were higher in untreated samples than in ozone-treated ones. Ozone-enriched atmosphere maintained constant the antioxidant activity (DPPH) during the storage compared to the untreated samples (Fig.2c).

Therefore, 'Soreli' kiwifruits answered better than 'Hayward' to the storage at 4 °C under ozone atmosphere. Whereas, the firmness dropped significantly during the storage, already after 15 days; the ozone-enriched atmosphere was not able to delay the softening, confirming that firmness retention is a critical point for yellow flesh-kiwifruits.

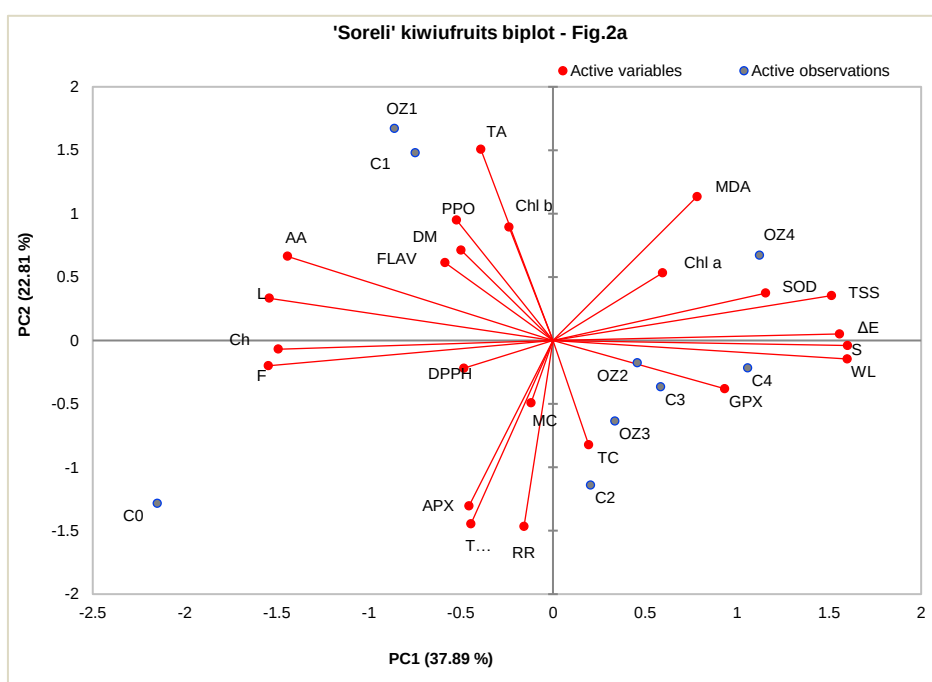


Figure 2a The PCA biplot of 'Soreli' kiwifruits stored at 4 °C under gaseous ozone (OZ) and on air (C) over a period of 60 days (T0, T1, T2, T3, T4). Sampling was carried out every 15 days. TC: total carotenoids; AA: ascorbic acid; TP: total phenols; Chl a: chlorophyll a; Chl b: chlorophyll b; FLAV: flavonoids; MC: microbial count; TA: titratable acidity; L: lightness; Ch: Chroma; F: firmness; S: softening; DM: dry matter; RR: respiration rate; WL: weight loss; TSS: total soluble solids; ΔE: difference color; DPPH: antioxidant capacity; SOD: superoxide dismutase; APX: ascorbate peroxidase; PPO: polyphenol oxidase; GPX: glutathione peroxidase; MDA: malondialdehyde content.

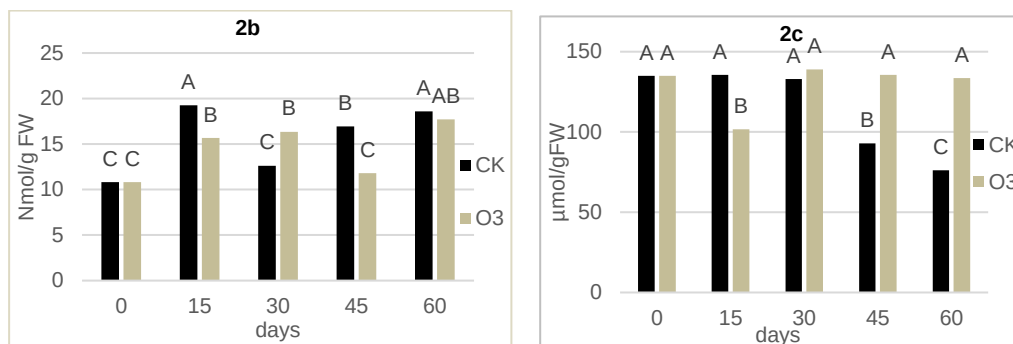


Figure 2b Malondialdehyde content of 'Soreli' untreated (CK) and treated (O₃) kiwifruits during cold storage (60 days).

Figure 2c Radical scavenging activity of 'Soreli' untreated (CK) and treated (O₃) kiwifruits during cold storage (60 days).

3A) The first activity of third trial highlights that 'Soreli' showed different physico-chemical and nutraceutical behaviors correlated to storage temperatures and ozone postharvest treatments. Ozone treatment induced the ripening process and the elicitation of the kiwifruit's defense mechanism with a decrease in bioactive compounds during the first period of cold storage (15 days) at the two tested temperatures. Ozone treatment controlled the microbial growth at the two tested storage temperatures. 4 °C is not suitable for preserving kiwifruit quality during storage compared to 2 °C, in 'Soreli', because 4 °C was less effective at preserving microbial decay and involved the synthesis of bioactive compounds, such as carotenoids, in the defense against oxidative stress. The gaseous ozone treatment at 300 ppb in continuous flow combined to 2 °C can be used as an effective postharvest treatment of 'Soreli'. It delayed the microbial decay, affected the reduction of sugars (SSC) and it controlled better the natural decay of bioactive compounds (Fig.3a).

Postharvest oxidative stress occurs during fruit storage, causing an imbalance between the production and the removal of ROS from tissues. In order to study the effect of ozone in the protection of 'Soreli' cells from oxidative stress, the levels of antioxidant enzymes which scavenge ROS were investigated, in the second main activity of third trial. Superoxide dismutase (SOD) is the first enzyme involved in antioxidant defense during oxidative stress. It increased during curing and during the first 15 days of cold storage. Ozone-treated fruits showed significant higher values compared to untreated samples. CAT, involved in the detoxification of H₂O₂, increased its activity after curing compared to pre-curing (harvest). Ozone-treated samples showed higher values. Moreover, curing and cold storage induced a decrease in APX activity in untreated and treated samples. The H₂O₂ content exhibited a constant trend during storage time. Ozone-treated samples showed a significant lower H₂O₂ content compared to untreated samples. GPX catalyses the oxidation of several antioxidant compounds. Curing treatment induced a significant increase in GPX activity, whereas, a lower GPX activity in untreated kiwifruits and at the end of storage have been registered. Two-way ANOVA (Fig.3c) showed the effects of ozone treatment and the storage time and their interaction on the antioxidant enzymes of 'Soreli' during cold storage. In 'Soreli' kiwifruits, curing induced an increase in antioxidant enzymes activities, our results are in agreement with previous studies. Therefore, ozone action on enzymatic browning and membrane damage showed that PPO activity increased after curing with no meaningful differences between ozone-treated and untreated kiwifruits during cold storage. The analysed MDA content and LOX activity

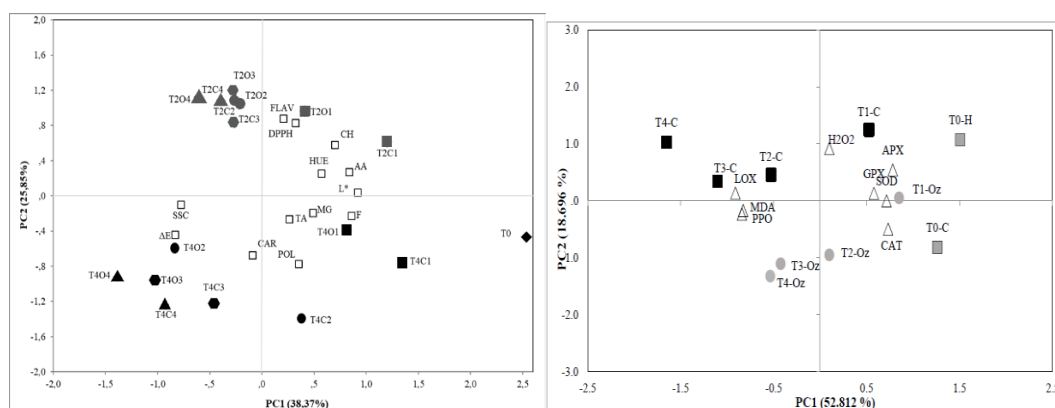


Figure 3a Two-dimensional principal component analysis plot of qualitative and nutraceutical traits in “Soreli” kiwifruits, at harvest (T0), after 15 days (C1 control fruit, O1 ozone-treated fruit), 30 days (C2 control fruit, O2 ozone-treated fruit), 45 days (C3 control fruit, O3 ozone-treated fruit) and 60 days (C4 control fruit, O4 ozone-treated fruit) of storage at two different temperatures T2 (2 °C) and T4 (4 °C). F: firmness; SSC: soluble solid content; TA: total titratable acidity; L: lightness; HUE: hue angle; CH: Chroma; ΔE: color difference; MG: total microbial growth; POL: polyphenol content; FLAV: flavonoid content; AA: acid ascorbic content; CAR: carotenoid content DPPH: radical scavenging activity). The symbols represent the different storage time (◆ is T0, ■ is C1 and O1, ● is C2 and O2, ● is C3 and O3 and ▲ is C4 and O4); the different colours of the symbols indicate the two different temperatures: black represents T4 and grey T2.

Figure 3b Two dimensional principal component analysis plot of antioxidant system in untreated (C) and treated (Oz) kiwifruits 'Soreli' at harvest (T0-H) and after curing (T0-C) and at 15 days (T1), 30 days (T2), 45 days (T3) and 60 days (T4) of cold storage at $2^{\circ}\text{C} \pm 0.05$. Superoxide dismutase activities -SOD, catalase -CAT, ascorbate peroxidase -APX and H_2O_2 content - H_2O_2 , guaiacol peroxidase -GPX, polyphenol oxidase -PPO, lipoxygenase activities -LOX and malondialdehyde content -MDA.

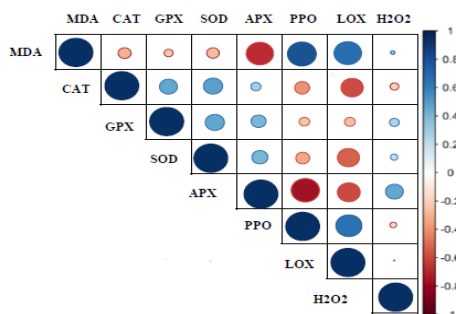


Figure 3c Pearson correlation matrix between superoxide dismutase activities (SOD), catalase (CAT), ascorbate peroxidase (APX) and H₂O₂ content (H₂O₂), guaiacol peroxidase (GPX), polyphenol oxidase (PPO), lipoxygenase activities (LOX) and malondialdehyde content (MDA). The correlation coefficients are proportional to the circular size and color intensity. Positive correlations are displayed in blue and negative in red.

4. Conclusions and Future Perspectives

Ozone postharvest treatments have great commercial interest due to its no residual property and flexibility of application methods. The combination of 2 °C and 300 ppb of continuous gaseous ozone allowed to improve the antioxidant system to maintain the cellular compartmentalization and preservation of nutritional components and antioxidants. Thus it is valid tool in postharvest to improve the storability of fruits.

The postharvest physiology of kiwifruit requires more attention at the genetic and molecular levels in order to understand the developmental process and better implementation management techniques.

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Development of Encapsulated Olive Oil and Olive Extracts as Innovative Natural and Health Food Ingredients

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This PhD project is aimed to develop ingredients with encapsulated olive bioactives (olive leaf extract) rich in phenolic compounds with health properties, with special attention to the main compound oleuropein. Several encapsulation techniques have been applied, namely freeze-drying, co-milling and liposomes, to produce products with different properties. Encapsulation efficiency, quality, stability and technological functionality were evaluated in both simple and complex model systems.

Sviluppo di incapsulati a base di olio ed estratti di oliva per la produzione di ingredienti funzionali e salutistici

Questa tesi di dottorato ha riguardato lo sviluppo di ingredienti incapsulati di composti bioattivi di olivo (estratti di foglie) ricchi di composti fenolici con proprietà salutistiche, con particolare attenzione al composto principale, oleuropeina. Diverse metodiche di incapsulamento sono state usate: liofilizzazione, milling e liposomi, con caratteristiche diverse. L'efficienza d'incapsulamento, la qualità e la stabilità e le proprietà tecnologiche e funzionali, sono state valutate sia in sistemi modello semplici che in sistemi complessi.

Key words: microencapsulation, olive leaves, oleuropein, freeze-drying, amorphous carbohydrates, liposomes

1. Introduction

In accordance with the PhD thesis project previously described (González Ortega, 2017), this oral communication reports the main results of the following four activities directed to:

A1) Development of encapsulated polyphenolic olive extracts using different techniques;

A1.1. Freeze-drying encapsulation (University of Teramo);

A1.2. Liposome encapsulation (University of Ljubljana);

A2) Evaluation of technological functionality and stability of produced encapsulated extracts, including encapsulation efficiency, microstructure and morphology (microscopy), thermal stability (calorimetry), water sorption, bioactive release and stability;

A3) Determination of stability, health functionality and bioavailability in real food systems: development of model and formulated emulsified and gelled products.

2. Microencapsulation

Throughout last decades significant changes occurred in food production, now not only oriented to meet basic nutritional needs and sensory properties but increasingly focused on prevention of nutrition related diseases and well-being of consumers

(Roberfroid, 2002). Main attention is given to presence and concentration of minor compounds able to exert bioactivity of various nature and health-promoting properties in humans, whose interest is increasing also for the likely exploitability as therapeutic agents and nutraceuticals. This interest has powered the research and development of new technologies to keep and enhance bioactive compounds in food products, to improve their bioavailability as well as to protect them against stressing environmental and matrix factors (McClements, Decker, Park, & Weiss, 2009). Micro-encapsulation or nano-encapsulation is nowadays representing an interesting strategy to enhance the functionality of bioactives and other biomolecules. From a food technological perspective, it is essential to maintain physicochemical quality and bioactivity of such compounds during prolonged storage and various storage conditions. It is also more convenient and applicable to benefit from bioactive phenolic extracts in powdered form that can be easily handled during storage and transportation and applied to in food and pharmaceutical purposes (Fang & Bhandari, 2012)

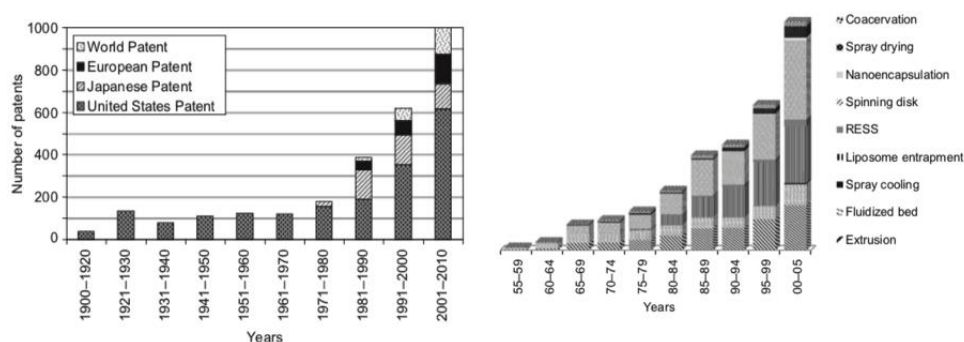


Figure 1 Trends in microencapsulation and number of patents during last decades. Note: Sourced from Sobel, R., Versic, R., & Gaonkar, A. G. (2014). Introduction to microencapsulation and controlled delivery in foods. In *Microencapsulation in the Food Industry* (pp. 3-12).

2.1. Freeze-drying encapsulation and effect of processing variables

Freeze-drying is a widely applied technique to encapsulate phenolic compounds and other heat-sensitive compounds, by means of co-lyophilization of a solution containing the bioactive of interest and a carrier/wall material, resulting in a dry, porous structure (Fang & Bhandari, 2012).

The application of freeze-drying requires a preliminary optimisation of the process factors such as wall material selection, amount of bioactives-wall, drying conditions to understand and tune the qualitative and technological properties of the microencapsulated powders. Commonly used wall materials are polysaccharides such as chitosan and alginate, and other carbohydrates like starch, maltodextrins and smaller oligosaccharides, thanks to their ability to form amorphous glassy matrices held up by hydrogen bonds (Kilburn, Claude, Schweizer, Alam, & Ubbink, 2005) in which the core bioactive is entrapped. Molecular weight and type of carbohydrate as well as presence of a second or third component in the amorphous solid matrix are known to affect the microstructure and physical properties (i.e. glass transition temperature), that governs molecular mobility and diffusion-controlled reactions (Roos & Drusch, 2015). Several studies have addressed encapsulation of vegetable and fruit extracts with maltodextrins with varying molecular weight (i.e dextrose equivalent). However, limited is the information about the impact of freeze-drying process variables on encapsulation

efficiency, powder microstructure, micro-distribution of bioactives and glass transition of resulting microencapsulated powders.

Response surface methodology is a useful design tool employed to optimize and study processes that have several factors and factor interactions influencing the responses, and has been commonly applied in encapsulation (Paulo & Santos, 2017).

2.2. Liposome encapsulation

Liposome particles are highly functional delivery systems for drugs and bioactive compounds. They are vesicles formed by lipid bilayers usually by using phospholipids and have remarkable advantages mainly due to their biocompatibility and possibility to tune their physicochemical properties. Encapsulation of food bioactive compounds with liposomes proved to improve stability, slower release and higher radical scavenging capacity *in vitro*, compared to free form of phenolic compound. Despite these effects and the possibility to prepare them using only natural food grade components, their application in food systems is still limited due to time consuming manufacturing processes and high costs. Recent studies are evaluating the encapsulation of food bioactive compounds using the proliposomes method (Perrett, Golding, & Williams, 1991) in which undesired solvents and expensive procedures are avoided, allowing scalability and food grade applications.

The behaviour and interaction of phenolic compounds with model lipid membranes and its effect on their physical and chemical state can provide valuable information to understand the techno-functional properties of encapsulated bioactive. Moreover, liposomes can serve as valuable models to understand protective effect of phenolic compounds against oxidative damage of lipid membranes (Balanč et al., 2015).

3. Experimental Procedure

In this PhD thesis dissertation, two encapsulation techniques were applied to encapsulate an olive leaf phenolic extract (OLE). Firstly, freeze-drying was applied and the effect of formulation variables on encapsulation efficiency, microencapsulates microstructure and distribution of bioactives in the amorphous matrix and glass transition. A response surface methodology was applied using a central composite to optimize the design of experiments and information obtained.

Secondly, liposome encapsulation was used, and two studies were carried out. A study on the effect of oleuropein (as main OLE bioactive compound) on model lipid membrane physicochemical properties was carried out to gain insight on the role of oleuropein in membrane stability and fluidity, its impact on techno-functionality, and its role against lipid peroxidation. A parallel study dealt with a further applicative approach in which OLE-liposomes were prepared and characterized with regard to encapsulation of main phenolics, size and charge and morphology. Oleuropein stability and release from liposomes was studied at different pH and temperature condition and in real/model lemonade drink.

4. Materials and Methods

A standardized olive leaf extract with a high content of oleuropein (~40 wt.%) kindly provided by OLEAFIT S.r.l (Italy), was used as bioactive core material.

Table 1 Overview of expected bioactive components and wall carrier materials.

Encapsulation method	Bioactive core	Wall/carrier material
Freeze-drying	OLE powder	Maltodextrin, Trehalose and their mix
Liposomes	OLE powder	Soy lecithin (Phospholipon 90G)
	Oleuropein	Dipalmytoylphosphocholine (DPPC)

Freeze-drying encapsulation: A design of experiment (DoE) and a central composite design with inscribed star points and response surface methodology was applied to produce OLE encapsulates. Three formulation parameters were taken as independent variables to produce encapsulates, namely: (V1) % total solids, (V2) composition ratio of matrix components and (V3) core:wall ratio. Microencapsulated dried powders were tested for: encapsulation efficiency, antioxidant capacity, colour, glass transition temperature (T_g) (DSC), surface morphology (SEM) and microdistribution of OLE (CLSM).

Liposome encapsulation: Pure oleuropein was used to study polyphenol-lipid membrane interaction and the effect on physicochemical properties and stability of the corresponding model membranes (liposomes). Thermal phase transitions were evaluated with a N-Differential scanning calorimetry (N-DSC); membrane fluidity and ordering by using fluorescence polarisation. Membrane lipids peroxidation was determined with BODIPY 581/591 C11 fluorescent probe and TBARS assay.

5. Results and Discussion

5.1 Freeze-drying encapsulation

The statistical analysis of the results of the TPC and oleuropein content indicated that the encapsulation efficiency (EE) of OLE was most significantly affected by matrix composition and ratio core: wall. Higher EE were obtained as the percentage of maltodextrin as carrier material increased, indicating that maltodextrin exert a better entrapping capsule material compared to trehalose. Additional factors that could be implied in this result include also potential interaction between the OLE phenolic compounds and the larger carbohydrate molecules of the maltodextrins and trehalose, affected by the specific charge, solubility and molecular mobility. It has been suggested that the plasticizing role of small sugars in amorphous polysaccharides is related to a reduction in the number of molecular entanglements without significantly changing the structure or packing of the glass (Kilburn et al., 2005). The ratio of bioactive core to matrix carrier (core: wall, X_3) also was a significant factor. The lower the ratio, i.e., the higher the matrix fraction in relation to the bioactive core material, the higher the encapsulation efficiency.

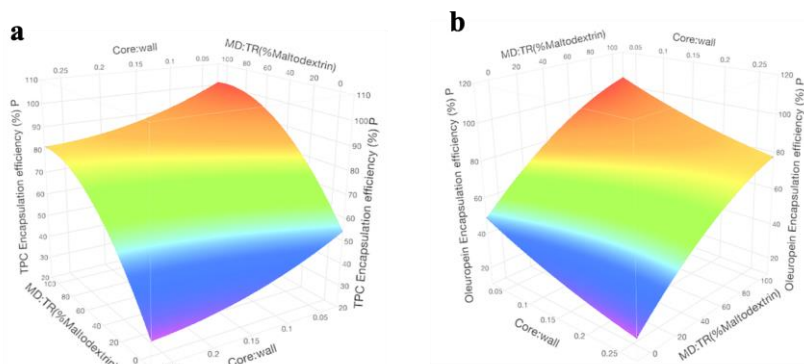


Figure 2 Response surface 3D plots for encapsulation efficiency of (a) total phenolic content (TPC) and (b) oleuropein as a function of changes in factors V2 (ratio MD:TR) and V3 (ratio core: wall).

Matrix composition affected also the microdistribution of OLE in the powders as shown by the CSLM images (Fig. 3). In particular, a higher fluorescence signal was observed in sample with higher trehalose content. This may be due to higher surface content of phenolics in these sample, as observed in encapsulation efficiency, since highly entrapped phenolics may have impaired or quenched fluorescence emission as compared to surface fraction.

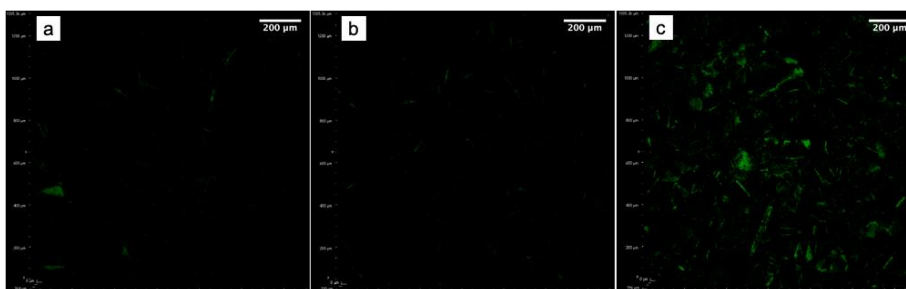


Figure 3 CSLM micrographs of microencapsulated powders at OLE:Matrix ratio 0.05 and (a) 100% maltodextrin, (b) 50%-50% maltodextrin-trehalose and (c) 100% trehalose as wall material.

The glass transition temperature of a system and its variation as a consequence of either matrix modification or enrichment with bioactive components represents one of the most important landmark for predicting the physical and chemical stability of low-moisture foods (Roos & Drusch, 2015). A single glass transition was observed in binary matrix samples (MD+TR), confirming its miscibility. OLE affected the Tg of the powders in which it has been physically entrapped by decreasing that of the corresponding carrier system without the extract. This effect may be a result of a plasticizing effect of the OLE low molecular weight components

5.2 Liposome encapsulation

DSC measurements were used to evaluate the changes in thermotropic properties of the liposomes membranes in the presence on encapsulated or titrated oleuropein. DPPC was used as a pure and saturated phospholipid with a sharp phase transition that can be easily monitored (Fig. 4). The changes in enthalpy (ΔH), gel-to-liquid phase

transition (T_m) and its related pre-transition (T_{pre}) were measured as a function of oleuropein:lipid molar ratios. It was observed an oleuropein concentration-dependent decrease in ΔH of T_{pre} and a broadening and shifting to lower temperatures of T_m without a significant change in ΔH . When oleuropein was encapsulated, this trend was amplified, showing an eventual disappearance of T_{pre} and a notable decrease of T_m from 41.6°C to 40°C.

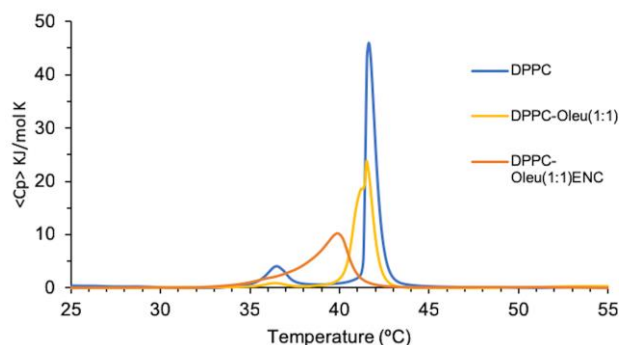


Figure 4 DSC thermograms of DPPC liposomes in the absence and presence of oleuropein at 1:1 molar ratio, added during liposome formation (ENC) or after liposome formation.

Results highlight that when oleuropein was encapsulated, it was partially intercalated in the phospholipid membrane and has stronger impact on membrane fluidity acting as a spacer.

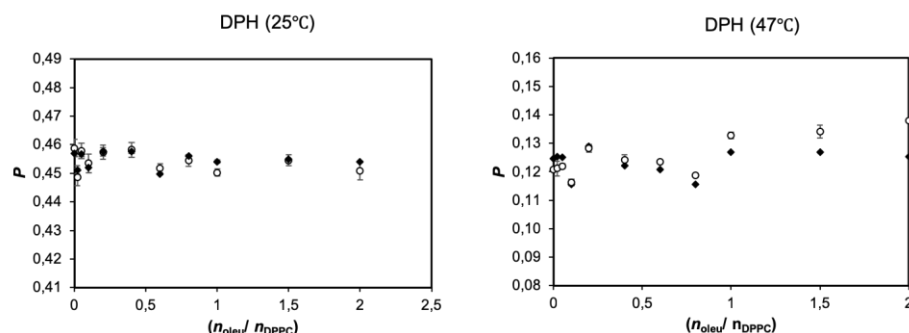


Figure 5 Fluorescence polarisation of DPH in liposome suspensions and titrated with increasing concentrations of oleuropein, below (25°C) and above (47°C) the T_m .

Fluorescent labels added to liposomes with ability to track changes in the inner apolar part of the membrane (DPH) or more superficial interaction with polar head groups of phospholipids (TMA-DPH) (Fig. 5). When probes are exposed to a more polar aqueous environment the polarization is quenched.

It was observed that polarisation values were higher at 25°C where lipids are in a more ordered and rigid state with lower fluidity. Oleuropein did not seem to significantly alter the membrane fluidity at this temperature. Above the T_m , presence of oleuropein at higher concentrations ($> 1:1$ molar ratio) increased polarization, meaning that interaction with phospholipids head groups may stabilize the membrane fluidity above the gel-to-liquid transition. This effect was more remarkable in samples with encapsulated oleuropein (data not shown). Membrane lipids peroxidation was determined with BODIPY 581/591 C11 fluorescent probe and TBARS assay. Oleuropein showed a strong

antioxidant potential to inhibit lipid peroxidation, and therefore promote membrane oxidation stability.

6. Conclusions and Future Perspectives

Due to the complexity of food matrices and encapsulating systems, optimisation and knowledge of the processing variables is needed in order to understand and predict behaviour of systems. As part of this PhD project, freeze-drying encapsulation of phenolic extract from olive leaves was optimised in terms of encapsulation efficiency as function of several processing variables showing that the physicochemical characteristics of wall material and the amount of core bioactive in relation to wall material play a major role.

Liposomes are biocarriers that can be manufactured in a cost-efficient manner for the food industry. However, their effectiveness and quality and stability can vary depending on their interaction with the compound of interest to be encapsulated. Therefore, it is important to understand how both components interact to predict their technofunctional and health potential. Liposomes can also be used as model membranes to study the potential antioxidant capacity to prevent free radical formation and lipid peroxidation, with implications for a food preservation and biological membranes.

This project will be completed with additional studies on extrusion and co-milling encapsulation, and incorporation of selected encapsulated ingredients in model food systems.

7. References

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Microencapsulation of Cocoa Polyphenols by Spray-Drying

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This PhD thesis consists in the microencapsulation of polyphenols, extracted from cocoa shells, by using spray drying technique. The optimal experimental conditions were evaluated in order to obtain bioactive compounds that will be added in cocoa liquor to produce functional cocoa's products.

Microincapsulazione di polifenoli del cacao con spray drying

Questa tesi di dottorato ha riguardato lo sviluppo di microcapsule di polifenoli estratti dal pericarpo delle fave di cacao con spray dryer. Sono state ricercate le condizioni sperimentali ottimali per l'ottenimento di componenti bioattivi da inserire in una miscela di cioccolato per la produzione di prodotti funzionali a base di cacao.

Key words: Cocoa shells; polyphenols; spray drying; encapsulation.

1. Introduction

Food lost and waste is considered one of the most serious economic and environmental problem of the modern society. For this reason, a lot of food industries are interested into recovery of a waste for the recapture of functional compounds and their use into same food chain (Galanakis, 2012).

For instance, cocoa shells, that are usually considered a by-product of chocolate manufacturing, were used like a source of natural polyphenols which include catechins, epicatechins and procyanidins (Miller et al., 2006). These compounds confer many advantages to human healthy due their antioxidant property. However, polyphenols are very sensitive to light, heat and oxygen so their concentration decrease during food processing. Furthermore, only a small part of them is absorbed into gastrointestinal tract because of their low solubility and permeability. Lastly, polyphenols confer astringent and bitter taste to food that are usually undesirable for the consumers (Munin & Edwards-Lévy, 2011).

Microencapsulation technique is used to overcome these drawbacks. Microencapsulation process consists in the covering of a liquid, solid or gas compound (core) with a coating material (shell) (Gibbs et al., 1999). In food industry, this technology is used to assure the protection of sensitive nutrients and their following controlled release (Onwulata, 2011). These capsules will be added in cocoa liquor in order to produce a functional chocolate bar, enriched of polyphenols.

2. Spray Drying

Spray-drying is a technique used to produce a dry powder from a liquid phase in pharmaceuticals and food technology. The main advantage of spray drying technique is the production of stable and high quality particles (Munin & Edwards-Lévy, 2011) with a

specific size and moisture content (Keshani et al., 2015). Each spray dryer is composed by: a source of hot air that includes an air supply fan, an air heater and an air distribution system; an atomizing system; a drying chamber and a powder recovery system. First an emulsion of core and coating agent was prepared. Then, the feed is load into the chamber where it is divided into small droplets by the nozzle. At the same time, the liquid gets in touch with the hot air stream, leading the water evaporation. After the drying process food particles leave chamber and the product is separated from air in a cyclone separator. The characteristics of the formed capsules depend on several factors that could be distinguished in operating conditions of the drying process (the inlet and outlet temperature and the flow feed), the properties of the feed (nature of coating agent and core:coating agent ratio) and structural features of the spray dried (Handscomb et al., 2009). These parameters affect the particle size and morphology, their storage stability, process yield, crystalline state, moisture content, the density (Kemp et al., 2016) and the bioavailability of the encapsulated substances.

3. Experimental Procedure

This PhD thesis is composed by three many steps: the extraction of natural polyphenols from cocoa shell using a green method to assure the food safety of the final product; the microencapsulation of the obtained polyphenols using spray drying technique; the addition of microcapsules in cocoa liquor in order to produce a stable, healthy and tempting chocolate bar.

4. Materials and Methods

4.1 Raw material and chemicals

Cocoa shells were collected by Dolceamaro SRL (Monteroduni (IS), Italy). Maltodextrin (dextrose equivalent (DE) 17.0 – 19.9) was purchased from CHIMPEX INDUSTRIALE SPA (Caivano (NA), Italy). Ethanol (96% v/v), methanol (99.8% v/v), acetic acid (99.8% v/v), gallic acid, calcium carbonate, DPPH and Folin-Ciocalteu reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

4.2 Extraction polyphenols

Polyphenols were extracted using green extraction method: 2 g of defatted samples were mixed with 50 ml of ethanol: water (1:1 v/v) solution and placed in a ultrasonic bath for 15 min at room temperature. The extracted liquid was separated from solids by centrifugation at 5000 rpm for 10 min. Ethanol was removed by rotary evaporator and the remaining solution was lyophilized until its use.

4.3 Encapsulation process

Polyphenols extract were encapsulated using maltodextrin as coating material. Coating solutions (10% w/v) were prepared 1 day before the encapsulation process to obtain full dissolution in the solvent (water). For encapsulation, 1 g of lyophilized polyphenols were mixed with 1:5, 1:10 and 1:15 core to coating ratio and the mixture were homogenized at 7000 rpm for 10 min using a highspeed homogenizer (DH. WHG02118, DAIHAN Scientific CO., Ltd. Korea) until obtaining a good dispersion.

4.4 Spray drying

The microencapsulation was carried by Mini Spray Dryer B-290 (BuchiLabortechnik AG, Switzerland) equipped with a nozzle two-fluid co-current. The

mixture was fed at two different speeds of 6 ml/min and 9 ml/min, respectively. The drying was performed at two different air inlet temperatures (120 and 150°C). The spray drying process was carried on in 15 min and 20 min for speeds of 6 ml/min and 9 ml/min, respectively.

4.5 Yield

The dried yield was calculated using Eq. (1)

$$Y(\%) = (\text{Mass of microcapsules [g]} / \text{Total mass of feed solution [g]}) \times 100 \quad 1)$$

4.6 Moisture content

1 g of capsules were kept in a hot-air oven at 105°C until they reached a constant weight. The moisture content was calculated in terms of the weight loss (AOAC, 2000).

4.7 Total phenolic content (TPC) and surface phenolic content (SPC)

During the spray drying process only a part of polyphenols was entrapped in the capsules. Some of them, being a matrix system, were placed on the surface and will be lost during the following process conditions. In order to evaluate the concentration of the incorporated material (Encapsulation Efficiency), the polyphenols content was quantified whether on the external part of the capsules or in the whole particles by using different solvents mixture, in which they could be dissolved.

The total phenolic content (TPC) was determined following the methods of Saénz et al., (2009). One hundred milligrams of the prepared microcapsules were dispersed in 1 ml ethanol, acetic acid, and water solution (50:8:42 v/v/v). This dispersion was agitated using a Vortex (1 min) and then sonicated in an ultrasonic bath (Bandelin Electronic, Sonorex RK 100H, Germany) for 20 min. The supernatant was centrifuged at 6000 rpm for 10 min and then filtered. The amounts of phenolic compounds were quantified by the Folin–Ciocalteu method (Singleton & Rossi, 1965).

For the determination of surface phenolic compounds (SPC), 100 mg of microcapsules were treated with 1 ml of a mixture of ethanol and methanol (1:1 v/v). These dispersions were agitated in a Vortex at room temperature for 1 min and then filtered (0.45 µm Millipore filter) (Robert et al., 2010). The amounts of phenolic compounds were quantified by the Folin–Ciocalteu method (Singleton & Rossi, 1965) and expressed as gallic acid, after calibration curve of the standard.

The encapsulation efficiency (EE) was calculated using Eq. (2):

$$EE (\%) = ([\text{TPC} - \text{SPC}] / \text{TPC}) \times 100 \quad 2)$$

4.8 Antioxidant activity

Antioxidant activity (AA) was determined using DPPH method (Tolun et al., 2016) with some modifications.

100 mg of phenolic powder was dissolved in 3 ml ethanol:acetic acid:water mixture (50:8:42, v/v/v). The solution was agitated using a vortex for 1 min and sonicated at room temperature for 20 min. The emulsion was filtered through a filter with a pore size of 0.45 µm. The samples were diluted 20 times with ethanol:acetic acid:water mixture (50:8:42, v/v/v) after filtration. 100 µl of each solution were mixed with 3.9 ml of 25 ppm DPPH radical solution (2.5 mg DPPH in 100 ml MeOH) and left in the dark at 25°C for 1 hour. For control, 100 ml of ethanol:acetic acid:water mixture (50:8:42 v/v/v) were used

instead of samples. Finally, for each solution the absorption was measured at 517 nm by using a UV/VIS spectrophotometer T 70 (PG Instruments LTD, UK). The percentage inhibition (%) of the free radical by the sample was calculated using the eq. (3)

$$\text{Percentage inhibition (\%)} = \frac{(\text{Abs}_{517\text{control}} - \text{Abs}_{517\text{sample}})}{\text{Abs}_{517\text{control}}} \times 100 \quad (3)$$

5. Results and Discussion

5.1 Yield

The yield of the microcapsules under different process conditions were investigated (Table 1). Generally powder's yield showed an increase with the rising temperature at different core:coating material ratios (Table 1). An opposite tendency was observed with the increase of flow feed from 6 ml/min to 9 ml/min. Usually, high temperature and low flow feed leads a greater efficiency in heat and mass transfer, improving the final yield (Tonon et al., 2011). According to Tolun et al. (2016) the yield also rises increasing the ratio core:coating agent because of maltodextrin reduces the deposition of particles on the wall of drying chamber (Quek et al., 2007) and allows a greater trap of the bioactive substance during the drying process. The best results were obtained for S3 (78.05%), S5 (73.32%) and S11 (80.68%) using 1:5, 1:10, 1:15 core:coating ratio, respectively.

Table 1 Yield of microcapsules at different core:coating agent ratios, inlet temperature, outlet temperature and flow feed conditions.

Sample	Ratio core:coating agent	Inlet Temperature [°C]	Outlet Temperature [°C]	Flow Rate [ml/min]	Yield [%]
S1	1:5	120	67	6	71.94
S2	1:5	120	51	9	73.98
S3	1:5	150	87	6	78.05
S4	1:5	150	71	9	59.44
S5	1:10	120	62	6	73.32
S6	1:10	120	50	9	61.15
S7	1:10	150	87	6	68.69
S8	1:10	150	72	9	68.77
S9	1:15	120	62	6	77.96
S10	1:15	120	57	9	70.93
S11	1:15	150	73	6	80.68
S12	1:15	150	60	9	76.30

5.2 Moisture

The moisture is one of the most important parameters evaluated to have useful information about the stability of the capsules. In 1:10 and 1:15 core:coating ratio, the moisture decreases with an increase of the drying temperature. This phenomenon is influenced by the greater drying rate of water evaporation at high temperature. On the other hand, increasing the feed flow rate the difference between inlet and outlet temperatures rises (Table 1), loading a higher residual moisture content in the particles. About the core:coating agent ratio, increasing from 1:5 to 1:15 the moisture content

decreases. This result confirms the data proposed by Singthong et al. (2015). Maltodextrin with high degree of polymerization (17-19.9) promotes the particles drying reducing the residual humidity in the final product. Furthermore, additives, like maltodextrin, reduce the glass transition temperature (T_g) of the product that provides less resistance to mass transfer (Pasrija et al., 2015). T_g is the temperature above the structure of feed pass from glassy state to rubbery state and it depends on the coating agent nature. According with the previous discussion the lowest value of moisture was found in S3 (3.92%), S7 (3.57%) and S11 (2.44%) for 1:5, 1:10 and 1:15 core:coating agent respectively (Fig. 1).

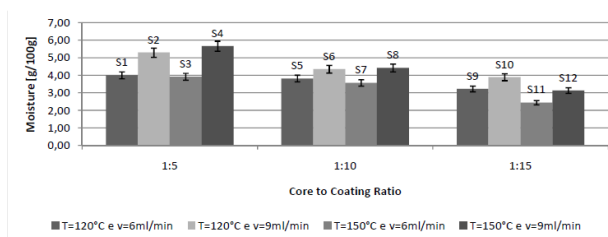


Figure 1 Moisture of microcapsules at different core:coating agent ratios, inlet temperature and flow feed conditions.

5.3 Total polyphenol content (TPC), surface polyphenol content (SPC) and encapsulation efficiency (EE)

The total polyphenol content aren't significantly influenced by the inlet temperature and feed flow rate (Fig. 2). Usually polyphenols are sensitive to high temperature but probably the range 120-150°C for shorter times isn't sufficient to reduce their amount. These results are according with those obtained by Tolun et al. (2016), who studied grape polyphenols microencapsulation at different inlet temperatures.

On the other hand the total polyphenol content is affected by the core:coating agent ratio. The amount of the latter decreases as a result of the increasing concentration of maltodextrin in the mixture. In 1:5 ratio, the highest performance (26.19) is obtained at 150°C and 6ml/min (S3) (Fig. 2). The same tendency could be observed for surface polyphenols, whose values aren't very high. Therefore, the encapsulation efficiency is correlated only to the core:coating agent ratio, with the highest values for S3 (94.63%), and S2 (91.30%) (Fig. 3). Finally, according with Poomkokrak et al. (2015) data suggests that encapsulation efficiency is not influenced by moisture content of the powder.

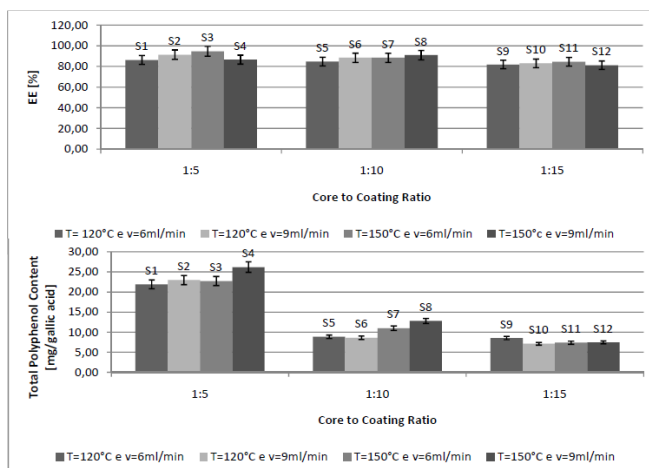


Figure 2 Total polyphenol content (a) and encapsulation efficiency (b) of microcapsules at different core:coating agent ratios, inlet temperature and flow feed conditions.

5.4 Antioxidant Activity

There is a positive correlation between polyphenols and antioxidant activity. For this reason, the AA% depends only with the core:coating ratio parameter. As shown in Fig. 4 the averages AA% values are about 26.90% for 1:15; 52.35% for 1:10 and 63.75% for 1:5. Antioxidant activity decreases when coating material concentration increases because maltodextrin doesn't contain any active radicals.

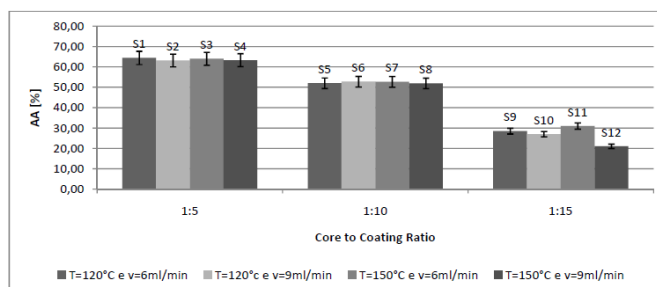


Figure 3 Antioxidant Activity at different core:coating agent ratios, inlet temperature and flow feed conditions.

6. Conclusions and Future Perspectives

The aim of this project is the recovery of high added value components, like polyphenols, from a waste of chocolate manufacturing, preserving their bioavailability during the shelf life of the final product. In the future, this recovery procedure is going to be scale up, to recognize if the technology result to be beneficial as it has been proposed in research level. For this reason, the future investigations should target to optimize the overall efficiency of suggested methodology improving the beneficial impact of recycling cocoa shells inside food chain.

7. Nomenclature

AA antioxidant activity (%); EE encapsulation efficiency (%); TPC total polyphenol content (mg/g gallic acid); SPC surphase polyphenol content (mg/g gallic acid).

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Microbes in Cocoa Beans: What Microbial Communities Can Do on the Formation of Cocoa Aroma during Fermentation

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Cocoa fermentation is a complex process addressed by a vast number of autochthonous microorganisms. The present dissertation assesses the microbial diversity found in fermented cocoa beans to determine effective parameters for optimizing this process. Two different cocoa varieties, fermented in Cameroon and Mexico were used to describe the physicochemical, microbiological, and sensorial quality of fermented cocoa beans. These biological samples were also used to establish the development and optimization of targeted molecular methods to assess fungal communities. Overall, this work highlights the perspectives and challenges of the standardization of the fermentation process and the selection of starter cultures for fermented cocoa beans.

Microorganismi nelle Fave di Cacao: Cosa le Comunità Microbiche Possono Fare sulla Formazione dell'Aroma del Cacao Durante la Fermentazione

La fermentazione del cacao è un processo complesso affrontato da un vasto numero di microrganismi autoctoni. La presente tesi analizza la diversità microbica trovata nelle fave di cacao fermentate con l'obiettivo di determinare parametri efficaci per l'ottimizzazione di questo processo. Due diverse varietà di cacao, fermentate in Camerun e in Messico, sono state utilizzate per descrivere la qualità fisico-chimica, microbiologica, e sensoriale delle fave di cacao fermentate. Questi campioni biologici sono stati anche usati per stabilire lo sviluppo e l'ottimizzazione del metodo molecolare mirato per valutare le comunità fungine. Nel complesso, questo lavoro evidenzia le prospettive e le sfide della selezione delle colture starter per la fermentazione delle fave di cacao.

Key words: Criollo, forastero, bacteria, yeast, quality, starter cultures

1. Introduction

In accordance with the Ph.D. thesis project previously described (Mota-Gutierrez, 2017), this oral communication reports the main results of the following five activities directed to:

- 1) describe the physicochemical, microbiological and sensory evolution of fermented cocoa beans in box and heap fermentations
- 2) assess the co-occurrence and/or co-exclusion relationships among the cocoa microbial communities through a pairwise correlation matrix adjusted for multiple comparisons
- 3) correlate the concentration of metabolites produced during cocoa fermentation and the presence of microbial communities
- 4) explore the potential metabolic pathways and interactions between bacterial communities
- 5) compare two different target regions through next-generation approaches to assess fungal communities

2. Functionalities of Microbial Communities in Fermented Cocoa Beans



Microbial communities are responsible for the unique functional properties of chocolate. The production of microbial metabolites in cocoa beans begins during fermentation, in this process, microorganisms, encompassing bacteria and yeasts, serve to confer taste, texture and desirable aromas to the final product. An effective cocoa fermentation develops when a correct microbial succession of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Mota-Gutierrez *et al.*, 2018; Nielsen *et al.*, 2007). The success of these dynamics is due to the nutrient content of the cocoa pulp that is used as an optimal substrate for the microbial growth, and yeasts are considered the first microorganisms growing at the beginning of this fermentation process, producing ethanol, organic acids, and VOCs, that contribute as precursors of chocolate flavor (Schwan & Wheals, 2004). For these reasons, yeasts have been widely used as starter cultures in cocoa beans with the aim to enrich the sensory quality of chocolate. However, the modulation of the remarkable complexity of microbial communities in cocoa beans to obtain an optimal flavor fingerprinting as well as understanding the metabolic and regulatory networks, concerning the production of secondary metabolites are still not clear. In this context, the present thesis aims to describe the diversity and evolution of the microbes during cocoa beans fermentation carried out spontaneously and in presence of yeasts starter cultures, either in boxes or heaps to acquire deeper knowledge about the relationship of microorganisms between each other and their surroundings. In this thesis, we also proposed the measurements of associations between microbial communities and the development of microbial volatile compounds. A better understanding of the microbial communities and physicochemical dynamics during box and heap fermentations will undoubtedly help the development

of new management procedures to produce high-quality cocoa.

3. Experimental Procedure

In this Ph.D. thesis a transdisciplinary approach was set up by performing in sequence the following independent tests, that is i) physicochemical analysis including the measurement of temperature, pH, and the identification and quantification of volatile and non-volatile compounds, using HPLC and GC-MS analysis; ii) classical and molecular microbiology analysis using plate counts and amplicon-based sequencing targeting 16S and 26S rRNA, respectively; iii) sensory evaluation by quantitative descriptive analysis; iv) correlation analysis between the chemical and molecular analysis and between microbial communities; v) metataxonomic comparison between two different target regions for the identification of fungal communities using next-generation sequencing. A sixth approach was added to explore the potential metabolic pathways and interactions between microbial communities to investigate the microbial metabolic activity and development of fermented cocoa beans.

4. Materials and Methods

Two different cocoa varieties (*Criollo* and *Forastero*) inoculated or non-inoculated from Cameroon and Mexico were used to identify the microorganisms that could contribute to the aroma generation of the cocoa flavor during box or heap fermentations (B and H). The lyophilized strains *Saccharomyces cerevisiae* ID67 and *Torulaspora delbrueckii* ID103 were provided by Lallemend, (Canada, Quebec, Montreal) and were used as

starter cultures in farmer-scale cocoa bean fermentations carried out only in Cameroon, while non-inoculated cocoa fermentations were assessed in Cameroon and Mexico. The dynamics of non-volatile and volatile organic compounds (VOCs) of fermented cocoa beans-pulp under different conditions were obtained applying chromatographic methods (Rodriguez-Campos *et al.*, 2011). The assessment of the microbial population was performed by culture-independent analysis described elsewhere (Klindworth *et al.*, 2013; Tedersoo *et al.*, 2015). Last but not least, a mock community containing twenty fungal species was prepared to validate the performance of the two target regions (Mota-Gutierrez *et al.*, 2019).

5. Statistical Analyses

Statistical analyses were carried out using generalized linear mixed-effects models for non-normally distributed data set and anova for normally distributed data. Mixed models were chosen for their ability to capture both fixed (fermentation condition: inoculated with S, ST and non-inoculated and fermentation time: 0-120 h) and random effects (fermentation type: B and H or cocoa varieties: C and F). The *P*-values were adjusted using Bonferroni's method when the test revealed significant differences ($P < 0.05$) the Duncan honestly significant difference (HSD) test was applied. Mixed models were built using R version 3.3.2.

6. Bioinformatics

Paired-end reads (2x250 bp) were first merged using the FLASH software (Magoč & Salzberg, 2011), with default parameters. Joint reads were further quality filtered (Phred < Q20) using the QIIME 1.9.0 software (Caporaso *et al.*, 2011). Chimeras were then removed with the adopted USEARCH version 8.1 software. Lastly, OTUs were picked at 99% of similarity by means of UCLUST clustering methods (Edgar, 2010) and representative sequences of each cluster were used to assign taxonomy. For the 26S data, each cluster was used to assign taxonomy using the Constructed 26S rRNA gene database and SILVA, while for the ITS dataset the UNITE rRNA ITS database version 2012, by means of the RDP Classifier. For the 16S data, each cluster was used to assign taxonomy by mapping against the Greengenes 16S rRNA gene database, version 2013 as described recently (Ferrocinio *et al.*, 2017). Sequences were double-checked using the BlastN search tool (<http://www.ncbi.nlm.nih.gov/blast/>) to confirm the taxonomy assignment. Weighted and Unweighted UniFrac distance matrices, as well as OTUs table, were used to find differences between fermentation processes (B and H), under different conditions (inoculated and non-inoculated) and between cocoa varieties by Adonis and Anosim statistical test in R environment in order to avoid biases due to different sequencing depths, all samples for each dataset were rarefied at the lowest number of reads after raw read quality filtering. QIIME was used to produce a filtered OTU table at 1% in at least 2 samples. The OTU table displays the higher taxonomy resolution reached when the taxonomy assignment was not able to reach the species level, genus or family name was displayed. As a measure of the association between microbial OTUs occurring in at least 2 samples and chemical variables, the Spearman's rank correlation coefficient was obtained through the function *corr.test* and *corrplot* and plotted through the *psych* and *corrplot* package of R, respectively. OTUs occurring in at least 2 samples from microbial communities were conglomerate by hierarchical clustering analysis using Ward's method acquired through the function *heatmap.2* plotted through the *gplots* package of R.

7. Results and Discussion

7.1 Physical and microbiological changes throughout cocoa fermentations

No significant difference ($P > 0.05$) between the conditions used (inoculated and non-inoculated) or cocoa varieties was observed through physical and microbiological analysis, while temperature and pH observed during B and H fermentations significantly increased the end of the fermentation (data not shown). During fermentation, cocoa beans constitute an ecological niche for a wide range of microbes. The advances in studying the dynamics of cocoa microbial communities have shown that the composition of these communities follows predictable patterns reporting a rapid decline in yeast counts after 48 hours when the sugars are depleted, the temperature rise and LAB and AAB increase.

7.2 Dynamics of non-volatile and volatile organic compounds during cocoa beans fermentation

The evolution of non-volatile compounds was determined during B and H fermentations of different cocoa varieties. No significant differences between inoculated and non-inoculated fermentations were observed through non-volatile and volatile compounds analysis at the end of the cocoa fermentation from Cameroon (data not shown). However, the volatilome Box fermentations showed a faster carbohydrate metabolism and higher production of organic acid compounds than in heap fermentations, which boosted the formation of alcohols and esters. As expected, statistical difference between the volatilome profile of two different cocoa varieties were observed (data not shown).

7.3 Performance of primers targeting ITS2 and 26S rRNA in mock communities

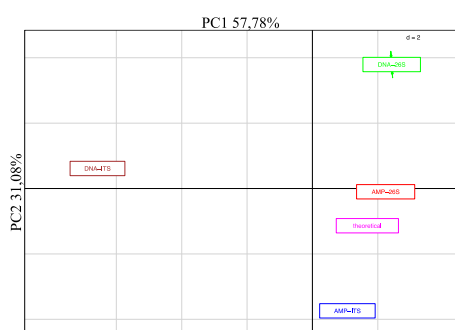


Figure 1 Principal component analysis (PCA) based on mock mycobacteria composition

A significant difference in mycobacteria composition ($P < 0.05$) by using the two target regions or mock communities (DNA or AMP) was observed by Principal Component Analysis (Figure 1). The relative abundances observed for ITS2 suggest that species with longer amplification fragments are underestimated and concurrently species that render shorter amplification fragments are overestimated. However, this correlation between amplicon length and estimation is not valid for all the species analyzed. The variability in the amplification lengths observed in our results might be contributed to the preferential amplification phenomenon.

7.4 Microbial communities and their correlation with microbial volatile compounds of cocoa beans during fermentation

Hanseniaspora opuntiae, *Saccharomyces cerevisiae*, and *Acetobacter pasteurianus* were the most abundant OTUs during the box and heap fermentation processes (B and H) from both countries. However, *Pichia pijperi* and *Lactobacillus fermentum* were also driven the cocoa fermentation in Cameroon, while *Lactobacillus cacaonum*, and *Lactobacillus plantarum* group for the Mexican fermentation. Microbial richness analysis showed that only the diversity of fungal species indicated a higher level of complexity in B compared to fermentations ($P < 0.05$) and also revealed a statistically significant difference between starter cultures initially inoculated from the Cameroon samples ($P < 0.01$). In contrast, the bacterial community showed a higher level of complexity in *Criollo* compared to *Forastero* fermentations from Mexico and a significant difference across fermentation time was observed ($P < 0.05$). The difference between the microbial communities found in two different cocoa-producing countries highlights the importance of the environmental conditions to determine the denominated restricted microbial cocoa species (Figure 2).

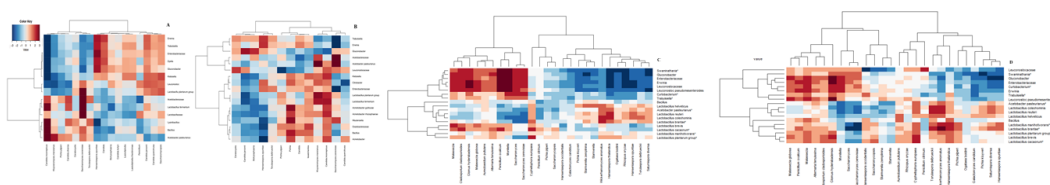


Figure 2 Significance associations between microbial OTUs observed with an incidence above $> 1\%$ in at least 2 samples are described. Samples are label according to fermentation method **A)** Forastero Box from Cameroon **B)** Forastero Heap from Cameroon, **D)** Forastero Box from Mexico **C)** Criollo Box from Mexico. The intensity of the colors represents the degree of correlation between yeast and bacterial OTUs as measured by the Spearman's correlation.

Overall, the microbial dynamics and associations between bacteria, yeast, and metabolites were found to depend on the type of fermentation and cocoa varieties, as shown in Figure 3. We observed that the major bacterial group found in our study increased the concentration of alcohols, esters, and acetaldehydes. Overall, the biochemical contribution in food ecosystems might change by the complexity of the microbial consortia (Fleet, 1999). Therefore, further research is needed to understand the role of other compounds such as free amino acids, oligopeptides, and polyphenols in the development of microbes and aroma compounds (Lima *et al.*, 2011; Thompson *et al.*, 2001).

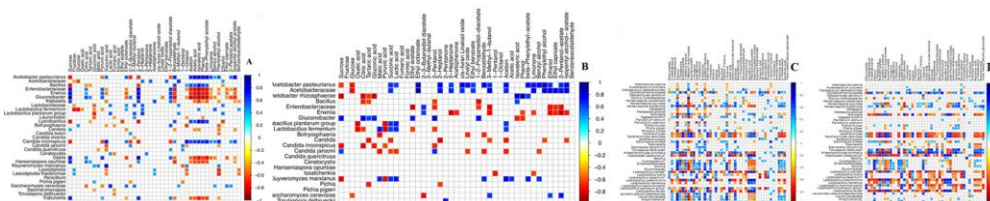
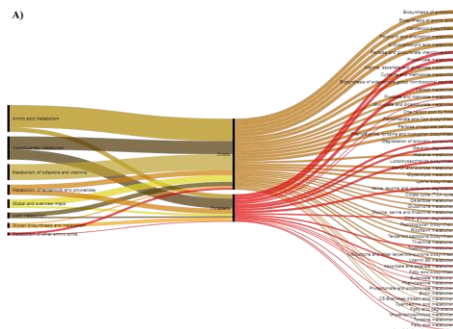


Figure 3 Correlation plot showing Spearman's correlation between microbial OTUs and metabolites observed with an incidence above $> 1\%$ in at least 2 samples. Samples are label according to fermentation method **A)** Forastero Box from Cameroon **B)** Forastero Heap from Cameroon, **D)** Forastero Box from Mexico **C)** Criollo Box from Mexico. The intensity of the colors represents the degree of correlation between microbial OTUs and metabolites as measured by the Spearman's correlation.

7.5 Potential metabolic pathways of fermented cocoa microbiota

The pathway enrichment analysis of the predicted metagenomes showed that the amino acid, carbohydrate, and energy metabolisms were the most abundant during cocoa fermentation and significant difference between cocoa varieties over a specific fermentation time was observed for the amino acid, aromatic compounds and fatty acids degradation pathways (Figure 4, $P < 0.05$). In addition, a clear separation between the type of variety and the metabolism of the main pathways are observed (Figure 4). The inferred metagenomes confirmed differences between the cocoa varieties and indicated that the minor bacteria group (*Erwinia*, *Gluconobacter*, *Curtobacterium*, *Trabulsella*, and *Leuconostoc*) have a negative association with the metabolism of short-chain fatty acids and amino acids.

Figure 4 Sankey diagram showing the carbohydrate, amino acids and lipid metabolic pathways activated for Criollo and Forastero varieties.



7.6 Sensory Evaluation

The quantitative descriptive analysis performed using a trained panel showed that no influences were observed between cocoa varieties, but the roasting process had a positive influence on the sensory profile of the cocoa beans (Figure 5). Interestingly, the sensory perception of fermented cocoa beans changes over fermentation time as a function of the microbial metabolic activity and development.

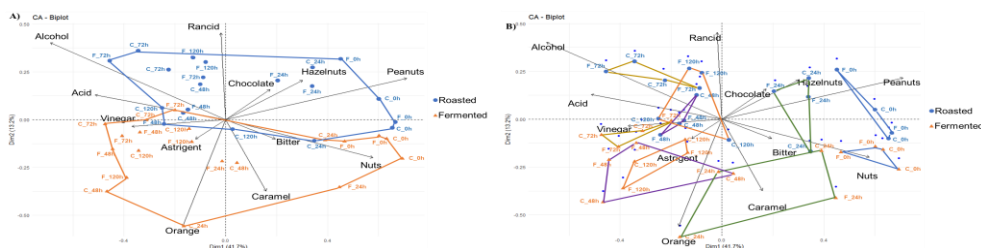


Figure 5 Correspondence analysis factor (CA) map showing the flavor profile of fermented and roasted cocoa beans **A)** Criollo box from Mexico and **B)** Forastero box from Mexico.

8. Conclusions and Future Perspectives

The experimental strategy following the evolution of microbial populations, physicochemical and quality parameters used in this study provides new information regarding the discrimination of microbial development and aroma formation of two different cocoa varieties over two different fermentation methods. We demonstrated that the degree of fermentation and bacterial composition is influenced by the type of cocoa variety and fermentation method used. The evolution of the different parameters used in this study has a potential value in the chocolate industry to determine new fermentation management to standardize desirable aroma and flavor cocoa development. In spite of the limited effectiveness of starter strains, the information so far available suggests that microbial communities have been an important factor in the evolution of aroma compounds. Understanding the pathways taken place during the formation of aroma by

microorganisms could be used to improve fermentation processes and to enhance chocolate quality. However, the challenges and opportunities in understanding the complexity of microbial diversity and interactions in fermented cocoa beans under a control fermentation system guided by a dominated microbial species might help us to obtain high-quality chocolate.

9. References

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Changes in Oral and Gut Microbiota Composition in Overweight Subjects upon Dietary Intervention Based on Mediterranean Dietary Model

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This PhD thesis dealt with the assessment of changes in gut and salivary microbiota composition upon a health-promoting nutritional intervention based on Mediterranean dietary pattern for 8 weeks in a cohort of overweight to obese subjects at risk of cardiovascular disease (CVD), without changing neither the daily energy intake nor their level of physical activity.

Cambiamenti nella composizione microbica orale ed intestinale in soggetti sovrappeso in seguito ad intervento nutrizionale basato sui principi della dieta Mediterranea

Questa tesi di dottorato ha riguardato la valutazione dei cambiamenti nel microbiota intestinale e salivare a seguito di un intervento nutrizionale durato 8 settimane e basato sui principi di un modello alimentare salutare come quello Mediterraneo in una coorte di soggetti sovrappeso tendenti all'obesità e a rischio di sviluppo di malattie cardiovascolari, mantenendo invariato sia l'assunzione giornaliera di calorie sia il livello di attività fisica.

Key words: Mediterranean diet; gut microbiota; metabolome; cardiovascular disease; personalized nutrition.

1. Introduction

The existence of the axis diet-microbiota-health is nowadays well established. The most recent researches are oriented towards a profitable use of diet as a cost-effective strategy for preventing and treating diseases through a modulation of the gut microbiota activities (Cani, 2018). Among others, obesity, defined as abnormal or excessive fat accumulation that may impair health status, is a worldwide recognized problem due to the Westernization of diet, enriched in fats, sugars and animal proteins.

Mediterranean diet (MD) is a dietary pattern recognized as healthy and useful for both prevention and treatment of several diseases. High-level of adherence to the MD is significantly and directly correlated to protection against the major chronic inflammatory disorders (Sofi et al., 2010) as well as cardiovascular disease (Corella et al., 2013) and it has been recently shown to have beneficial effects on gut microbiota composition and its related metabolome (De Filippis et al., 2016; Jin et al., 2019).

In order to further explore the effects of Mediterranean diet on human health, in this study we investigated the global changes in the gut and oral microbial communities of overweight to obese subjects (n=32, MedD) at risk of CVD upon an 8-weeks isocaloric intervention with MD. The MD-treated group was compared with a matched control group (n=30, ConD).

2. Materials and Methods

2.1 Selection criteria of study population

Selection of participants was based on the following inclusion criteria: both gender, age range 20-65 years, sedentary lifestyle, low alcohol consumption, absence of pre- and/or probiotics assumption, daily intake of fibers from fruit, vegetable or whole meal servings up to 3 portion/day and body mass index (BMI) ≥ 25 kg/m². Exclusion criteria included presence of hypertriglyceridemia, hypercholesterolemia, food-related allergies or intolerances (coeliac disease or lactose intolerance), gastrointestinal diseases, chronic or metabolic diseases, smoking, pregnancy or breastfeeding, hypertension, antibiotic and/or drug consumption, medication up to 3 months before the enrollment, previous surgical intervention or simultaneous attendance to other interventional studies. The food frequency questionnaires (FFQ) were provided to collect data and to evaluate both the frequency of consumption and the weekly dietary intake.

2.2 Dietary intervention and study design

Each participant in MedD received an isocaloric diet compared to the habitual one. They were provided suggestions to increase the consumption of fruit and vegetables, nuts, wholegrain products and to replace meat, eggs and dairy products with fishery and legumes as well butter with extra virgin olive oil, in order to increase their Italian Mediterranean Index score (MD index). On the contrary, subjects in ConD were asked to maintain their habitual diet unchanged. At baseline, after 4 (4w) and 8 weeks (8w), blood, urine, saliva and faecal samples of volunteers were collected along with anthropometric measurements and self-recorded food diaries to assess individual compliance to the intervention. The study design is presented in Fig. 1.

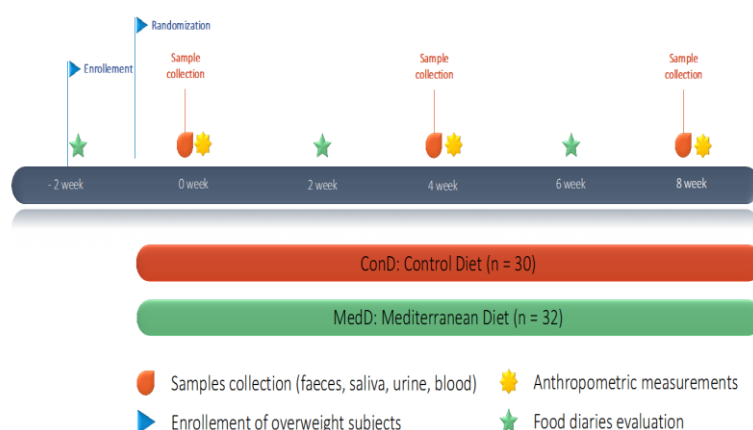


Figure 1 Study design schematization.

2.3 Metagenomics, targeted metabolomics and statistical analysis

DNA extraction from faecal and salivary samples was performed following IHMS SOP P7 V2 procedure. Amplicon-based sequencing was carried out on both faecal and salivary samples in order to define microbial composition. The primers S-D-Bact-0341F5'-CCTACGGGNGGCWGCAG and S-D-Bact-0785R5'-GACTACHVGGGTATCTAATCC

were used for the amplicon-targeted analysis on V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013). The PCR conditions were set as described by Canani et al. (2017). PCR products were purified following the Illumina 16S metagenomics sequencing library preparation and then quantified using a Plate Reader AF2200 (Eppendorf). After, amplicon library was normalized and pooled. Sequencing was carried out with an Illumina MiSeq system using the MiSeq Reagent kit v2. The output led to a 2X250 bp paired-end reads.

Bioinformatics analysis was performed as reported by De Filippis et al. (2016). Targeted quantification of carnitine, choline, creatinine and betaine in blood and urines as well as urinary urolithins were determined by LC-HRMS.

Statistical analysis and visualization were carried out in R environment version 3.4.2 (<https://www.r-project.org>).

3. Results and discussion

3.1 Compliance to the nutritional intervention

At baseline, no significant differences were observed between the ConD and MedD groups concerning both anthropometric measures and clinical parameters. However, adherence to the MD significantly increased only in the MedD group at 4w and 8w compared to baseline (Fig. 2A).

Significant percentage changes in dietary and metabolic variables are shown in Figure 2B. After 4 weeks of dietary intervention, MedD subjects significantly increased the intake of dietary fibers (129%) and of the vegetable/animal proteins ratio (128%) compared to ConD group ($p < 0.005$). Moreover, a significant reduction of saturated (27%) and increase of polyunsaturated fats intake (111%) was also achieved for MedD compared to ConD ($p < 0.005$) after 8 weeks. These changes of nutrient intake were more likely due to an increased consumptions of fruit, vegetables, nuts, wholegrain cereals and fish products and a simultaneous reduction of dairy and meat products. Indeed, the significant decrease in biomarker of intake of these foods confirmed this observation. Indeed, levels of plasma carnitine significantly decreased after 4 (14%, $p < 0.05$) and 8 weeks (11%, $p < 0.005$) of intervention in MedD compared to ConD. Consistently, a significant decrease of total blood cholesterol was assessed in MedD group (8% after 4w, $p < 0.05$).

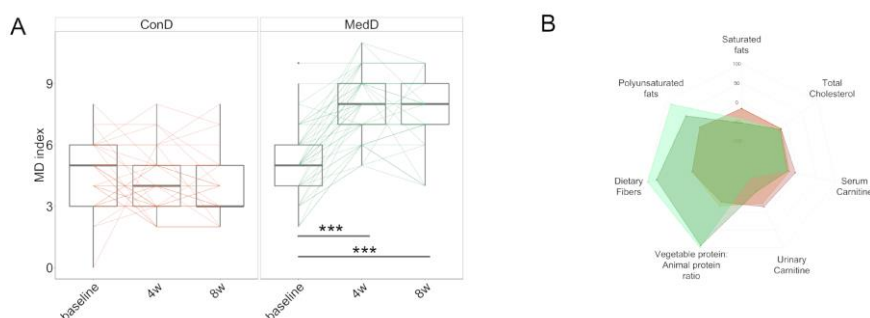


Figure 2 Evaluation of compliance and changes in dietary and metabolic variables. Box plots showing differences in MD index score between ConD and MedD through the intervention (A). Spider chart representing percentage changes in levels of dietary and metabolic variables (B).

Orange color refers to ConD and green to MedD subjects, respectively. Baseline, 0 weeks; 4w, 4 weeks; 8w, 8 weeks of nutritional intervention. (post hoc Friedman-Nemenyi test, *** $p < 0.001$).

3.2 MD-mediated changes in salivary and gut microbiota composition

Although there were no significant differences in the overall gut and salivary microbiota composition between MedD and ConD groups in all time points, we found specific microbial signatures affected by dietary intervention. In particular, a significant decrease in the relative abundance of the oral OTUs *Treponema denticola* (A) *Porphyromonas gingivalis* (B) and *Prevotella intermedia* (C) was assessed for MedD compared to ConD group (Fig. 3).

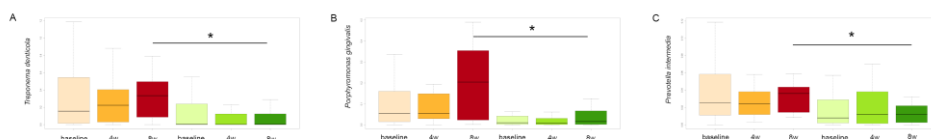


Figure 3 Box plots showing the relative abundance of oral *Treponema denticola* (A), *Porphyromonas gingivalis* (B) and *Prevotella intermedia* (C) OTUs detected throughout the intervention. Colors state diets (green for MedD and orange for ConD) and time points (light, medium, dark shades for baseline, 4w and 8w, respectively). * indicates $p < 0.05$ for variation at the specific time point compared to baseline in MedD vs ConD by unpaired Wilcoxon rank-sum tests. Baseline, 0 weeks; 4w, 4 weeks; 8w, 8 weeks of intervention.

These microorganisms are non-saccharolytic bacteria and responsible of oral periodontal lesions (Haigh et al., 2017). Since the MD pattern is characterized by a high intake of fibers and a lower intake of animal proteins, the reductions observed might due to lower levels of nitrogen source for bacteria, resulting in an improved oral health for MedD patients.

The increased adherence to MD in the first 4 weeks of intervention tended to be anti-correlated to gut microbiota similarity in the MedD group (Spearman rho= -0.3, $p=0.1$). This change was not observed in the ConD group during the same time-interval (Spearman rho= 0.1, $p=0.9$) suggesting a specific MD-induced rearrangement of the gut microbial composition. Moreover, *Faecalibacterium prausnitzii* OTU was significantly enriched in the MedD group, after 4 ($p < 0.05$) and 8 weeks ($p < 0.1$) of intervention, along with several fibre-degrading OTUs such as *Roseburia inulinivorans* ($p < 0.05$ after 8 weeks), a trend of increase concerning *Eubacterium* genus at 4w and members of *Lachnospiraceae* family at 8w ($p < 0.01$). These taxa are known as members of a healthy gut microbiota supporting the hypothesis of the impact of a Mediterranean-based dietary pattern shaping and positively affecting microbial ecology.

3.3 Changes in targeted metabolomics from diet influence

We measured urinary urolithins levels, metabolites associated to gut microbial metabolism in order to further investigate the potential effect of MD on host clinical profile. Urinary levels of total urolithins significantly increased in the MedD compared to ConD group both at 4w ($p=0.0034$) and at 8w ($p=0.03$). Interestingly, the increase was positively correlated to several members of the urolithin producers *Eggerthellaceae* family ($p < 0.05$) and to the consumption of nuts, fruit, vegetables and legumes while simultaneous

negative correlations with lipids and proteins from snacks, meat and dairy products occurred (Fig. 4). The clinical involvement on host health was highlighted by a negative correlations of such molecules with the marker of inflammation hs-CRP and some risk factors of cardiometabolic disorders like serum triglycerides, body fat mass, body weight and BMI ($p < 0.05$).

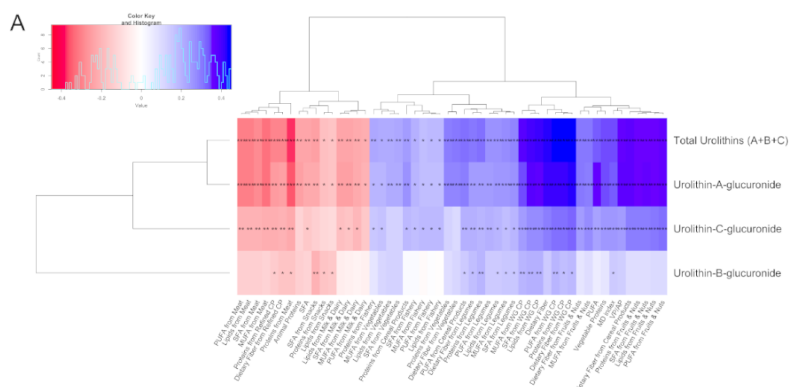


Figure 4 Correlations between urolithins and food variables. Heatmap showing hierarchical Ward-linkage clustering of urolithins levels based on Spearman's correlation with dietary variables. The color scale represents the scaled version of Spearman's rho coefficients, with red indicating negative and blue indicating positive correlations. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

4. Conclusions and Future Perspectives

The Mediterranean diet is a recommended dietary pattern and it is recognized as cultural heritage by UNESCO. It has been demonstrated that adherence to MD dietary model can be beneficial for treatment of dysmetabolic and chronic syndromes among others obesity and cardiovascular diseases (CVD). This interventional study aims to investigate and to widen the knowledge about the diet, microbiota and human health interplay. Most of the recent researches focused on dietary intervention based on calorie restriction in order to assess a direct gut microbiota-driven influence on clinical profiles of individuals. Such dietary modifications may promptly cause a sudden change of the gut microbiota composition, resulting in a complicate puzzle to recompose and probably disguising signals from long-term host-diet-microbial interactions.

Here, we demonstrated how isocaloric and individual dietary recommendations finalized to increase the adherence level to MD led to changes in oral and gut microbiota composition in overweight subjects with unhealthy dietary style. Our results highlighted such a beneficial impact by reducing the relative abundances of oral proinflammatory species and by enriching the gut microbial ecology of MedD group in specific taxa able to synthesize secondary metabolites which in turn may affect host health.

Mediterranean diet might be proposed in future strategies of personalized medicine as a suitable starting point for customized diets in order to positively influence microbial ecology, to establish diet-based host homeostasis and to prevent the onset of diet-related pathologies.

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Are New Ancient Wheats Suitable for Breadmaking?

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This project was part of my PhD thesis and aimed at assessing the baking performance of a set of seven wheats comprising two monococcums, two new emmers, one landrace and two old wheats by analyzing their gluten composition, starch damage, alpha-amylolytic activity, pasting properties, CO₂ production during fermentation, dough rheology and bread volume and textural analysis.

I “Nuovi” Cereali antichi sono adatti per la panificazione?

Il progetto presentato è stato una parte della mia tesi di dottorato che ha riguardato la valutazione delle proprietà di panificazione di 7 varietà di grani tra cui 2 *Triticum monococcum*, 2 *Triticum dicoccum*, 1 landrace e 2 vecchie varietà attraverso l'analisi della componente glutinica, amido danneggiato, attività amilasica, proprietà viscosimetriche, proprietà fermentative, proprietà reologiche degli impasti e del pane attraverso l'analisi volumetrica e di struttura.

Key words: gelatinisation, gluten, wheat, texture, bread.

1. Introduction

In the last decade, consumers interests in organic and artisanal bakery products with improved health properties increased significantly leading Italian bakers and farmers to rediscover ancient and old wheats known for their suitability in low input farming system and for their better health benefits (Hidalgo & Brandolini, 2014; Longin 2015) and wheat breeders to concentrate on the genetic improvement of baking quality which became one of their biggest challenges (Michel et al, 2018).

However, ancient wheats such as einkorn and emmer are known to have low technological quality due to their poor qualitative gluten alleles composition as high protein content is not a quality trait per se as long as the protein quality is poor which restrains their use to a limited number of food products, such as biscuits and traditional cakes. This stimulated breeding programs in Italy for releasing and registering improved genotypes obtained by either selection from landraces and local population as the case of the einkorn varieties monlis and norberto (used to be known as ID331) which were claimed to be suitable for bread-making or even by crossing landraces with durum wheat (*Triticum durum* Desf.) varieties as in the case of padre pio (PP) and giovanni paolo (GP) which are the results of emmer x durum wheat (referred to in the study as new emmers) and were claimed to be suitable for pasta making (Terzi et al, 2007; De Vita et al, 2006; CREA, 2006 & 2018). New varieties were created with a good adaptability to different environments and resistance to diseases and lodging. One study conducted on bread-making properties of new einkorn varieties confirmed that monlis and norberto are potentially suitable for making bread (MonICA, 2008) whereas no study on new released emmers were made.

We believe that the use of ancient and old wheats could represent a strategy for local communities to cope with agriculture sustainability while improving food security, diversity and quality. The aim of the study was to assess the suitability of two improved einkorn and emmer varieties for bread making by analyzing all aspects of their bread

performance along with other three cultivars; kamut, cappelli and the emmer landrace garfagnana.

2. Material and Methods

2.1 Plant Materials and field trial

A total of seven varieties cultivated under the same environmental conditions in Ottava (Sardinia, Italy, 41°N; 8°E; 80 m above sea level), were included in this study. Two *T. monococcum* subsp. *monococcum* cultivars monlis and norberto (used to be known before 2017 as IDD 331), two emmer cultivars *T. dicoccum* shubler cultivars padre pio and giovanni paolo selected by using the pedigree-selection method from the population obtained from cross between *T. dicoccum* schubler line selected from the molise population x *T. turgidum* subsp. *durum* Desf. cv simeto. (modern durum variety), one emmer landrace, *T. turgidum* subsp. *dicoccum*, garfagnana, one old durum wheat *T. turgidum* subsp. *Durum*, senatore cappelli (cappelli) and kamut wheat, originated from Egypt, is known to be a genotype of Khorasan wheat and still has uncertain original botanical identification. KAMUT[®], is a registered trademark of Kamut International, Ltd. confusedly identified as *T. turgidum*, subsp. *polonicum*, *T. turgidum*, subsp. *uranicum*, *T. turgidum* related to emmer and modern durum wheats or hybrid between *T. durum* and *T. polonicum*. Raw material was ground using an industrial mill and sifted to collect the resulting flour.

2.2 Parameters analyzed

Sequential extraction of gliadin and glutenin for reverse phase-high performance liquid chromatography (RP-HPLC) analysis

The sequential extraction of protein from wheat flour was done as described by Wieser et Seilmeier (1998) using a Chain HPLC equipped with a UV detector and an oven (50°C) with a column C18 type ACE1-221-2502 (250*2.1, 5µm, 300Å). Albumin and globulin are first extracted two times using a phosphate based alkaline buffer. Gliadin is extracted three times using 60% ethanol while glutenins are extracted two times from the pellet using a solution of 50% propanol, 2M urea, TRIS HCL 50mM and 1% DTE at pH 7.5. Gliadin percentage (GLI%) is calculated by dividing and multiplying by 100 the total chromatogram peak area ((area, mVmin⁻¹) per mg of flour) for 60% ethanol extracts to the sum of total chromatogram peak area for 60% ethanol extracts, the total chromatogram peak area for propan-1-ol+Tris HCL+ dithiothreitol+ Urea extracts and the the total chromatogram peak area for phosphate extract. Glutenin percentage (GLU%) is calculated by dividing and multiplying by 100 the total chromatogram peak area for propan-1-ol+Tris HCL+ dithiothreitol+ Urea extracts to the sum of total chromatogram peak area for 60% ethanol extracts, the total chromatogram peak area for propan-1-ol+Tris HCL+ dithiothreitol+ Urea extracts and the the total chromatogram peak area for phosphate extracts.

Pasting properties and stirring number

Pasting curves and stirring number (SN) were measured using the Rapid Visco Analyser (RVA), models 3CR, 3D. Pasting curves, including peak viscosity, breakdown, set back and final viscosity value were obtained by AACC Method 76-21 approved method while stirring number was obtained by AACC Method 22-08 approved method.

Starch damage

Starch damage was measured using Chopin SDmatic and values were obtained by AACC 76-31 approved method.

Flour water absorption

Farinograph curves were obtained using a model 810105001 Brabender instrument (Brabender OHG, Duisburg, Germany). Water absorption, mixing time, degree of stability and softening index (12 min after peak time) were measured according to AACC Approved Method 54-21. Only water absorption values are mentioned in the below results.

Dough development and CO₂ production during yeast activity

The maximum height reached by the dough, the total volume of gas produced and retained during fermentation following the addition of yeast at 2% and salt at 1.8% to the flour and water were measured using a Chopin Rheo F4. Curves and values were obtained by AACC 89-01,01 approved method.

Dough rheology

Alveograph measurements, strength of the dough (W), tenacity (P), extensibility (L) and the ratio of tenacity (P) to extensibility (L), were determined using a model MA87 Chopin alveograph (Group Tripette & Renaud, Villeneuve- La-Garenne, France).

Bread preparation and textural analysis

Dough was prepared by mixing flour (100%), water, salt (1.8%) and yeast (2%) in a 10-kg spiral mixer (Sigma srl, Italy) for 10 min at low speed. The amount of water used to prepare each sample was calculated according to the Farinograph values. Bread loaf volume was measured using the small seeds displacement method (AACC Standard 10-05.0133). The specific volume was calculated as bread volume (mL) over bread weight (g). Bread mechanical properties (hardness, cohesiveness, springiness and chewiness) were recorded in a TA-XTplus Texture Analyzer (Stable Micro Systems) using a 36-mm cylindrical probe (P36R), a speed rate of 1mm/s, 40% penetration depth, and a gap of 30 s between compressions on three central slices (thickness 20 mm) of 1 loaf. The yellow color index (YI) of flour and bread crust and crumb was determined using a model CR 300 colorimeter (Minolta, Osaka, Japan).

2.3 Statistical analysis

All data were subjected to analysis of variance (ANOVA), analysis of regression, correlations and principal component analysis (PCA), as appropriate, using R software (R Core Team).

3. Results and Discussion

3.1 Flour quality

Cappelli had the highest GLU% (28.9%) and the lowest GLI% together with kamut (47.9 and 51.1% respectively). Garfagnana had the highest GLI% (65.4%) and the lowest GLU% together with norberto (14.7 and 15.2%). GP had the lowest value of SN which means the highest alpha amylase activity (Collar et al, 2016) and the lowest values of peak and final viscosity, breakdown and set back. The lower the breakdown viscosity is the higher the stability of the final product is and the lower the setback value is the lower the rates of starch retrogradation and syneresis are (Ragaei & Abdel-Aal, 2006; Collar, 2016). These two parameters are a predictable index of bread staling properties as a low breakdown and set back viscosity indicate a reduced bread staling. Both einkorns and kamut had the highest SN. Einkorns had the highest peak viscosity, norberto had the highest final viscosity, followed by monlis, kamut and PP. Monlis followed by garfagnana, norberto and PP had the highest breakdown. Kamut and Garfagnana had the highest set back. Cappelli and Kamut had the highest SD while both einkorns had the lowest one. All our varieties had a SD below 7 to 7.5% considered the threshold for a good baking for durum wheat by Sisson (2008) (Table 1).

Table 1 *Gluten composition; glutenin and gliadin percentage (GLU% and GLI%), dough viscometric properties; peak viscosity, final viscosity, breakdown, set back and stirring number (SN) and starch damage (SD).*

	GLU%	GLI%	Peak Viscosity (cP)	Final viscosity (cP)	Breakdown (cP)	Set Back (cP)	SN (RVU)	SD %
Cultivars	***	***	***	***	***	***	***	***
Cappelli	28.86 a	47.9 b	1740 d	2219 d	665 c	1144 c	158 d	3.26 a
Garfagnana	14.7 b	65.4 a	2504 b	2981 b	953 b	1430 a	183 c	2.23 b
Giovanni Paolo	21.4 ab	53.3 ab	986 e	1223 e	456 d	693 d	110 e	2.59 b
Kamut	23.5 ab	51.1 b	2195 c	2966 b	662 c	1433 a	199 b	3.26 a
Monlis	23.4 ab	56.9 ab	3035 a	2975 b	1134 a	1156 c	230 a	1.57 c
Norberto	15.2 b	61.1 ab	2990 a	3195 a	950 b	1156 c	211 b	1.18 c
Padre Pio	21.3 ab	58.3 ab	2219 c	2620 c	913 b	1313 b	181 c	2.25 b

Means with the same lowercase letter are not statistically different by Tukey's test for $P < 0.05$ *** $P < 0.001$

Table 2 *Water absorption, rheofermentometer and alveograph measurements; the maximum dough height (Hm), total volume of CO₂ produced (V tot CO₂), volume of CO₂ retained (V retained C=2), tenacity over elasticity ratio (P/L) and Energy (W).*

	Water absorption (ml)	Hm (mm)	V tot CO ₂ (ml)	V retained CO ₂ (ml)	P/L	W (J)
Cultivars	***	***	***	***	^	^
Cappelli	64.1 b	52.7 b	1547 b	1315 b	2.23	84.0
Garfagnana	54.7 de	50.0 bc	1456 b	1276 b	0.53	23.9
Giovanni Paolo	67.7 a	58.2 a	1716 a	1434 a	3.76	302
Kamut	61.4 c	48.8 bc	1530 b	1310 b	1.68	91.3
Monlis	53.6 ef	37.5 e	1090 c	1007 b	0.85	39.8
Norberto	53.2 f	42.2 de	979 c	936 c	0.66	47.0
Padre Pio	55.3 d	45.9 cd	1476 b	1276 b	0.73	40.9

Means with the same lowercase letter are not statistically different by Tukey's test for $P < 0.05$ *** $P < 0.001$; ^ The values of P/L and W were calculated from the mean of the three measured graphs. Consequently, they were not subject to statistical analysis

3.2 Dough quality

According to the Farinograph, GP had the highest water absorption (67.7 ml) while norberto followed by monlis had the lowest water absorption (53.2 – 53.6 ml). In fact, a negative correlation was established among water absorption and peak and final viscosity ($r = -0.94$, $p < 0.002$; $r = -0.85$, $p < 0.01$), breakdown ($r = -0.96$, $p < 0.001$) and SN ($r = -0.84$, $p < 0.01$). The rheofermentometer revealed that GP had the highest Hm (58.2 mm),

V Tot of CO₂ produced (1716 ml) and retained (1434 ml) while the einkorns had the lowest values (Hm 37.5-42.2 mm), (V tot 978-1090 ml) and (V retained 936-1007 ml). To note that a higher gas production capacity means a higher content of total water-soluble sugars. A negative correlation was obtained between SN and both volume of CO₂ produced and retained ($r=-0.82$, $p<0.02$ for both). GP had by far the highest P/L (3.76) and W (302 J) and Garfagnana the lowest ones (0.53 and 23.9) (Table 2). According to Sisson (2008), durum wheat should have a P/L>1.5 and an energy W of more or less 200 J for a good breadmaking process. None of our studied varieties had the right value of energy W and only cappelli, GP and kamut had a P/L ratio higher than 1.5. All the other cultivars had a P/L<1 meaning that their extensibility overcomes their tenacity. A negative relationship was found between all the RVA measurements and P/L and W (Table 3).

Table 3 Correlations among tenacity over extensibility ratio (P/L) and the energy (W) and RVA measurements; peak and final viscosity, set back and stirring number (SN). Significance is equal to $P<0.05$ (according to Tuckey test) when r is higher than 0.75.

	Peak viscosity	breakdown	Final visco	Set Back	SN
P/L	-0.92**	-0.90**	-0.92**	-0.78*	-0.84*
W	-0.86*	-0.82*	-0.90**	-0.84*	-0.83*

* $P < 0.05$; ** $P < 0.01$

Table 4 Bread quality; bread specific volume (V), hardness, springiness, cohesiveness, resilience and crust and crumb yellow index (YI).

	V (ml/g)	Hardn ess (N)	Springine ss	Cohesivin ess	Chewine ss (N)	Resilien ce	YI crust	YI crumb
Cultivars	***	***	***	***	***	***	***	***
Cappelli	2.82 a	17.6 c	0.96 ab	0.82 a	13.8 cd	0.450 a	20.6 d	17.5 g
Garfagnana	2.75 ab	21.2 b	0.95 ab	0.76 c	15.3 b	0.383 d	29.9 b	18.2 f
Giovanni Paolo	2.58 bc	18.0 c	0.92 c	0.76 c	12.6 d	0.364 e	26.9 c	20.4 d
Kamut	2.43 cd	20.0 b	0.94 bc	0.79 b	14.8 bc	0.413 b	29.3 b	22.5 c
Monlis	2.29 d	33.0 a	0.97 a	0.78 b	25.0 a	0.399 c	37.3 a	32.8 a
Norberto	2.37 d	34.6 a	0.96 ab	0.79 b	26.1 a	0.399 c	36.8 a	30.4 b
Padre Pio	2.63 ab	20.1 b	0.94 bc	0.79 b	14.9 bc	0.403 bc	28.9 bc	19.3 e

Means with the same lowercase letter are not statistically different by Tukey's test for $P < 0.05$ *** $P < 0.001$

3.3 Bread quality

Cappelli had the highest bread specific volume (2.82 ml/g) followed by Garfagnana and Padre Pio (2.75 and 2.63 ml/g respectively) while einkorns had the lowest ones (2.29-2.37 ml/g respectively). GP had the highest Hm and CO₂ production and retention and was supposed to have the highest bread specific volume however its high energy (W) and very high P/L, indicating a greater (in the case of GP, it is more than three times) dominance of tenacity over extensibility, limited the bread specific volume raising during cooking. Monlis and Norberto had the highest values for bread hardness

(33 N and 34.6 N respectively) and chewiness (25 N and 26 N respectively) while cappelli (17.6 N for hardness and 18N for chewiness) and GP (12.6 N for hardness and 13.8 N for chewiness) had the lowest values. Hardness and chewiness were positively affected by peak viscosity ($r = 0.81$, $p < 0.02$ for both) and negatively affected by SD ($r = -0.88$, $p < 0.009$ and $r = -0.85$, $p < 0.015$) and rheofermentometer measurements ($r > 0.85$, $p < 0.015$ for all). Cappelli had the highest springiness, cohesiveness and resilience while GP had the lowest springiness and resilience and the lowest cohesiveness along with Garfagnana. Einkorns had the strongest crust and crumb YI (30.4 – 32.8) while Cappelli the lowest one (17.5).

3.4 Biplot of studied parameters

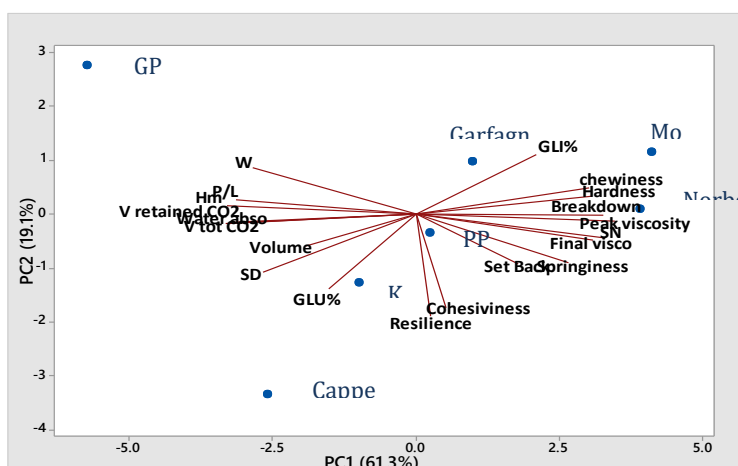


Figure 1 Biplot of flour, dough and bread quality characteristics obtained from the seven cultivars studied; glutenin and gliadin percentage (GLU% and GLI%), peak viscosity, breakdown, set back, final viscosity, stirring number (SN), starch damage (SD), water absorption (water abso), maximum dough height (Hm), total and retained volume of CO₂ produced (V tot CO₂ and V retained CO₂), tenacity over elasticity ratio (P/L), Energy (W), bread specific volume (Volume), hardness, springiness, cohesiveness and resilience.

The biplot in figure 1 illustrates the first two axes explaining the 80.4% of the variation of a PCA based on flour, dough and bread quality parameters of our varieties. PC1 represented 61.3 % of the variability and was associated positively with RVA parameters, springiness, hardness and chewiness and negatively with water absorption, rheofermentometer parameters, P/L and SD. The varieties that contributed the most to the variation were GP, cappelli and both einkorns. For our cultivars studied, GLU% was associated positively to bread quality, being linked to bread volume, resilience and cohesiveness, and to SD while GLI% was associated positively to hardness and chewiness. Bread specific volume was linked positively to water absorption and volume of CO₂ produced. Alpha amylase activity (through SN) had a greater effect than GLU% and GLI% on dough and bread quality. In fact, numerous studies in the past till now have highlighted the importance of alpha amylase in breadmaking. Einkorn varieties were both grouped together next to chewiness and hardness.

4. Conclusions and Future Perspectives

The grain characteristics of seven different wheat varieties showed significantly different effect on dough pasting, fermentation and bread properties. Einkorns are

suitable for making non-fermentating staple food due to the poor fermentation properties but higher viscosity than the other cultivars. The new emmer GP showed higher gas production capacity and low gelatinization properties but it was so tenacious to make bread with good volume, springiness and cohesiveness. While PP made a bread similar to that of kamut and garfagnana. Cappelli made the best quality of bread among our studied varieties. Given the greater sustainability of their cultivation, ancient and old wheats are the best choice to be used in organic farming and finding their right end-products is of importance. Thereafter, our seven varieties will be tested for their suitability for pasta making, a staple food in the Italian cuisine.

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Aroma Volatile Compounds of Coffee: Role in Authenticity, Traceability and Well-Being of Consumers

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This PhD thesis dealt with unravelling the innovative role of aroma volatile compounds for the purpose of authenticity and traceability of coffee from different geographical origins. For this purpose, an analytical method was optimised and applied to determine presence and concentration of 3-Alkyl-2-methoxypyrazines (MPs) in green coffee and roasted as key quality index. Furthermore, a study to investigate the effects of coffee aroma on cognitive performance and autonomic nervous system functions in humans was conducted. In particular, this latter investigation was carried out to evaluate psychophysiological responses based on the olfactory stimulus induced by the aroma of coffee on human participant in the study of chemosensory analysis.

Composti volatili del caffè: ruolo nell'autenticità, tracciabilità e benessere dei consumatori

Questa tesi di dottorato ha l'obiettivo di studiare il ruolo innovativo dei composti aromatici volatili ai fini dell'autenticità e della tracciabilità del caffè di diversa origine geografica. A tale scopo, un metodo analitico è stato ottimizzato ed applicato per determinare la presenza e la concentrazione di 3-alkil-2-metossipirazine (MP) nel caffè verde e tostato come elemento caratterizzante di qualità. Inoltre, è stato condotto uno studio per studiare gli effetti dell'aroma del caffè sulle prestazioni cognitive e sulle funzioni del sistema nervoso autonomo nell'uomo. A tale scopo, lo studio è stato effettuato per valutare le risposte psicofisiologiche basate sullo stimolo olfattivo indotto dall'aroma di caffè nell'uomo mediante analisi chemosensoriale.

Key words: green coffee, potato taste defect PTD, HS-SPME-GC-MS, alkyl-methoxypyrazine, geographical origin, cognitive performance, coffee odor, autonomic nervous system.

1. Introduction

In accordance with the PhD thesis project previously described (Mutarutwa, 2017), this oral communication reports the main results of the following activities directed to:

A1) Optimisation of extraction techniques and identification of MPs in a range of food vegetable matrices and subsequent development of quantitative method and validation.

A1) Application and optimisation of the developed method to quantify MPs in coffee from different geographical origins and coffee insect damaged.

A3) Investigation of the effects of coffee aroma on cognitive performance and autonomic nervous system functions in humans.

Therefore, the results of the thesis will be reported in two parts, the study of MPs and the chemosensory study.

2. Study 1: MPs quantitation

Green coffee aromatic profile is characterised by greenish notes, and among them 3-alkyl-2-methoxypyrazines (MPs) constitute an important class of volatile organic compounds (VOCs). However, when present in high amounts, they are perceived as defects: the so called “potato taste defect” (PTD) typical of East African countries, mainly Burundi and Rwanda (Bouyjou *et al.*, 1999). Since the presence of PTD can drastically reduce the quality of the entire batch, for the coffee Industry that has as consequence rejection back to producers of the entire lot of coffee, and this result, in the other hands, in disproportionate consequences for farmers and for the entire economy of countries where coffee plays a very important role in their economy.

Consequently, further studies to assess and control the factors leading to PTD, as well as quantitative methods for MPs, which are present in both defective and non-defective beans, becomes of paramount importance to avoid the possibility of onset and the resulting economic damage to coffee producers.

The analysis of these volatiles is then remarkably important, and it may be very challenging as confirmed by the scarce literature focused on MPs, particularly with respect to quantitative data. In literature, very few works have investigated the presence and quantitation of MPs in Coffee regarding PTD affected beans and in particular with focus on IPMP (Becker *et al.*, 1988; Jackels *et al.*, 2014). In the attempt to fill this gap, in this thesis, presence and quantification of MPs was developed and optimised in four different vegetables, in which these MPs are known to be characteristic, namely, green bell pepper (*Capsicum annuum* L. var *grossum*), green pea (*Pisum sativum* L. and *Pisum* spp), carrot (*Daucus carota* subsp *sativus*), and cucumber (*Cucumis sativus* L.) have been investigated.

The analytical method was initially optimised on different vegetables in which MPs are generally present and contribute to the aroma pattern, then applied to quantify MPs in green and roasted coffee. In addition, this study was the first to study the occurrence of MP in coffee from different origins, in order to unravel its role and with greater attention to PTD affected.

2.1. Materials

In the present work, the optimisation of the method to determine MPs was carried out on four fresh vegetables, i.e., green bell pepper (*C. annuum* L. var *grossum*), green peas (*P. sativum* L. and *Pisum* sp), carrot (*D. carota* subsp *sativus*), and cucumber (*C. sativus* L.).

The optimised method was then applied to 27 coffee samples from different commercial batches of green Arabica beans from 11 different geographical origins (Burundi, Rwanda, Tanzania, Kenya, Ethiopia, Brazil, Guatemala, Colombia, Nicaragua, Honduras and India), 2 green coffees (*C. canephora* Pierre) and 1 sample of green coffee (*C. arabica* L.) from Rwanda damaged by insects were analysed.

Pure standards of 3-Isopropyl-2-methoxypyrazine (97%), 3-secbutyl-2-methoxypyrazine sBMP (99%), and 3-Isobutyl-2-methoxypyrazine IBMP (97%) were used as reference compounds for identification and quantitation; pure standards of 3-methyl-2-methoxypyrazine (MMP) (99%) and 2-methoxypyrazine (MP) (99%) were used

as internal standards for quantitative analysis. All pure standards were purchased from Sigma Aldrich (Milan, Italy).

2.2. Methods

Two different volatile extraction techniques (solid phase microextraction [SPME] using 75 μm CAR/PDMS (Supelco) and simultaneous distillation-extraction [SDE]) in conjunction with VOC qualitative analysis by gas chromatography-mass spectrometry (GC-MS) methods were applied.

Sample analyses were performed with an Agilent 6890 Gas Chromatograph equipped with a 5975 Agilent Mass Spectrometer (Agilent, Palo Alto, USA) and an autosampler MPS2. A volume of 1 μl of SDE extract and the SPME fiber were analysed. Compounds were separated using a 60 m ZB-WAX plus capillary column (film thickness 0.25 μm and 0.25 mm of internal diameter) (Phenomenex, Bologna, Italy). GC injector was set in split mode (split ratio 4:1). The oven temperature, initially set to 50°C for 3 minutes, was increased to 200°C at 4°C/min, then raised to the final temperature of 240°C at a rate of 20°C/min, hold for 5 minutes. Quantitation was achieved using selected ion monitoring (SIM).

2.3. Results

SPME coupled with GC-MS in SIM mode (single ion monitoring) showed the best performance in terms of sensitivity to detect MPs in different vegetable matrices. Therefore, a quantitative SPME-GC-MS quantitative method in head space (HS) was developed and optimized and the dominant MPs were quantified. 3-Isopropyl-2-methoxypyrazine (IPMP), 3-sec-butyl-2-methoxypyrazine (sBMP), and 3-isobutyl-2-methoxypyrazine (IBMP) have been detected and quantified in all the vegetables. 3-Isopropyl-2-methoxypyrazine was the prevalent MP in peas and cucumber while IBMP in bell pepper. These MPs were detected for the first time in carrot and cucumber. Finally, sBMP has been confirmed to be the most important MP in carrot. The relative standard deviation ($n = 3$) of the MP data ranged between 4% and 13% in the different vegetables with good data reproducibility. The concentration of MPs ranges between 0.04 and 60.53 ng/g, higher than the determined limit of quantitation, which confirms the adequacy of the proposed method for quantification of these compounds in vegetables (Mutarutwa *et al.*, 2018).

Results highlighted the feasibility to further applications of the analytical approach to determine MPs in coffee samples, where their excess is perceived as defect. Therefore the method was applied and optimized to determine MPs in green and roasted coffee.

The presence of all 3 MPs was found in all coffee samples and they were all higher than the LOQ values, with good reproducibility values (n plus 3) for all MPs. The concentrations of IPMP and sBMP are consistently lower than those of IBMP. This later is the most abundant in all the samples and has shown a very high variability range between different geographical origins, and might play a very important role in the aroma of coffee. sBMP has been quantified for the first time in the coffee aroma and confirmed its role in the aroma of coffee, and in general the green herbal character, along with IPMP and IBMP already reported in the literature.

For coffee sample from Rwanda severely damaged by insects and clear PTD defect, the amount of IPMP varied significantly in the same batch with different samplings, from concentrations closed to non-defected coffee to very high amount up to 1000 times in highly defected coffee. These results show different degree of contamination or different PTD contribution between beans of the insect coffee damaged sample. It is also

important to point out that in our samples, the coffee beans were insect damaged, and it was not necessarily by antesia.

2.4. Conclusions and Future Perspectives

HS-SPME, coupled with GC-MS in Single Ion Monitoring (SIM) mode, in addition to its greater ease of use and the least amount of sample required, was shown to be more efficient for isolating, detecting and quantifying metoxypyrazine in the different vegetable matrices and also in green coffee samples.

The application of the optimised method has confirmed its reliability in the study of coffee and therefore constitutes a useful analytical tool to deepen the quantitative evaluation of MPs in coffee samples as their excess is undesired and generates a serious PTD defect.

The current study provided additional knowledge on alkyl-methoxypyrazines in coffee and their greater impact on coffee quality; in particular, the high variability of IBMP seems to depend on geographical origins. However, other factors that could affect MPs presence in coffee need to be understood and further studies are needed.

3. Study 2: Psychophysiology analysis under coffee aroma

The aim of this study was to assess the effects of coffee aroma on the psychophysical state of consumers. The main goal was to assess whether coffee olfactory stimulation can increase cognitive information processing (increased attention, alertness, accuracy). Although the effects of caffeine are well recognised on the psychophysical state, the effects of aroma are still unknown. In this way, this study objectively evaluates these effects of coffee aroma during a task, while simultaneously measuring different parameters of the autonomic nervous system (ANS).

3.1. Methods

A within-subject design was utilized in this study. Thirty healthy students (15 female and 15 male), aged 18–26 years (mean age = 22.97 years, SD = 2.36) participated in the experiment. Their mean body mass index (BMI) was 22.50 kg/m² (SD = 2.36). All participants signed a written informed consent form. The Ethics Committee of the Medical Faculty of the University of Erlangen-Nürnberg approved the experimental protocol of the study according to the ethical standards of the Declaration of Helsinki.

In the present experiment, the odor of ground coffee (*C. Arabica*) aroma (20 g commercial ground coffee, illy blend for Moka preparation) supplied by illycaffè (Trieste, Italy) was compared to an odorless blank (20 g demineralised water).

The experiment took place in a laboratory darkened with blinds ensuring constant lighting through the ceiling lamps at Fraunhofer Institute IVV. Participant sat comfortably in front of a monitor and a normal keyboard.

3.2. Experimental paradigm

Participants were asked to perform the Stroop task two times under coffee odor presentation and no odor with 5 min break between the two tasks. Each Stroop task took ~14.5 min each. During the Stroop task, physiology was measured, and each task began with 2 min baseline to normalize the physiological parameters. In order to avoid habituation, the order was counterbalanced between participants and between genders so that half began with coffee and half with water. Among other tasks within this experimental session, the subjects were asked to evaluate each odor on 4 dimensions:

Intensity, arousal, familiarity and Pleasantness (from 1=not at all; to 9=extremely) each dimension.

3.3. Autonomic nervous system measures

Heart rate (beats per minute (BPM) and electrocardiogram (ECG)), skin conductance response (SCR), breathing and skin temperature were recorded simultaneously. They were recorded using sensors (ADInstruments Ltd) placed on the non-dominant hand, and an electrocardiogram (ECG) (biosignal amplifier g.BSamp with 3 electrodes Ag/AgCl surface electrodes with gel (Vivomed GmbH, Germany)). All instructions and presentation of the odors were accomplished using E-prime 2 Psychology Software Tools, Inc. (Sharpsburg, USA). Birhinal olfactory stimulation was accomplished using a computer-controlled olfactometer (Lundstrom *et al.*, 2010) trough tube system with manifold with nose pieces connected to the PowerLab Spirometer Pod (PowerLab Spirometer Pod ML311, connected to PowerLab 8/35 system, ADInstruments Ltd.) that was also used to measure the breathing (Hoffmann-Hensel & Freiherr, 2016).

3.4. Stroop task

Participants received instructions before the task started. They were asked to indicate the color in which the color name was printed appearing on the screen as quickly and accurately as possible via button press. Answers were given with index, middle ring finger and little finger of the dominant hand on a keyboard with four buttons marked. Each stimulus started with the presentation of a white fixation cross on a black background with a duration that varied between 200–1400 ms. Then, a color name in German appeared at the center of the screen until the button press and for a maximum of 3000 ms.

The results of subjective perception responses, and cognitive test were collected with E-prime software. The physiological results were processed with the Ledalab Software. All data were analyzed using SPSS software.

3.5. Results

For all physiological responses and cognitive test, the main effects of aroma were calculated using ANOVA /T-test And Friedman/Wilcoxon test. The SPSS general linear model (GLM) procedures were used for interactions. For all analysis significance threshold was set to $\alpha = .05$.

The main effects of odor on cognitive performances were measured by evaluating reaction time (RT) and accuracy during stroop task by comparing the values under coffee aroma with that while receiving water as control. The results of the analysis mainly did not reach statistical significance ($p > .05$) (Mutarutwa *et al.*, under submission).

The interactions between odors (coffee vs. control) and order of stroop were found to be significant for reaction time ($p < 0.05$). SPSS general linear model (GLM) analysis of covariance ANCOVA procedures was therefore used for controlling the order of stroop. Were examined using repeated measures Within subjects variables and order stroop as covariate. There was a significant effect of odor after controlling the the order of stroop for RT. RT decreased during exposure to coffee aroma i.e. when participant performed the stroop task under coffee aroma for second, compared to control.

For each odor condition, the physiological responses for Heart rate (beats per minute (BPM) and electrocardiogram (ECG)), skin conductance response (SCR), breathing and skin temperature, were measured. The main effects of odor were obtained only for skin conductance (SCR) that showed significant responses for Electrodermal Activity that elicited significantly higher electrodermal responses during coffee odor

presentation compared to control. Further analysis with GLM repeated measures confirmed interactions between physiological responses of odors (coffee vs control) and order of stroop significant ($p < .05$) for heart rate, skin conductance and respiration.

These results show that the aroma of coffee increase performances, especially when fatigue or attention is considered i.e. when in the second test participant was exposed to coffee aroma. Given that effects of coffee aroma on performance and on expectations have been found in the study of Madzharov et al. (2018), the current study provides additional information that the effects of coffee do not depend only on expectations that coffee aroma will be associated with the positive effects that caffeine is known to have on alertness and performance, despite tiredness, there are arousing effects of coffee aroma. Furthermore, for the first time, this was supported by physiological responses in particular in the first 5 min.

Besides skin conductance, significant variations of heart rate and respiration probably more related to the pleasantness of coffee aroma have been observed (Bensafi et al., 2002).

3.6. Conclusions

Our results thus demonstrate an increase of performance, and not only based expectations, in particular when participants are tired there are effects and these findings were partially supported by physiological responses.

This study extends thus the knowledge on coffee volatiles aroma beyond the already known pleasantness attributes that are one of the most appreciated for consumers, and demonstrates that coffee aroma plays a role in psychophysiological state by inhaling coffee aroma. Since numerous studies focused on stimulating effects of caffeine, these findings of increasing of cognitive performance by coffee aroma could have great interest for its physiological effects on well being of consumers and given the higher consumption of coffee worldwide, could have several applications not only for the coffee industry but also to those study related to scents.

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Drying of Fruits and Vegetables: Improvement of Quality and Process Modelling

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This Ph.D. thesis deals with the optimization of the drying process of fruits and vegetables to improve their quality. The effects of innovative and natural pre-treatments during conventional hot air drying at different temperatures were investigated on quality of dried and rehydrated some foodstuffs. The drying and rehydration processes were studied. In this report the study on the “Annurca” apple is described. The drying process was performed at different temperatures, evaluating the main quality parameters. Suitable mathematical model was developed to predict the transport phenomena parameters of the hot air drying and to obtain high quality products in terms of physico-chemical and nutritional properties.

Essiccazione di frutta e verdura: miglioramento della qualità e modellazione matematica

Lo scopo del progetto di dottorato è di ottimizzare il processo di essiccazione di prodotti ortofrutticoli per migliorarne la qualità. Per limitare gli effetti di imbrunimento e di perdita delle principali caratteristiche sensoriali sono stati testati ed ottimizzati dei pretrattamenti con sostanze naturali su alcuni frutti (mela “Annurca”, pesca cv Terzarola, caco cv Rojo Brillante, pera cv Rojo Brillante). In particolare in questo studio si riportano i risultati relativi al processo di essiccazione e reidratazione della mela “Annurca”. È stato inoltre sviluppato un modello matematico per predire i parametri del trasporto di materia caratteristici del processo di essiccazione per ottenere prodotti di alta qualità.

Key words: “Annurca” apple, pre-treatment, hot air drying, process modelling, shrinkage, total phenolics, rehydration.

1. Introduction

In accordance with the PhD thesis project previously described (Önal, 2018), this communication reports the main results of the following four activities directed to:

A1) the influence of innovative and natural pre-treatment during hot air drying at four different temperatures (50, 55, 60 and 65° C) on drying time and quality of “Annurca” apple in terms of shrinkage, colour, total phenolic content, antioxidant activity, microstructure and sensorial evaluation;

A2) development of mathematical model for describing the drying process;

A3) evaluation of rehydration conditions (such as rehydration temperatures at 30 and 70°C) and used innovative pre-treatment on the rehydration kinetics and characteristics of dried Annurca apples.

2. Drying and rehydration of fruits and vegetables

Drying is well-known one of the most important preservation techniques and it is commonly applied for prolonging shelf life of fruits and vegetables and improving food stability by reducing moisture content, microbial growth and minimising chemical

deterioration. Nevertheless, shrinkage, fading of natural color or discoloration, decreased flavor and unappetizing texture are the most evident deficiencies of dry products. Moreover, poor rehydration ability and reduced nutritional quality are also evidence of disadvantageous influence of drying on food. In this context, drying combined with pre-treatments have been proposed with the aim to reduce various adverse changes. A wide variety of pre-treatments have been utilised depending upon the type of food to be dried, food composition and its availability. Examples of pre-treatments are immersion in chemical solutions, hot water blanching and physical pre-treatments. Chemical pre-treatments, usually comprise sulfiting, immersion in sodium chloride, potassium carbonate, organic acids or sugar, use of surface active agents and impregnation with biopolymers. Rehydration is a complex process and its main purpose consists of restoring the properties of dried foods. The capacity of the dried material to be rehydrated depends on certain intrinsic properties of the vegetal tissue and also, on how the rehydration process is carried out (process conditions) and on the previous pretreatments applied to the product.

3. Materials and Methods

Initial moisture content of “Annurca” apples (*Malus x domestica* Borkh. cv Annurca Rossa del Sud) was 83.85 ± 1.44 % on wet basis (5.19 kg water/kg db). Cylindrical slabs (diameter 30 ± 0.22 mm and thickness 5 ± 0.01 mm) were prepared using several raw materials. Two kind of samples were compared: untreated slabs (UTR) and pre-treated slabs with innovative aqueous carbohydrate/salt solution (TR), as reported in (Önalet al., 2019). Drying experiments were performed in a convective dryer (Zanussi FCV/E6L3) at four temperatures: 50, 55, 60 and 65°C at air velocity of 2.3 m/s until the constant mass was reached (about 0.04 kg water/kg db). The weight loss of the samples was recorded online every 5 min by using a balance sensor (Phidgets INC., Canada) and the results were reported in terms of M_t/M_0 , where M_t was the moisture content (kg water/kg db) at a given drying time and M_0 was its initial value. Dehydrated samples were rehydrated in distilled water at 30°C and 70°C by using water bath to estimate their rehydration capacities. The approximate ratio between the weight of dried fruits and water was kept 1:100. Samples were weighted every 15 min in the initial phase of rehydration process (up to 120 min) and then every 30 min. At fixed time, samples were removed from the rehydration media, dried gently with tissue paper and weighted by using an electronic balance. The total rehydration times at 30°C and 70°C for UTR and TR dried samples were determined as 270 and 210 min, respectively.

3.1. Drying Kinetics and Mathematical Modelling

Drying is a process characterized by two simultaneous phenomena: heat and material transport phenomena. Water removal causes structural changes of the matrix, both at microscopic (porous) and macroscopic (shrinkage) levels which have to be taken into account. The mathematical model was obtained from following simplified hypotheses that the material is homogeneous and isotropic, the shape and size characteristic is constant and the initial moisture content is uniform (C_0). The following Eq. (1) describes the evolution of water content in the material.

$$\frac{\partial c}{\partial t} + \frac{\partial uc}{\partial x} = \frac{\partial}{\partial x} \left(D \frac{\partial c}{\partial x} \right) \quad (1)$$

where c is the dimensionless water content in differential volume, t is the temporal coordinate (s), x is the spatial coordinate along which material transport takes place (cm), D is the effective coefficient (cm^2/s).

The parameter u in the Eq. (2) is the shrinkage velocity, here introduced to take into account shrinkage phenomena.

$$u = u_0 \frac{\partial c}{\partial x} \quad (2)$$

where u_0 is the shrinkage velocity coefficient (cm/s).

The initial condition (I.C.) and boundary conditions (B.C.) (uniform initial water content profiles are imposed) are:

$$\text{I.C.: } t=0 \quad c(x,0)=1 \quad x=\left[-\frac{L}{2}; \frac{L}{2}\right] \quad (3)$$

$$\text{B.C.1: } x=0 \quad \frac{\partial c}{\partial x}=0 \quad (4)$$

$$\text{B.C.2: } x=\frac{L}{2} \quad \frac{\partial c}{\partial x}\bigg|_{x=\frac{L}{2}} = -k_m c\bigg|_{x=\frac{L}{2}} \quad (5)$$

where L is the size of sample and k_m is the overall transport coefficient (Brasiello et al., 2013). The I.C. is related to the assumption of uniform and constant initial distribution of water content, B.C.2 represents the interface condition. Model parameters were estimated by non-linear regression method using Matlab Software.

3.2 Analyses

Colour parameters (H° , WI and ΔE) was evaluated before and after drying. The volume ratio (V_t/V_0) as a function of the relative moisture ratio (M_t/M_0) (Adiletta et al., 2016) was evaluated during drying to monitor shrinkage. The total phenolic content and the antioxidant activity of dried and fresh apple (both UTR and TR) extracts were determined by Folin-Ciocalteu colourimetric method and DPPH radical scavenging method. Microstructure changes of apple samples were observed by scanning electron microscopy (SEM) according to Proietti et al. (2018) before and after drying. Rehydration characteristics were determined according to Barreta et al. (2016) to describe the behavior of foods submitted to rehydration: water absorption capacity (WAC), dry matter holding capacity (DHC), rehydration ability (RA), water holding capacity (WHC).

4. Results and Discussion

4.1 Drying Kinetics and mathematical modelling of “Annurca” Apple Drying Process

The pre-treatment is important for influencing the drying time. TR slabs were found to have shorter drying time compared with UTR ones. In Fig.1(a,b) the evolution of theoretical and experimental moisture ratio (M_t/M_0) at 65°C is compared for UTR (a) and TR (b) apples. Shrinkage velocity values (u) and the effective diffusion coefficient of apple (dried in the range $50\text{--}65^\circ\text{C}$) were reported in Table 1. The lowest value of u is observed for both TR and UTR cases at 65°C . This result means that at such temperature the drying process is close to a pure diffusive phenomenon. Because the parameter u defines the distance of drying process from a pure diffusive process due to shrinkage phenomenon, we can assess that low u value corresponds to low shrinkage. As expected D_{eff} values indicate an increase with increasing drying temperature. Based on these results, TR samples had the highest D_{eff} compared to UTR ones. The developed mathematical model is able to predict with sufficient accuracy the evolution of water content for both samples at given temperature.

Table 1 Shrinkage velocity values of applied drying and effective diffusion coefficient of UTR and TR samples at different drying temperatures.

Samples	u (cm ² /sec)				D_{eff} (m ² /s)			
	50°C	55°C	60°C	65°C	50°C	55°C	60°C	65°C
UTR	$6.08 \cdot 10^{-2}$	$5.38 \cdot 10^{-2}$	$4.25 \cdot 10^{-2}$	$5.57 \cdot 10^{-3}$	$9.15 \cdot 10^{-5}$	$1.10 \cdot 10^{-4}$	$1.33 \cdot 10^{-4}$	$1.58 \cdot 10^{-4}$
TR	$1.94 \cdot 10^{-2}$	$1.44 \cdot 10^{-1}$	$1.02 \cdot 10^{-1}$	$9.53 \cdot 10^{-4}$	$1.54 \cdot 10^{-4}$	$1.83 \cdot 10^{-4}$	$2.17 \cdot 10^{-4}$	$2.56 \cdot 10^{-4}$

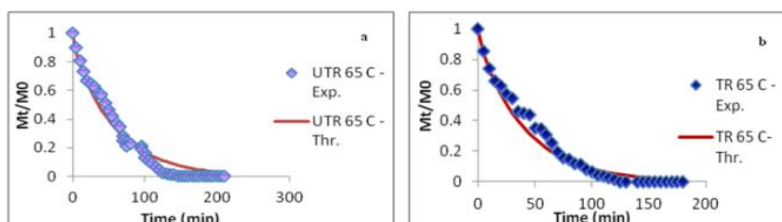


Figure 1 Experimental and theoretical drying kinetics for UTR (a) and TR (b) slabs at 65°C.

4.2. Shrinkage and Colour Evaluation

Shrinkage has negative consequences on the quality of dried product and increases with the volume of removed water (Adiletta et al., 2016). As an example V_t/V_0 as a function of M_t/M_0 was given at 60°C and 65°C (Fig. 2). TR slabs showed lower shrinkage respect to UTR ones. According to this results, pre-treatment has significantly influence on the shrinkage of apple slabs. Regarding to the effect on colour, the Hue angle (H°), white index (WI) and total colour differences (ΔE) of fresh and dried samples were reported in Fig. 3. Results demonstrated that pre-treatment and drying process have remarkable effect on the colour of apples (Fig. 4). TR samples had always lower ΔE values and higher H° and WI values.

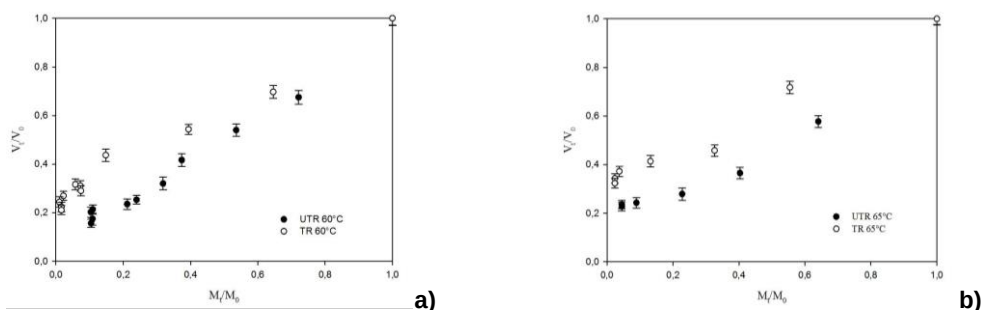


Figure 2 Experimental data of volume shrinkage of "Annurca" apple during drying at 60°C (a) and 65°C (b).

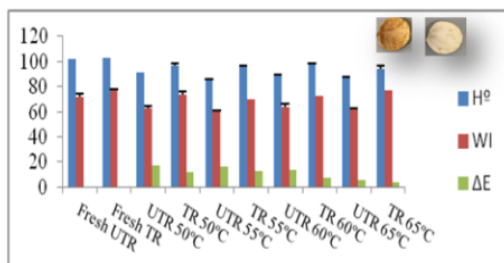


Figure 3 Colour parameters H° , WI and ΔE of fresh and dried UTR and TR apple samples at 50, 55, 60 and 65 °C.



Figure 4 Annurca apple slabs: fresh UTR (a), fresh TR (b), dried UTR (c) and dried TR (d) at 65 °C.

4.3 Total Phenolic Content (TPC) and Antioxidant Activity (AA)

The TPC of fresh UTR and TR apple slabs was 419.46 and 499.80 mg GAE/100 g db. It can be possible to see from Fig.5 that UTR samples after drying had a significant reduction of TPC respect to fresh ones, but the increase of drying temperature did not significantly affect the polyphenols content. In contrast, there were no statistical differences ($p > 0.05$) between TR fresh samples and TR dried ones up to 60°C. Only at the highest temperature (65°C) a significant reduction in TPC was observed.

The lowest EC_{50} values (the highest antioxidant activity) were determined as 16.06 and 18.66 mg/ml db in TR and UTR fresh samples, respectively. For TR samples at lower drying temperatures (50 and 55°C) the antioxidant activity was better retained than at higher drying temperatures. This trend of AA it may be associated with modifications of the chemical structure of the main antioxidant compounds in apple (i.e. chlorogenic acid, quercetin etc.) or interactions between antioxidant compounds and other apple constituents, such as proteins at higher temperatures.

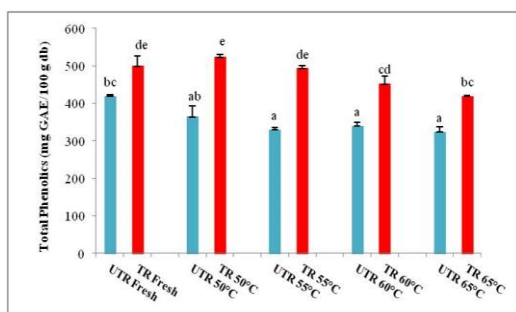


Figure 5 Total phenolic content in fresh and dried apple samples (50, 55 60 and 65°C).

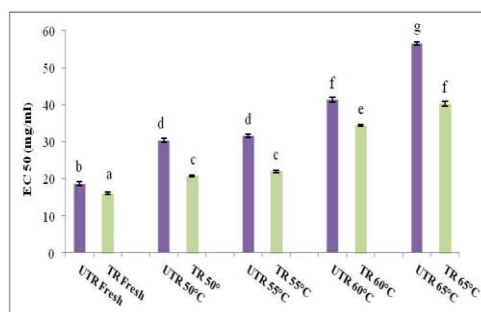
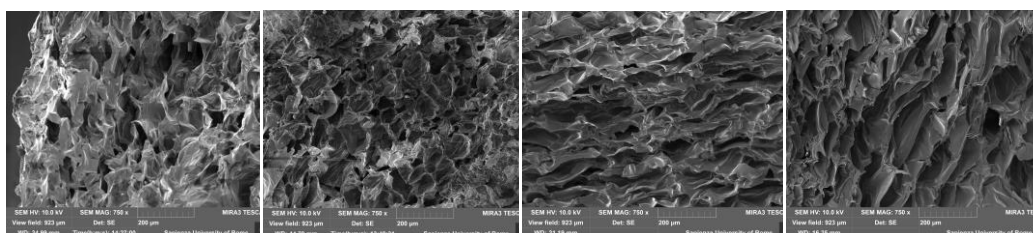


Figure 6 Antioxidant activity of fresh and dried samples (50, 55, 60 and 65°C).

4.4 Microstructure Analysis

SEM analysis was used to examine the structure of both UTR and TR dried apples. Typical SEM images of apple dried 50, 60 and 65°C were reported in Fig. 7 (a-f) for UTR and TR samples, respectively. According to SEM images, a cell breakage was observed in the UTR samples, especially at higher temperatures, emphasising loss of turgor and rupture of cell membranes from the breakage zone. This turgor loss is due to degradation of cell walls (mainly attributed to pectins) and causes a possible shrinkage phenomena in the cell walls. The UTR dried samples at 50°C presented a contracted-closed microstructure, broken and non-uniform pores (both larger and smaller pores) with

respect to the treated dried samples. At higher temperatures (65°C) the cell volume reduction and tissue collapse caused the formation of a lamellar structure (Fig.7c), which resulted more porous and with larger pores after pre-treatment (Fig. 7d).



a) UTR 50°C b) TR 50°C c) UTR 65°C d) TR 65°C
Figure 7 SEM images of UTR – TR dried apples at 50°C (a,b) and 65°C (c,d).

4.5 Rehydration kinetics and characteristics

Rehydration is a complex process of moistening of dry products and it cannot be interpreted as a reversible process of drying. The dried samples were rehydrated at 30°C and 70°C until the moisture content reached equilibrium. The weight gain (%) (at two rehydration temperatures: 30°C and 70°C) of samples dried 65°C were shown in Fig. 8a-b). All rehydration tests showed similar behaviour: the TR samples exhibited higher rehydration capacity with respect to UTR ones. Less and slower rehydration of UTR samples was probably due to the tissue collapse by longer drying times. In this case, this innovative solution was proven to be a useful pre-treatment to preserve the apple cellular structure during the rehydration process.

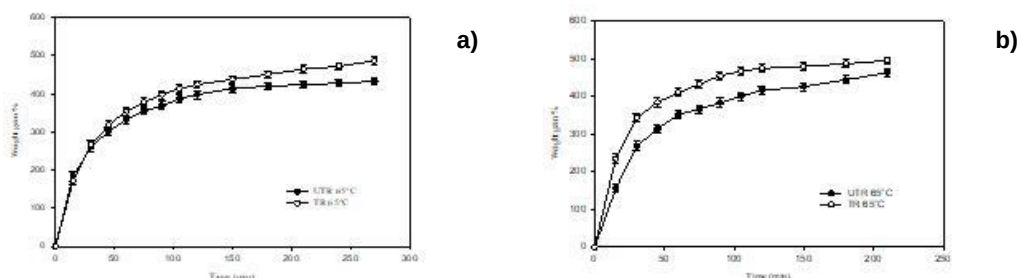


Figure 8 Rehydration kinetics at 30 °C (a) and 70°C (b) of UTR and TR apples dried at 65°C.

In order to have complete information about the amount of water absorbed or the amount of solutes removed during the rehydration process, the following four indices were used: water absorption capacity (WAC), dry matter holding capacity (DHC), rehydration ability (RA) and water holding capacity (WHC) were calculated to investigate the effect of drying temperatures, pre-treatment and rehydration temperatures on rehydrated apples. The WAC and DHC indexes showed higher values for all treated samples at 30 and 70°C. This trend indicated that the treated samples were able to absorb more water during rehydration with respect to untreated ones. The highest ability to rehydrate (RA) was at 30°C for treated samples dried at 60 and 65°C. This result can be explained by the development of a porous structure at higher drying temperatures, while the lower rehydration temperature (30°C) reduces the cellular damage promoting greater rehydration capacity and lower shrinkage.

Table 2 Rehydration characteristics of both UTR and TR dried samples (50, 55, 60 and 65°C) of rehydration temperatures at 30 °C (a) and 70°C (b).

Rehydration at 30°C (a)					Rehydration at 70°C (b)				
	WAC	DHC	RA	WHC		WAC	DHC	RA	WHC
UTR 50°C	0.781 ± 0.03	0.233 ± 0.025	0.182 ± 0.02	0.794 ± 0.013	UTR 50°C	0.767 ± 0.02	0.222 ± 0.02	0.177 ± 0.005	0.807 ± 0.02
TR 50°C	0.806 ± 0.02	0.243 ± 0.03	0.196 ± 0.009	0.822 ± 0.02	TR 50°C	0.811 ± 0.023	0.236 ± 0.003	0.191 ± 0.012	0.825 ± 0.02
UTR 55°C	0.784 ± 0.024	0.240 ± 0.02	0.188 ± 0.005	0.796 ± 0.06	UTR 55°C	0.799 ± 0.03	0.213 ± 0.003	0.170 ± 0.02	0.813 ± 0.14
TR 55°C	0.827 ± 0.03	0.247 ± 0.015	0.204 ± 0.014	0.859 ± 0.002	TR 55°C	0.822 ± 0.02	0.237 ± 0.02	0.195 ± 0.03	0.823 ± 0.03
UTR 60°C	0.816 ± 0.014	0.241 ± 0.02	0.197 ± 0.03	0.844 ± 0.03	UTR 60°C	0.801 ± 0.02	0.218 ± 0.014	0.181 ± 0.02	0.821 ± 0.02
TR 60°C	0.847 ± 0.02	0.268 ± 0.03	0.227 ± 0.02	0.902 ± 0.015	TR 60°C	0.830 ± 0.013	0.241 ± 0.02	0.200 ± 0.003	0.870 ± 0.015
UTR 65°C	0.831 ± 0.02	0.243 ± 0.008	0.202 ± 0.009	0.877 ± 0.02	UTR 65°C	0.790 ± 0.015	0.240 ± 0.02	0.190 ± 0.004	0.843 ± 0.009
TR 65°C	0.853 ± 0.013	0.274 ± 0.014	0.234 ± 0.012	0.911 ± 0.02	TR 65°C	0.817 ± 0.01	0.239 ± 0.012	0.195 ± 0.008	0.879 ± 0.005

5. Conclusions and Future Perspectives

In the third year of Ph.D. activity, an improved drying and rehydration process of apple, peach, kaki and pear samples have been analysed. In this paper, drying and rehydration process' results of "Annurca" apple are presented. A suitable and innovative pre-treatments were applied to each fresh product. The analyses have been carried out both experimentally and theoretically. From the experimental point of view, several parameters have been taken into account to assess quality of the dried materials: colour, shrinkage, total phenolic content, antioxidant activity, microstructure and rehydration capacity. This study may be useful to provide information about the development of innovative and natural pre-treatment conditions before drying of fruit and vegetables. Therefore, the combination of dipping pre-treatment and hot air drying at 65°C seems to be a suitable process to produce high quality (i.e. the lowest colour changes, the better structure preservation with less shrinkage and higher rehydration capacity etc.) and healthy dried apple snacks for both consumers and food industry. A diffusive mathematical model of the dehydration process has also been developed. Through the mathematical model it has been shown this pre-treatment could be used with the purpose to reduce the intensity of the thermal treatments and its impact on the quality of vegetables and fruits.

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Development of Barley Based Couscous with High Nutritional Value and Functional Properties

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In order to develop functional couscous with high nutritional value, different balanced formulations, containing semolina and β -glucan enriched barley flour, have been studied and two mixtures were selected for the following handmade production. On the obtained couscous a complete chemical-nutritional characterization was carried out together a bioactive compounds evaluation and cooking quality assessment. The final product is turned out to be improved in nutritional and health properties preserving the rheological/technological characteristics of commercial couscous.

Sviluppo di couscous a base di orzo con alto valore nutrizionale e proprietà funzionali

Allo scopo di sviluppare un couscous ad alto valore nutrizionale/funzionale, sono state studiate diverse formulazioni a base di semola e sfarinato d'orzo arricchito in β -glucani. Due miscele sono state quindi selezionate per successive prove di produzione artigianale. Il couscous ottenuto è stato poi caratterizzato in termini di composizione chimico-nutrizionale, componente bioattiva e qualità di cottura. I risultati ottenuti evidenziano che il prodotto finito non solo risulta essere migliorato da un punto di vista nutrizionale/salutistico, ma preserva anche le caratteristiche reologiche/tecnologiche tipiche di un couscous commerciale.

Key words: couscous, barley, β -glucan, phenolic compounds, functional foods.

1. Introduction

In accordance with the PhD thesis project previously described (Oriente, 2017), this oral communication reports the main results obtained from the following activities:

A1) development of an appropriate systems for the enrichment of barley flour in bioactive compounds (β -glucan);

A2) production of semolina and barley based couscous, using traditional process, and their complete chemical-nutritional characterization;

A3) evaluation of thermal treatment effects on couscous samples;

A4) determination of free and bound phenolic compound and alkylresorcinols in couscous samples and assessment of cooking quality of final products.

2. Unconventional raw materials for the production of innovative couscous

Nowaday, a major challenge for cereal technologists is to produce traditional foods, like couscous, pasta, etc, using non conventional raw materials. In this regards, barley (*Hordeum vulgare* L.) is certainly one of the most suitable cereal for the development of functional foods, as it is characterized by the precence of many bioactive compounds, such as dietary fibre, β -glucans, arabinoxylans, phenolic compounds, tocols and others (Panfili et al., 2008), which are recognized for their important physiological properties linked to the ingestion of soluble dietary fibre and to the effects linked to the

reduction of glucose and cholesterol levels in the blood with consequent reduction in the risk of cardiovascular diseases.

β -glucan are a type of soluble dietary fibre located in the endosperm cell wall of cereal grains. Chemically they are high molecular weight polysaccharides formed by linear chains of glucose linked by β -1-3 and β -1-4 type glycosidic bonds and they play an important role for human health due to their capacity to lower plasma cholesterol, improve lipid metabolism, and reduce the glycaemic index (Wood, 2007). In this regard, already in 1997, the US Food and Drug Administration had set values for β -glucan contents (0.75 g/portion and 3 g/day) to allow foods to bear health claims on lowered risk of coronary heart diseases (FDA, 1997). Phenolic compounds are characterized from one or more aromatic rings with one or more hydroxyl groups and they can be found free as well as bound to fibre. Free phenolic fraction of barley is constituted especially by flavanols (Gangopadhyay et al., 2016), while phenolic acids are the main compounds of the bound phenolic fraction (Abdel-Aal et al., 2012). A particular class of phenolic compounds is represented by alkylresorcinols which are one of the major groups of phenolic compounds in wheat, rye and barley grains. The interest towards these compounds is due to their antioxidant, antimicrobial and antifungal properties, and their effects on biological membranes due to their amphiphilic nature as well as anticancer activity.

3. Experimental procedure

This work is structured in three main experimental activities: E1) use of air classification system for the production of barley flour enriched in β -glucan; E2) production of barley based couscous, through traditional process, their complete characterization and evaluation of thermal treatment effects; E3) evaluation of bioactive compound of couscous samples and assessment of cooking quality.

4. Materials and methods

The waxy, hullless barley grain cultivar, CDC Alamo (β -glucan 7.6% d.m.), was grown in experimental fields of the University of Molise.

For the production of barley flour enriched in β -glucan, the kernels were milled by a model 8/B (Beccaria S.r.L. Italy) and micronized by a model TMX-500 (100–200 kg/h, Separ Micro System, Brescia, Italy). The obtained flour was then subject to air classification process (Messia et al., 2019) through turbo-separating system (Separ Micro System s.a.s. BS, Italy) resulting in two fractions which were defined as coarse and fine fractions, with regard to the dimension of the flour particles (Figure 1). Micronized barley and fractions were characterized.

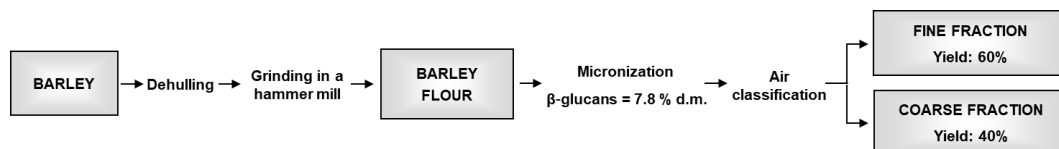


Figure 1 Flowchart of the air classification process

Couscous was produced using the handmade traditional procedure. Durum wheat semolina or mixtures of semolina and barley coarse fraction were moistened with water (about 40%) in a large wooden dish, stirred by hand and rubbed with the palm of the hand until granules are formed. The granules were then compressed between the hand and the container, and handled for fifteen minutes. Successively, the granules were calibrated using a metal sieve and stem cooked for ten minutes. The couscous was then placed into a dish, further handled and steamed again. This procedure was carried out

three times. After cooking, the couscous grains were dried using a static dryer (Namad Impianti, Roma, Italy) set at low temperature (30 °C for 24 h) (Messia et al., 2019).

Obtained couscous were analysed in terms of particle size evaluation, (stainless steel certified sieves with mesh sizes ranging from 2.36 mm to 0.85 mm), colour analysis (reflectance colorimeter CR200 Chromameter, Minolta, Japan) and bulk density (Debbouz & Donnelly, 1996). On couscous sample, proximate composition was evaluated and further chemical analyses have also been made: dietary fibre (Prosky et al., 1988); total β -glucans (K-BGLU assay kit, Megazyme, Ireland); total and gelatinized starch (K-TSTA and K-SDAM spectrophotometric assay kits, Megazyme, Ireland); furosine (Resmini et al., 1990); phenolic compound (Verardo et al., 2018); alkylresorcinols (ethyl acetate extraction and quantification by fluorescence). Finally, cooking quality assessment was carried out. Specifically, Cooking Loss (CL) and Swelling (Ounane et al., 2006), Water Absorption Index (WAI) and Water Solubility Index (WSI) (Oliveira et al. 2015) have been determined.

Statistical analysis was carried out using IBM SPSS Statistics 23. The data reported for all parameters are the average values of three different results. Analysis of variance was performed to determine significant differences (LSD test $*P \leq 0.05$) between means.

5. Results and Discussion

Analyses of barley fractions showed that coarse fraction had high total dietary fibre (25.5% d.w.) and β -glucans (14.4% d.w.) contents and a lower total starch content (51.8% d.w.) compared to semolina and fine fraction, thus being a functional ingredient with high amounts of bioactive compounds and a reduced caloric intake. For this reason, and on the basis of preliminary studies, two different percentage of barley coarse fraction were selected for the production of functional couscous (70/30 CC, couscous made with 70% semolina and 30% barley coarse fraction; 80/20 CC, couscous made with 80% semolina and 20% barley coarse fraction) and compared to control couscous 100% semolina (CTR CC). The chosen ratios (70/30 and 80/20) facilitated product functionality with a minimum replacement of semolina.

5.1 Physical characteristics of couscous samples

The results obtained from sieving tests shows that the couscous produced experimentally had a granulometry of 1400 μ m - 850 μ m, resulting medium grain-type couscous (Codex Standard, 1995) (Oriente, 2018). Regarding colour analysis, values of L^* (+49.01), a^* (-2.0) and b^* (+22.50) of control couscous were in the range reported by Guezlane et al. (1998) and Debbouz & Donnelly (1996), while functional couscous (70/30 CC and 80/20 CC) was darker than semolina couscous. This outcome does not penalize this type of product since the consumer is already accustomed to identify fibre-rich products by darker coloured products. Moreover, bulk density values for the functional couscous (0.56 and 0.60 g/cm³ for 70/30 CC and 80/20 CC, respectively) were lower than the values obtained for control couscous made with 100% semolina (0.69 g/cm³) (Oriente, 2018).

5.2 Functional couscous characterization and evaluation of thermal treatment effects

Regarding proximate composition, the presence of barley-enriched fraction in barley based couscous resulted in an increase in ash and fat content, compared to

semolina couscous, while no significant differences were found in the protein content of both functional couscous and CTR CC (Messia et al., 2019).

Table 1 shows β -glucan and dietary fibre content of couscous samples. Compared to CTR CC, barley based couscous had high levels of total dietary fibre (9.8 and 7.4% for 70/30 CC and 80/20 CC, respectively) and β -glucan content (4.09 and 2.79% for 70/30 CC and 80/20 CC, respectively). According to Reg. UE 432/2012 the amount of β -glucan found in functional couscous permits the assertion of the health claim, "*beta-glucan contributes to the maintenance of normal cholesterol levels in the blood*", as the innovative products contain at least 1 g of β -glucan from barley per quantified portion (50 g). Moreover, β -glucan content of both barley based couscous, did not change after cooking. In table 1 the evaluation of thermal treatment effects, on couscous sample, is also reported. In this regards, functional couscous shows gelatinized starch values in the range 51-60% and therefore comparable with what is reported by Debbouz & Donnelly (1996) which indicates values of gelatinized starch of 60% as a good compromise to have couscous with good water absorption, high absorption capacity and good consistency. In addition, functional couscous and control couscous shows furosine value (24.2, 25.1 and 29.0 mg/100 g protein for 70/30 CC, 80/20 CC and CTR CC, respectively) significantly lower than the average value found in commercial couscous (79.2 mg/100 g protein) (Messia et al., 2019), highlighting a reduced development of Maillard's reaction due to a milder heat treatment than those generally used in industrial plants (55°C for 17 h or 95°C for 3h).

Table 1 β -glucan and dietary fibre content of couscous samples and evaluation of thermal treatment effects (g/100 g as is)

Sample	β-glucan		Dietary fibre	Gelatinized starch	Furosine (mg/100 g protein)
	per serving (50g)				
70/30 CC	4.09 ± 0.071 ^a	2.05	9.8 ± 0.24 ^a	51.2 ± 0.75 ^a	24.2 ± 0.88 ^a
80/20 CC	2.79 ± 0.078 ^b	1.40	7.4 ± 0.81 ^b	53.7 ± 0.72 ^b	25.1 ± 0.24 ^a
CTR CC	0.18 ± 0.071 ^c	0.09	3.6 ± 0.11 ^c	59.9 ± 0.01 ^c	29.0 ± 0.41 ^b

70/30 CC, couscous made with 70% semolina and 30% barley coarse fraction; 80/20 CC, couscous made with 80% semolina and 20% barley coarse fraction; CTR CC, control couscous made with 100% semolina

Different superscript letters within a column indicate statistically significant differences at $p < 0.05$, by ANOVA LSD

5.3 Determination of free and bound phenolic compounds and alkylresorcinols in couscous samples

In Table 2 is reported the total free and bound phenolic content of couscous sample. A total of thirty-five free phenolic compound were identified in couscous sample but only twelve in CTR CC, this means that the remaining twenty-three compounds derive exclusively from barley. Overall the final amount of free phenolic compound is 361 μ g/100g for CTR CC and, this amount, increase significantly when enriched barley flour is added reaching value of 2444 μ g/100g for 80/20 CC and 3024 μ g/100g for 70/30 CC with an increase of more than six and eight times respectively compared to CTR CC. Specifically, this increase is due to the barley contribution of flavonoids, such as flavan-3-ols, that represent the 74% and the 80% of total free phenolic compound for 80/20 CC and 70/30 CC respectively.

Also twenty-nine bound phenolic compound were found in semolina and barley based couscous with relevance of phenolic acid that represent, in all samples, more than 99% of bound phenolic compound (Table 2), in accordance to Martini et al. (2017) that reported higher concentration of bound phenolic acids in micronized kernels respect

semolina obtained from traditional milling process although the micronization preserves the global natural endowment of total phenolic compound.

Overall, the total amount of bound phenolic compound is 5282 µg/100g for CTR CC, and this amount, increases proportionally with the partial substitution of semolina with barley coarse fraction reaching value of 19599 µg/100g and 27140 µg/100g for 80/20 and 70/30 couscous respectively.

Table 2 Content of total free and bound phenolic compound and relative classes in couscous samples (µg/100g as is)

CLASS OF PHENOLIC COMPOUND	Free phenolic compounds			Bound phenolic compounds		
	CTR CC	80/20 CC	70/30 CC	CTR CC	80/20 CC	70/30 CC
Flavan-3-ols	n.d.	1812.6 ± 41.3	2435.5 ± 5.7	n.d.	11.7 ± 1.5	54.8 ± 0.01
Phenolic acids	273.9 ± 2.4	516.4 ± 18	464.0 ± 5.1	5281.2 ± 146	19586.3 ± 783.1	27084.8 ± 1619.3
Flavones	87.5 ± 4.5	115.3 ± 2.7	125.5 ± 0.3	1.11 ± 0.23	1.02 ± 0.09	0.37 ± 0.08
Total	361 ± 7	2444 ± 21	3024 ± 10	5282 ± 146	19599 ± 782	27140 ± 1619

CTR CC, control couscous made with 100% semolina; 80/20 CC, couscous made with 80% semolina and 20% barley coarse fraction; 70/30 CC, couscous made with 70% semolina and 30% barley coarse fraction

n.d.: not detected

In Table 3 is reported the concentration of alkylresorcinols in samples of analysed couscous. The obtained results show that, among alkylresorcinols, C23:0 and C25:0 increase slightly with the use of higher percentage of barley flour while, C19:0 and C21:0 do not show great difference between the two barley based couscous although their content is higher than the control. Overall the content of alkylresorcinols increased from 0.92 mg/g for CTR CC to 2.25 mg/g for 70/30 CC and this is probably due to the fact that the barley fraction used, it has not been subjected to excessive refining processes and still retains part of the external layer of kernel confirming what reported by Giambanelli et al. (2018).

Table 3 Content of alkylresorcinols compound in couscous sample (mg/g as is)

ALKYLRESORCINOL COMPOUND	CTR CC	80/20 CC	70/30 CC
C19:0	0.46 ± 0.04 ^a	0.60 ± 0.03 ^b	0.548 ± 0.006 ^{a,b}
C21:1	0.049 ± 0.004 ^a	0.0916 ± 0.0006 ^b	0.0787 ± 0.0002 ^c
C21:0	0.30 ± 0.01 ^a	0.481 ± 0.008 ^b	0.49 ± 0.02 ^b
C23:0	0.111 ± 0.002 ^a	0.227 ± 0.002 ^b	0.254 ± 0.007 ^c
C25:0	L.O.Q.	0.550 ± 0.007 ^a	0.88 ± 0.03 ^b
Total	0.92 ± 0.05	1.95 ± 0.01	2.25 ± 0.05

CTR CC, control couscous made with 100% semolina; 80/20 CC, couscous made with 80% semolina and 20% barley coarse fraction; 70/30 CC, couscous made with 70% semolina and 30% barley coarse fraction

Different superscript letters within a line indicate statistically significant differences at p<0.05, by ANOVA LSD;

L.O.Q. limit of quantification

5.4 Cooking quality assessment

About physicochemical-cooking properties, the various samples did not show great differences preserving the quality of the cooked product (Table 4). In detail, the obtained results show, for barley based couscous, higher values of Water Absorption Index (WAI) and Swelling than CTR CC, denoting the good hydration capacity of cooked functional couscous. This feature is due to the presence of β-glucans, which has a well-established capacity for water absorption (Ahmed & Thomas, 2015). Regarding Cooking Loss (CL), it is a characteristic related to the cooking behaviour of dry couscous and, generally, low values of this parameter are indicative of a high quality product. In this

study, slightly higher values of cooking loss were found in 70/30 CC and 80/20 CC compared to CTR CC but always in line with what was indicated by Demir et al., (2010) to obtain good quality couscous. Finally, the Water Solubility Index (WSI), which expresses the extent of couscous disintegration during water absorption, was in the range of 4–16%, which has been indicated by Abecassis et al. (2012) as classical values for a semolina couscous.

Table 4 *Cooking quality of functional and control couscous*

Sample	Cooking Loss CL (%)	Water Absorption Index WAI (g/100 g)	Water Solubility index WSI (%)	Swelling (mL/100g)
70/30 CC	17.2 ± 1.14	489.0 ± 4.11	5.0 ± 0.08	478 ± 5.35
80/20 CC	16.8 ± 0.56	497.9 ± 3.01	4.3 ± 0.02	489.7 ± 11.13
CTR CC	11.5 ± 0.40	415.3 ± 6.48	3.0 ± 0.16	419.8 ± 10.0

70/30 CC, couscous made with 70% semolina and 30% barley coarse fraction; 80/20 CC, couscous made with 80% semolina and 20% barley coarse fraction; CTR CC, control couscous made with 100% semolina

6. Conclusions and Future Perspective

The produced couscous shows quantities of fibre (at least 6 g/100g) and β -glucans (at least 1 g per served portion) that are compatible with the provisions of the EC Reg. 1924/2006 and the EU Reg. 432/2012, fulfilling the nutritional and health claims related to bioactive compounds. Moreover, the final product result to be enriched also in free and bound phenolic compounds and alkylresorcinols, permitting to introduce, in the diet, different compounds useful for human health to help prevention of chronic diseases related to oxidative stress.

In conclusion, air classification can be considered a useful green technology to obtain new ingredient for the production of functional foods, in fact, the innovative couscous, produced with different percentage of barley coarse fraction, can be considered functional products with improved nutritional value and good cooking qualities that preserve simplicity and versatility typical of traditional couscous.

Therefore, the innovative couscous can be considered a possible successful product on the market with economic advantages for the companies that would produce and commercialize functional couscous that preserve all the characteristic typical of a classical commercial couscous but with a higher content of bioactive compounds.

On the basis of what has been said so far, it is advisable to extend the concept of functional food to that of functional diet, in order to promote the intake of different types of food (couscous, pasta, biscuits, bread) characterized by significant amounts of bioactive compounds. Only in this way is possible to reach, during the different occasions of food intake (breakfast, lunch, snack, dinner), the three grams / day of beta-glucans necessary to have the desired physiological effects (Cubadda & Marconi, 2008; Bruno & Marconi, 2012).

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Advanced Solutions for Authenticity and Quality of Virgin Olive Oils

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This PhD project aims to develop and improve analytical solutions for the control of quality and authenticity of virgin olive oils (VOOs). The activities developed concerned *i)* the study of new analytical approaches and the revision, with in-house validation, of the official method for the determination of ethyl esters fatty acids to detect blends of extra virgin olive oils with lower quality oils, *ii)* the application of a Flash-GC Electronic Nose for the discrimination of VOOs with a different geographical origin, *iii)* the development and in-house validation of a rapid electrochemical method for the determination of the free acidity.

Soluzioni avanzate per la qualità e la genuinità degli oli di oliva vergini

Questo progetto di dottorato mira allo sviluppo e miglioramento di soluzioni analitiche per il controllo della qualità e genuinità dell'olio d'oliva vergine. Le attività hanno riguardato *i)* lo studio di nuovi approcci analitici e la revisione, con validazione in-house, del metodo ufficiale di determinazione degli etil esteri degli acidi grassi per l'individuazione di miscele di oli extra vergini di oliva con oli di qualità inferiore, *ii)* l'applicazione di un Flash-GC Electronic Nose per la discriminazione di oli d'oliva vergini con diversa origine geografica, *iii)* la messa a punto e validazione in-house di un metodo rapido per la determinazione dell'acidità libera.

Key words: Virgin olive oil, fatty acid ethyl esters, soft-deodorization, geographical origin, quality, free acidity.

1. Introduction

In accordance with the PhD thesis project previously described (Palagano, 2018), this oral communication reports the main results of the following activities:

A1) development and in-house validation of an HPLC-UV-Vis/GC-FID (with a PTV injector) method for the fatty acids ethyl esters (FAEEs) determination, revising both the preparative step and the GC injector required by the official method;

A2) study of new analytical approaches (GC-IMS, difference between experimental and theoretical content of diglycerides (DAGs)) to detect blends of extra virgin olive oils with lower quality oils;

A3) application of a FGC E-Nose and of a chemometric data elaboration for a rapid screening of virgin olive oils characterized by a different geographical origin;

A4) development and in-house validation of a portable system for the evaluation of the free acidity in VOOs.

All the activities of this PhD project are developed in the context of the project OLEUM “Advanced solutions for assuring authenticity and quality of olive oil at global scale”, funded by the European Commission within the Horizon 2020 Programme (2014–2020, GA no. 635690).

2. State of the art

Considering the world production of vegetable oils, olive oil is only at the ninth place with about 3 million metric tons produced during the 2018/19 harvest (USDA, 2019). Nevertheless, virgin olive oil is a product with a high commercial value, mainly linked to its chemical composition and sensory characteristics, and this makes the control of its quality a very complex and evolving issue (Clodoveo et al., 2014).

The chemical and sensory characteristics that have to be evaluated for the control of quality and authenticity of VOOs are defined by the European Union (CEE Reg. 2568/91 and following amendments) that establishes both the analytical method to determine them and the legal limits for the definition of the commercial category. However, despite the EU legislation is among the most advanced in the field, the category “fats and oils”, that includes also olive oil, is at the third place in the classification of food categories according to the number of fraud cases detected (EU Food Fraud Report, 2016). For this reason, the EU legislation, among the most advanced in the field, continuously chases after the emerging frauds. The process of proposing new methods or reviewing those current is constantly in progress, to ensure the robustness and the clarity required by official standardized procedures (Conte et al., 2019).

3. Experimental Procedure

In this PhD thesis different experimental procedure have been followed according to the different specific objectives of the project. Concerning the activities (A1) and (A4), about the development of a revised method for the FAEs determination and for the rapid measure of the free acidity, in the first step different tests were carried out in order to define the best analytical conditions to be applied. Then, both methods have been in-house validated evaluating those parameters that are generally considered to study the performance of an analytical method. About the activities (A2) and (A3), existing methods (GC-IMS, DAGs determination and FGC E-Nose) have been applied but following approaches not investigated until now.

4. Materials and Methods

4.1 Development and in-house validation of a method for FAEs determination

The scheme and the analytical conditions of the method developed for the FAEs determination is presented in Figure 1.

1. SAMPLE PREPARATION	2. HPLC INJECTION	3. FAEs RECOVERY	4. GC-FID ANALYSIS
➤ 75 mg of oil ➤ 0.5 mL of IS solution (methyl heptadecanoate 0.01% in n-heptane) ➤ 0.75 mL of Sudan I solution (0,00025% in n-hexane) ↓ Filtration (polyamide filters 0,20 µm)	Column: silica (l 25 cm; id 4.6 mm; ps 5 µm) Mobile phase: n-hexane/MTBE (95/5 v/v) in isocratic mode Flow: 1 mL/min UV-Vis detector: 460 nm Injection volume: 100 µL Analysis time: 30 min.	➤ Collection of the FAEs fraction (till the Sudan elution) ➤ Dryness under nitrogen ➤ Addition of 300 µL of n-heptane	Column: TG-5SILMS (l 15 m, id 0.32 mm, ft 0.25 µm) Gas carrier: helium, 1 mL/min Injection volume: 1 µL, splitless mode PTV temperature: 70 °C; sample transfer: 3 °C/s → 300 °C (1 min) Oven temperatures: 80 °C (1 min), 20 °C/min → 140 °C, 5 °C/min → 335 °C (20 min) FID temperature: 340 °C

Figure 1 Scheme and analytical conditions of the method for the FAEs determination.

The peaks identification was realized injecting directly in GC-FID a solution of a mix of standards. The quantification step was carried out according to the EU Reg. 61/2011. The parameters considered for the in-house validation of the method were: linearity, limit of detection (LOD) and quantification (LOQ), recovery, robustness,

accuracy and precision intra-day and inter-day. For the evaluation of these parameters (except for accuracy and intra-day precision), samples were prepared following the steps described before but using refined olive oil and replacing the IS solution with 0.50 mL of mix of standards solution in *n*-hexane. To study the accuracy and the intra-day precision of the revised method, 3 VOOs with a different FAEs content (low, medium, high) were analyzed by both the official method and the revised one.

4.2 Study of new analytical approaches to detect blends of extra virgin olive oils with lower quality oils

A set of samples composed by 2 extra virgin olive oils (EVOO), 16 defective olive oils (DOO) before and after the soft-deodorization (SDOO) and 60 blends of them were analyzed by Flavourspec[®], a gas chromatograph equipped with an ion-mobility detector (GC-IMS). The procedure consists in analyzing the headspace of a sealed sample (5 g of oils weighted in a 20 mL vial, heated at 60 °C for 1 hour) in order to evaluate its volatile compounds that are pre-separated by GC, then inserted in the atmospheric ionization region and separated depending on the fact that ions travel at atmospheric pressure versus a flow of inert drift gas (N₂) and need to pass a fixed distance (drift tube) in a defined electric field. The following conditions have been applied: column with a stationary phase 5% phenyl polysiloxane (l. 30 m, i.d. 0.32 mm, f.t. 0.25 µm); carrier gas (N₂) flow at 1 mL/min from the beginning to 2 min, then increases to 10 mL/min within 15 min and remains constant till the end (30 min); syringe and injection temperature: 80 °C; column temperature: 40 °C; detector temperature: 45 °C.

On the same set of oils, also the free acidity (determined according to the European regulation) and diglycerides (DAGs) determination were carried out in order to evaluate the relation between these two parameters in this kind of oils. The total content of diglycerides (DAG_{exp}) was determined by SPE (diol phase)/GC-FID, the theoretical amount (DAG_{theo}) was mathematically calculated from the free acidity value.

4.3 Application of a Flash GC-E-Nose for the discrimination of VOOs with a different geographical origin

A set of 174 VOOs were collected and analysed (Table 1).

Table 1 Number of samples for each category considered and detail of their geographical origin. EU: oils from countries members of European Union; Extra-EU: oils from countries outside the European Union; Blends: oils obtained mixing EU oils or EU and extra-EU oils.

Category	Tot.	Origin
EU	116	29 Spain, 28 Italy, 20 Croatia, 16 Greece, 12 Portugal, 11 Slovenia
Extra-EU	40	21 Turkey, 15 Morocco, 3 Tunisia, 1 Chile
Blends	18	12 EU blends, 6 EU/Extra-EU blends

Each sample was analyzed by the FGC E-Nose Heracles II applying the conditions previously described (Palagano, 2018). About the chemometric elaboration of results with a non-targeted approach, after a data pre-treatment, a PLS-DA model was built and validated (cross and external validation).

4.4 Development and in-house validation of a portable system for the free acidity (FA) determination in VOOs

The working principle of the portable system is based on the measure of the electrical conductance of an emulsion between a hydroalcoholic solution and the oil sample. A 50 mL Falcon tube, modified to feature a couple of cap-shaped stainless-steel electrodes (6 mm in diameter, spaced by 12 mm one from the other) required for the electrical characterization, is used as sensor to realize the measurement. The first step of the analysis is a “reagent test” to check the electrical properties of the hydroalcoholic solution to avoid an overestimation of the oil free acidity linked to the conductance of the solution. For this purpose, the tube is filled with 9 mL of ethanol and 6 mL of distilled water and its conductance is measured. If the reagent passes the test, 1 mL of the oil to be tested is added and the mixture is manually shaken vigorously for about 20 sec to create an emulsion, whose conductance is measured to estimate the oil FA. The instrument is equipped with a temperature sensor to measure the room environmental temperature during the analysis. The emulsion electrical conductance and the temperature are measured, and these values are used to calculate the emulsion conductance at the calibration temperature (23.5 °C). Then the free acidity of the sample is estimated using the calibration function stored inside the microcontroller non-volatile memory. In order to ensure the method quality, the following parameters were evaluated as described in Grossi et al., 2019: LOD and LOQ, intra-day and inter-day precision and accuracy. Moreover, before the application of the portable system on olive oil samples and in order to verify the correlation between the concentration of the analyte and the response of the instrument, the system was calibrated (Grossi et al., 2019). The determination of free acidity by titration was applied according to the European regulation (CEE Reg. 2568/91 and following amendments).

5. Results and Discussion

5.1 Development and in-house validation of a method for FAEs determination

A revised protocol for the FAEs determination in virgin olive oils was studied. The method is based on the application of an HPLC-UV-Vis as alternative for the traditional liquid chromatography applied in the preparative phase for the FAEs determination, and the use of a PTV injector as an alternative to the OC one that is the GC injector required by the official method. The analytical conditions (Figure 1) applied were chosen in order to apply the same analytical principle of the official method: normal phase liquid chromatography in isocratic mode. As in the official method, also in this case the Sudan I was used as dye to visually check the right elution of the fraction containing FAEs and waxes since it is characterized by an elution time intermediate between waxes and triglycerides. About the in-house validation of the method, results of linearity (considering a range between ~ 2.5 and 50 ppm) and recovery are reported in Table 2.

Table 2 Values of linearity (expressed as R^2 between the area values and the concentrations of each compound considering a mix solution at 5 different concentrations between 2.5 and 50 ppm). Recovery percentages for FAEs and methyl heptadecanoate measured at 3 different concentrations (approximately 10 – 25 – 50 ppm).

Compound	Linearity (R^2)	Recov. at 10 ppm (%)	Recov. at 25 ppm (%)	Recov. at 50 ppm (%)
Ethyl palmitate	0.97	70.7	95.3	89.0
Ethyl linoleate	0.97	67.4	88.0	84.4

Ethyl oleate	0.97	71.0	90.0	87.3
Ethyl stearate	0.98	82.9	102.5	93.3
Methyl heptadecanoate	0.97	72.6	97.6	86.8

LOD and LOQ values for each FAEES examined were lower than 1 mg/kg and 1.5 mg/kg, respectively. About the robustness of the method, the standards solution at 25 ppm was injected in HPLC at two different mobile phase flow (1 mL/min and 0.7 mL/min) and no significant differences between the two methods (Student's test, $p \leq 0.05$) were evidenced. To evaluate the inter-day precision the sample prepared with the solution of mix of standards at 25 ppm was analyzed in three different days and, comparing the areas of each compound in the different days, no significant differences (One-way ANOVA, $p \leq 0.05$) were found. Concerning the accuracy, the total content of FAEES determined in 3 virgin olive oil with a different FAEES content (low, medium, high) by both the official method and the revised one was compared and no significant differences (Student's test, $p \leq 0.05$) were found (Table 3). Finally, the intra-day precision was expressed as RSD and all the values obtained were lower than the 15%.

Table 3. Mean value and standard deviation of the content of fatty acids ethyl esters.

Sample	LC – Low content		MC – Medium content		HC – High content	
	Off. met.	Rev. met.	Off. met.	Rev. met.	Off. met.	Rev. met.
Total FAEES (mg/kg)	18.4 ± 1.1	17.8 ± 1.0	39.8 ± 0.7	38.7 ± 0.4	82.1 ± 2.8	85.1 ± 0.5

4.2 Study of new analytical approaches to detect blends of extra virgin olive oils with lower quality oils

About the application of a GC-IMS, for each sample a topographic plot was obtained: a tri-dimensional map in which Y axis represents the retention time in the chromatographic column (in seconds), X axis represents the drift time in the drift tube (in milliseconds), and Z axis the intensity value (in V) of each compound that results as a spot of different dimension depending on its concentration. A visual comparison of the spectra obtained for all the samples allowed to identify specific spots (compounds) than can be useful to discriminate the oils according to their quality. In general, spectra of defective olive oils were richer in signals than spectra of the respective soft-deodorized samples. A total of 25 spots were selected: some compounds were detected in all the samples EVOO, DOO, SDOO, even if with a different intensity, and others were detected mainly in defective OOs. Subsequently, considering the area values (calculated by the software) of the 25 spots selected a PCA with the extra virgin olive oils, the SDOO and the blends was realized (explained variance PC1-PC2 60%). A good separation of the samples according to their categories was evidenced and a satisfactory distribution of the blends respects to the EVOO has been obtained. Some preliminary results showed that the difference between the DAG_{exp} and the theoretical DAGs content is over 0 in the soft-deodorized samples and close to 0 or negative in the extra virgin olive oils.

5.3 Application of a Flash GC-E-Nose for the discrimination of VOOs with a different geographical origin

All the 174 VOOs sampled were analysed by the FGC E-Nose technique. The approach applied for the data elaboration allowed to build a satisfactory chemometric model able to discriminate the samples according to their geographical origin. The percentages values of the sample correctly classified for each category, applying two different validation process, are presented in Table 4.

The cross validation was realized considering the 75% of the sample for each category (casually sorted), instead of the external validation carried out on the remaining 25% of samples not used for building the chemometric model.

Table 4. Mean values (of 5 replicates) of the percentages of sample correctly classified for each category applying the cross validation and the external validation.

	Cross validation	External validation
EU	86% (~ 87/101)	80% (~ 26/33)
Extra EU	98.5% (~ 29/30)	92.5% (~ 9/10)
Tot.	89% (~ 116/131)	84% (~ 35/43)

5.4 Validation of an electrochemical method for the free acidity determination

A portable battery-operated electronic system for in-situ measurements of olive oil free acidity has been built to allow olive oil quality assurance directly in the oil mills or packaging centers. The dimensions of the instrument are 11x15x5 cm and can be powered by USB port or using batteries (3 AAA alkaline batteries 1.5 V) (Figure 2A).

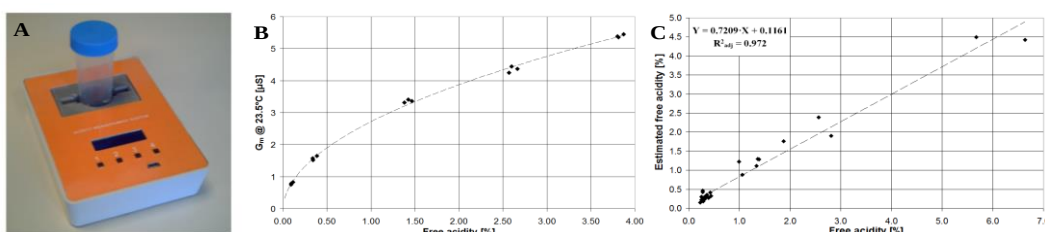


Figure 2 Pictures of the electronic system and the sensor for olive oil FA analysis (A); measured electrical conductance (G_m) at 23.5 °C vs FA for the calibration set (B); scatter plot of the estimated FA vs FS measured by titration for a set of 30 olive oil samples (C).

To calibrate the system, samples of refined sunflower oil added with different concentrations of oleic acid were analyzed by both the official method to determine the reference value of FA and by the portable system to measure the conductance value. Then the values were plotted (Figure 2B): in all cases the electrical conductance measured for the samples increases with its free acidity with a non-linear relation. The values obtained for LOD and LOQ were 0.02% and 0.07%, respectively. Next, a set of 30 olive oil samples with a different quality grade were analyzed in triplicate applying this new analytical approach in order to study its accuracy and intra-day precision. The portable instrument showed good precision results since all the obtained values were under the 15%. All the samples were also analyzed following the official method and the comparison between the values obtained by the two methods did not give statistically differences for the mean values. Moreover, the regression between the two series of values provided a coefficient R^2 of 0.97 (Figure 2C). Finally, considering the inter-day precision of the instrument, no significative differences (Student's Test, $p \leq 0.05$) were found among the results obtained for each analyzed sample in three different days.

6. Conclusions and Future Perspectives

A1) A revised method for the FAEs determination was developed and in-house validated with satisfactory results. The method, based on the application of an HPLC-UV-Vis/GC-FID (with a PTV injector), allows to realize the analysis in less time and with a

lower amount of solvents than the official one. An inter-lab validation of this method, involving several laboratories as participants, is expected to start very soon in order to confirm and strengthen the reliability and the good performances of the herein presented approach, in the view of its proposal to the normative bodies for its possible adoption.

A2) The new analytical approaches tested to detect blends of extra virgin olive oils with lower quality oils showed encouraging results representing promising approaches to discriminate this kind of oils. More samples need to be analysed to verify the effectiveness of these approaches.

A3) The application of a FGC E-Nose with a chemometric approach for the data elaboration represents a promising tool for the rapid screening of virgin olive oils characterized by a different geographical origin. Other samples collected from extra-EU countries and their blends with EU oils will be analyzed in order to improve the robustness of the model.

A4) A portable battery-operated electronic system suitable for in-situ measurements of olive oil free acidity was developed and in-house validated. The data obtained showed good performances. The procedure to realize the analysis is very quick and easy, this make the system suitable also for people without specific training. A next study will be focused on the evaluation of the performance of this portable system when applied to bottles stored for several months in dark/light conditions simulating possible different commercial conservation/exposure on shelves of groceries. This study will give indications regarding the maximum oxidation state of olive oil sample tolerable to use this portable system as accurate and reliable.

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The Production of an Innovative Ingredient Based on Leguminous Flour and Germinated Cereals for the Improvement of Baked Goods Obtained by Sourdough Technology

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This PhD thesis research project is aimed at setting up ingredients obtained from alternative vegetable matrices, using biotechnological protocols based on grains germination and fermentation with selected lactic acid bacteria, to develop baked goods with an improved sensory and functional profile.

Produzione di un ingrediente innovativo a base di farine di legumi e cereali germinati per il miglioramento dei prodotti da forno a lievitazione naturale

Questo progetto di tesi di dottorato prevede lo studio e la messa a punto di prodotti lievitati da forno con un migliorato profilo sensoriale e funzionale attraverso l'utilizzo di miglioratori, ovvero ingredienti ottenuti da matrici vegetali alternative, mediante protocolli biotecnologici basati sulla germinazione della granella e sulla fermentazione con batteri lattici selezionati.

Key words: germination, fermentation, lactic acid bacteria, sourdough, food improvers

1. Introduction

In accordance with the PhD thesis project previously described (Perri, 2017), this oral communication reports the main results of the following three activities directed to:

A1.1. Selection of raw materials (cereals, legumes and pseudo-cereals) and set up of protocols for grains germination;

A1.2. Study of the effect of germination on cultivable microbiota and microbioma of flours;

A1.3. Set up of biotechnological protocols on laboratory, pilot and industrial scale to obtain improvers and new baked goods.

2. Novel Insights on the Cereals, Legumes and Pseudo-cereals Fermentation

Cereals represent the most important food resources on the planet and wheat is the main constituent of the Mediterranean diet. It provides high amounts of carbohydrates but limited amounts of essential amino acids, fibers and minerals. Recently, there is a great interest of the scientific community, producers and consumers to use alternative vegetable matrices, to fortify and enrich the cereals products. Despite the high biological value of proteins, content of fibers, minerals and polyphenols, the use of pseudocereals and legumes in the food sector, is limited due to technological reasons and owing to the presence of anti-nutritional factors. The aim of this project is to obtain baked goods with an improved sensory and functional technological profile using innovative ingredients, obtained from alternative vegetable matrices, using biotechnological protocols based on grains germination and fermentation with selected lactic bacteria, developed between

University of Bari and University of Helsinki and then transferred on a pilot scale before, and industrial scale after near Puratos company.

3. Experimental Procedure

In this PhD thesis a six-step experimental procedure was set up by performing in sequence the following independent phases, that are: (i) selection of alternative vegetable matrices to be used to obtain the improvers; (ii) set up of experimental protocols for grain germination; (iii) culture-dependent and –independent microbiological characterization of flours obtained from selected whole or germinated grains; (iv) selection of lactic acid bacteria starters to be used for the fermentation of selected vegetable matrices; (v) set up of biotechnological protocols to produce new ingredients and new baked goods on laboratory scale (University of Helsinki); (vi) set up of biotechnological protocols to produce new ingredients and new baked goods on pilot and industrial scale (Puratos Italia, srl).

4. Materials and Methods

4.1 Sprouting process and flours obtaining

Sprouting process was carried out in according to Montemurro et al., 2019. Five different grains were selected for this study. Legumes (*Cicer arietinum* var. Pascià and *Lens culinaris*) and cereals (*Triticum durum* var. Simeto and *Hordeum vulgare* var. Nure) were purchased by Caporal Grani S.a.s. (Gravina di Puglia, Bari, Italy); *Chenopodium quinoa*, was supplied by Quinoa Marche Srls (Jesi, Ancona, Italy). The optimal time was chosen based on the speed of grains to achieve good rootlets length (ca. 3/4 of the medium seed length). After sprouting, the grains were carefully washed with distilled water (3×800ml) and dried in drying chambers (Binder GmbH, Tuttlingen, Germany) at 50°C for 25h. Flours were obtained from whole and sprouted grains by a laboratory mill Ika-Werke M20 (GMBH, and Co. KG, Staufen, Germany). After milling all the flours were sieved (mesh size 500µm) to remove the coarse fraction.

4.2 Cultivable microbiota, microbiome and community-level catabolic profile

Lactic acid bacteria enumeration was carried out in according to Minervini et al., (2012). Yeasts enumeration was carried out as as described in Di Cagno et al. (2014). Genomic DNA of lactic acid bacteria was extracted in according to Minervini et al. (2018). Three oligonucleotides, P4, P7, and M13, were used for biotyping lactic acid bacterium isolates. Reaction mixture and PCR conditions were those described by De Angelis et al. (2001) and Siragusa et al. (2009). DNA extraction, biotyping and identification of yeasts were carried out as described in Di Cagno et al. (2014). DNA extracted from each whole and sprouted grains was used as the template for Illumina MiSeq diversity analyses, which were carried out at the Research and Testing Laboratory (RTL; Lubbock, TX). Primers Firm350F and Firm814R were used to amplify a fragment of the 16S rRNA gene for analysis of diversity inside the phylum of Firmicutes, Tenericutes and Cyanobacteria (Minervini et al., 2015). Carbon source utilization patterns of microbial communities of flours obtained from selected whole and sprouted grains, were assessed by using BIOLOG 96-well Eco-Microplates (Biolog, Inc., Hayward, CA, USA) as reported in Siragusa et al. (2009).

4.3 Set up of biotechnological protocols to produce innovative improvers

Fifteen autochthonous and allochthonous strains of LAB, were tested for growth and acidification kinetics and ability to produce exopolysaccharides (EPS). Selected strains

at initial cell density of ca. 7 log cfu/g, were used to ferment doughs obtained by sprouted and whole grains, individually or in mix. Fermentations were carried out at 20°C, 25°C and 30°C for 24 h, dough yield 500 ($DY = [\text{flour weight} + \text{water weight}] \times 100 / \text{flour weight}$). Before and after fermentation process, LAB count, TTA and pH were detected (Yan xu et al., 2017). Viscosity values of doughs at different shear rates were measured directly at 20 °C before and after fermentation by a RheolabQC rheometer (Anton Paar, Graz, Austria) (Yan xu et al., 2017). Sourdoughs were analyzed for the quantification of *in situ* produced dextrans and for the analysis of mono and oligosaccharides before and after fermentation processes in according with Katina et al. (2009) and Yan xu et al. (2017).

4.4 Set up of biotechnological protocols to produce new baked goods and sensory profile analysis

Improvers were included in the recipe at 30% w/w during the preparation of final dough, to produce new baked goods containing semolina flour and baker's yeast, DY 160, fermented at 35 °C, U_r 75% for 50 minutes. At end of leavened process, doughs were mechanically shaped and divided into 250g blocks. The cooking was carried out using a rotating basket oven (Sveba Dahlen, Fristad, Sweden) at 220 °C for 15 minutes. Trials were carried out comparing experimental improver with one commercial puratos improver "sapore lucia". Bread obtained from only semolina was used as a control. Texture Profile Analysis (TPA) of bread crumbs was done with a texture analyzer (Stable Mycro Systems, Texture analysers) using a 25-mm diameter aluminum probe on days 1 and 6 of storage (Katina et al., 2009).

5 Results and Discussion

5.1 Sprouting process

Sprouting process was different among selected grains. Quinoa reached the good rootlets length in one day, while wheat, barley and lentil in three days. Chickpea completed this stage in five days. In all grains the germinability was higher than 90%. After the first year of activity, two selected matrices (lentil and barley) were further chosen, based on some important nutritional aspects, such as total amino acids and protein content and soluble dietary fiber and GABA for lentil and barley, respectively (data not shown). Developed sprouting protocols were able to reduce the amount of some anti-nutritional factors in lentil and barley flours such as *stachiose*, *verbascose* and *raffinose* (Table 1). According to the presented results, flours from sprouted cereals have better nutritional features than those from non-sprouted grains (Gobbetti et al., 2019).

5.2 Effect of sprouting on cultivable microbiota, microbiome and community-level catabolic profiles

Sprouting process caused an increase in the cellular density of the presumptive lactic acid bacteria, in almost all selected matrices, except for flours from sprouted wheat in which a significant ($P < 0.05$) decrease was observed on SDB and mMRS media in anaerobic condition. In all sprouted flours, presumptive LAB density was higher than 4 Log cfu/g (average value). The highest presumptive LAB density was observed for sprouted chickpea, 7.94 ± 0.18 log cfu/g on SDB medium in aerobic condition. For presumptive yeasts a significant decrease ($P < 0.05$) was observed in wheat after sprouting process from 5.33 ± 0.12 to 2.6 ± 0.06 Log cfu /g, while a significant increase ($P < 0.05$) was recorded for chickpea, as shown in (Table 2). Germination triggers α - and β -amylases, proteases and di-phenol-oxidases in grains, which break down starch, fibers, and proteins, which can explain the higher density of cultivable microbiota observed in sprouted flour (Gobbetti et al., 2019).

Table 1 Profile variation of mono-and oligosaccharides of flours obtained from lentil and barley grains before and after sprouting process (Gal, galactose; Glu, glucose; Suc, sucrose; Fru, Fructose; Mel, melibiose; Raf, raffinose; Sta, stachiose; Ver, Verbascose; Mal, maltose).

Flours	Gal	Glu	Suc	Fru	Mel	Raf	Sta	Ver	Mal
Whole lentil	0.96±0.02	nd	nd	nd	nd	0.40±0.02	2.2±0.1	1.7±0.02	nd
Sprouted lentil	1.66±0.07	nd	nd	nd	nd	0.42±0.02	nd	nd	nd
Whole barley	0.50±0	nd	nd	nd	nd	0.4±0.02	0.5±0.1	nd	0.7±0.02
Sprouted barley	1.3±0.03	nd	nd	nd	nd	nd	nd	nd	3.7±0.02

Table 2 Cell numbers (Log cfu/g) of lactic acid bacteria (LAB) and yeasts in flours from wheat (W), barley (B), chickpea (C), lentil (L) and quinoa (Q) grains before and after sprouting (S) process (A, Aerobic condition; An, Anaerobic condition).

	Medium		W	SW	B	SB	C	SC	L	SL	Q	SQ
LAB	MRS	A	3.5±0.0 ^a	6.1±0.1 ^b	3.1±0.0 ^f	5.1±0.1 ^c	3.2±0.0 ^f	7.4±0.1 ^a	5.1±0.1 ^c	6.0±0.1 ^b	5.1±0.1 ^c	4.5±0.1 ^d
	mMRS	An	2.7±0.0 ^a	2.2±0.0 ^b	3.2±0.0 ^f	4.3±0.1 ^e	4.6±0.1 ^d	7.9±0.1 ^a	5.2±0.1 ^c	6.2±0.1 ^b	5.2±0.1 ^c	4.4±0.1 ^e
	mMRS	A	4.8±0.1 ^f	5.8±0.1 ^d	4.2±0.0 ^g	6.4±0.1 ^c	4.2±0.0 ^g	7.6±0.1 ^a	6.2±0.1 ^{cd}	7.0±0.1 ^b	5.1±0.1 ^e	6.6±0.1 ^c
	SDB	An	2.8±0.0 ^f	2.4±0.0 ^g	3.2±0.0 ^g	4.4±0.1 ^d	4.5±0.1 ^e	7.9±0.1 ^a	5.1±0.1 ^d	6.1±0.1 ^b	5.6±0.1 ^c	4.5±0.1 ^e
	SDB	A	4.6±0.1 ^f	6.2±0.1 ^d	4.7±0.1 ^f	6.5±0.1 ^c	4.2±0.0 ^g	7.9±0.1 ^a	6.4±0.1 ^c	7.1±0.1 ^b	5.1±0.1 ^e	6.4±0.1 ^c
Yeasts	SDA		5.3±0.1 ^b	2.6±0.0 ^g	3.7±0.0 ^{de}	3.5±0.0 ^e	2.1±0.0 ^h	6.3±0.1 ^a	4.6±0.1 ^c	5.6±0.1 ^b	3.1±0.0 ^f	3.7±0.0 ^d

A total of 91 presumptive lactic acid bacteria (LAB) were isolated from whole and sprouted selected matrices. Isolates identified through culture-dependent methods almost reflected the lactic acid bacteria microbiota retrieved by total DNA analysis. Isolates belonged to *Enterococcus casseliflavus* (50%), *P. pentosaceus* (8.8%), *Weissella confusa* (2.2 %), *Enterococcus spp.* (38%) and *Leuconostoc mesenteroides* (1%). Yeasts belonged to *Clavispora lusitanae*. The total DNA extracted from whole and sprouted grains was amplified with specific primers and sequenced by NGS techniques. The analyses detect a significant ($P < 0.05$) difference in Phylum composition for both *Prokaryotic* and *Eukaryotic* populations. *Firmicutes* OTUs were the dominant prokaryotic phyla in almost all samples, except for whole lentil and wheat, in which *Cyanobacteria* and *Tenericutes* characterized the 82 and 64 % of total relative abundance, respectively. *Glomeromycota* and *Ascomycota* were the eukaryotic OTU at phylum level that dominated whole flours. Before sprouting process, the *Lactobacillales*, *Acholeplasmatales* and *Bacillales* orders were dominant in all samples except on lentil in which the *Oscillatorales* represented the OTU at the 82% of relative abundance. After sprouting process, only *Lactobacillales* and *Bacillales* OTUs were detected. Among all samples higher diversity of genera and species was found. OTU corresponding to *Candidatus* genera represented the 64 and 63%, respectively, of the relative abundance in whole wheat and barley. *Halospirulina* was the dominant OTU in whole lentil. *Enterococcus casseliflavus* and *E. mundtii* were species detected in all the samples. After sprouting process, *Lactococcus lactis* and *Peaenobacillus* became the OTUs dominant in wheat and quinoa respectively. No significant differences were observed for the relative abundance of the *Enterococcaceae* among samples after sprouting process. Whole wheat, and sprouted barley, contained a lower relative abundance of the *Alternaria* and *Fusarium* genera. The changes observed agreed with Landry et al. (2018). The community-level catabolic profiles (CLCPs), showed that sprouting process caused activation of microorganisms inside the analysed matrices. It was possible to estimate H', S, and E indices for each flour studied. Moreover, microbial communities of sprouted matrices, were faster to consume carbohydrates, polymers,

amines, carboxylic acid and amino acids sources than microbial consortia from whole flours.

5.3 Selected strains and fermentation processes to obtain new ingredients on laboratory scale

Based on growth and acidification ability, EPS production, and effects on doughs, *Weissella confusa* LGA8, *Leuconostoc mesenteroides* LGA1, *Weissella cibaria* DSM 20194, *Leuconostoc pseudomesenteroides* DSM 20193 were selected as starter to ferment whole and sprouted lentil and barley flours, alone and mixed each other at different concentrations (Table 3). The results were in according with Bounaix et al. (2009), confirms the ability of the LAB belonging to *Leuconostoc* and *Weissella* genera to produce EPS. Table 4 shows the profile of the mono and oligosaccharides of the six selected sourdoughs, before and after fermentation. the fermentation caused a significant ($P < 0.05$) decrease of oligosaccharides belonging to α -galactosides family, such as verbascose, stachiose and melibiose. Raffinose remained almost constant. After fermentation, the dextran yield was significantly higher in all six selected sourdoughs compared to control dough. The use of sprouted lentil flour was associated with the highest yield of dextran, with concentration ranging from $9.1 \pm 0.8\%$ (*Weissella cibaria* DSM 20194) to $10.1 \pm 0.04\%$ (*L. pseudomesenteroides* DSM 20193). When whole lentil and sprouted barley, grains were mixed and fermented with *L. pseudomesenteroides* DSM 20193, the lower yield in dextran was obtained ($2.3 \pm 0.9\%$) (Table 4). EPS production, viscosity and growth kinetics were carried out. When *W. confusa* LGA8 was used as starter, the highest and faster increase in viscosity was observed ($3.39 \text{ Pa} \cdot \text{s}$ after 14h of fermentation).

Table 3 Temperature of incubation, viscosity and TTA before and after fermentation of whole and sprouted flours with the selected strains. LG sprouted lentil flour; LNG, whole lentil flour; OG, sprouted barley flour; LGA8, *Weissella confusa* LGA8; LGA1, *Leuconostoc mesenteroides* LGA1; D20194, *Weissella cibaria* DSM 20194; D20193, *Leuconostoc pseudomesenteroides* DSM 20193.

Samples	T°	Pa*s T0	Pa*s T-24	$\Delta \text{Pa*s T0-T-24}$	TTA T-24	$\Delta \text{TTA T0-T24}$
LGA8LG	20	0.04±0.00	5.98±0.44	+5.94	11.93±0.15	+7.533
LGA8LNG	20	0.05±0.00	3.53±0.43	+3.48	9.48±0.007	+6.571
D20193LG	20	0.04±0.03	5.72±0.18	+5.68	11.63±0	+7.235
D20194LG	25	0.005±0.00	5.41±0.46	+5.39	12.54±0.57	+9.626
D20193LGOG	20	0.30±0.00	3.21±0.33	+2.91	12.79±0.01	+8.392
D20193LNGOG	20	0.18±0.00	2.81±0.15	+2.63	10.89±0.01	+7.974

Table 4 Profile variation of mono-and oligosaccharides in sourdough obtained before and after fermentation of whole and sprouted flours with the selected strains. LG sprouted lentil flour; LNG, whole lentil flour; OG, sprouted barley flour; LGA8, *Weissella confusa* LGA8; LGA1, *Leuconostoc mesenteroides* LGA1; D20194, *Weissella cibaria* (DSM 20194); D20193, *Leuconostoc pseudomesenteroides* (DSM 20193) (Gal, galactose; Glu, glucose; Suc, sucrose; Fru, Fructose; Mel, melibiose; Raf, raffinose; Sta, stachiose; Ver, Verbascose, Dextran, % in situ produced dextran on dry weight of doughs, CT, control samples).

Samples	Gal	Glu	Suc	Fru	Mel	Raf	Sta	Ver	Dextran
LGA8LG- CT	0.08±0.0 0	4.11±0.0 0	14.7±0.0 0	0.31±0.0 5	0.30±0.3 9	0.36±0.1 4	0.18±0.1 1	0.15±0.0 0	1,47±0.0 9
LGA8LG	0.17±0.0 1	nr	nr	8.8±0.3	nr	0.15±0.1	0.12±0.0 0	0.01±0.0 1	9,6±0,80
LGA8LNG-CT	0.06±0.0 0	nr	10.4±0.7	0.22±0.0 9	0.12±0.0 9	0.15±0.0 0	1.9±0.20	0.77±0.0 3	0,88±0,0 6
LGA8LNG	0.38±0.0 3	nr	0.04±0.0 0	9.2±0.20	nr	0.06±0.0 0	0.95±0.0 2	0.22±0.2 0	9,18±0,6 3
D20193LG- CT	0.08±0.0 0	4.11±0.0 0	14.7±0.0 0	0.31±0.0 5	0.30±0.3 9	0.36±0.1 4	0.18±0.1 1	0.15±0.0 0	1,41±0,0 5
D20193LG	0.05±0.0 0	nr	nr	7.57±0.0 0	nr	0.25±0.0 0	0.14±0.0 0	0.03±0.0 3	10,4±0,0 4
D20194LG- CT	0.08±0.0 0	4.11±0.0 0	14.7±0.0 0	0.31±0.0 5	0.30±0.3 9	0.36±0.1 4	0.18±0.1 1	0.15±0.0 0	1,37±0,2 0
D20194LG	0.14±0.0 1	nr	nr	8.61±0.8 7	nr	0.24±0.0 0	0.13±0.0 0	0.02±0.0 2	9,16±0,2 9
D20193LGOG-CT	0.04±0.0 0	nr	11.9±1	0.18±0.0 5	0.04±0.0 1	0.27±0.0 1	0.14±0.1 0	0.11±0.1 0	0,58±0,0 2
D20193LGOG	0.18±0.0 1	nr	nr	6.09±0.5 5	nr	0.34±0.0 2	0.11±0.0 0	0.04±0.0 0	2,38±0,6 9
D20193LNGOG-CT	0.05±0.0 0	nr	11.5±0.0 4	0.14±0.0 4	0.08±0.0 7	0.13±0.0 2	1.35±0.1 6	0.66±0.2 4	0,43±0,0 2
D20193LNGOG	0.40±0.0 0	nr	nr	5.89±0.0 9	nr	0.04±0.0 1	0.76±0.0 0	0.33±0.0 1	2,73±0,0 6

5.4 Biotechnological protocols to produce new baked goods

Sourdough obtained by sprouted lentil flour fermented with *W. confusa* LGA8; whole lentil flour fermented with *W. confusa* LGA8 and a mix of lentil and barley sprouted flours fermented with *L. pseudomesenteroides* DSM 20193 (LGA8-LG, LGA8-LNG and D20193LGOG) were selected for the pilot scale transfer of the biotechnological process at Puratos Italia s.r.l. Germination condition, cell density of presumptive lactic acid bacteria, pH, TTA, viscosity, EPS production and mono and oligosaccharides profiles were set-up at pilot scales. The results confirmed the data obtained at laboratory scale. In addition, as planned by the project proposal, nutrition label was made on each selected sourdough. Preliminary bakery tests using new ingredients shows interesting results. The new ingredients enriched in dextrans were able to increase the shelf life of experimental breads with a concomitant improvement of dough leavening properties (data not shown). This result is in according with Katina et al. (2009). We are evaluating the nutritional and functional aspects of new improvers and experimental breads on pilote scale developed, comparing those with commercial products. After this phase, two innovative biotechnological protocols will be transferred on industrial scale to produce novel ingredients collaborating with R & D section of Puratos Italia s.r.l.

6. Conclusions and Future Perspectives

This PhD thesis provides evidence that flour from sprouted grains can be considered as an ingredient for improving the technological performance of commercial flours. The preliminary data on bread obtained with improvers based on selected sprouted grains confirms that those flours are a promising and interesting ingredient for formulating baked products, avoiding the use of enzymatic improvers or malt with a positive impact on functional features and consumers' acceptance.

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Individual Differences in Cocktails Perception and Appreciation

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The aim of the PhD Thesis was to investigate consumer individual differences as well as the influence of product properties on alcoholic cocktail perception and appreciation by means of a multidimensional and multidisciplinary study. Results showed that more information for product optimization can be gained combining methodologies from different research fields such as sensory and consumer science, psychology and semiotics. Another innovative aspect of the thesis is the exploration of the concept of perceived complexity and its relationship with liking in alcoholic cocktails, which could booster the development of product innovation models.

Differenze individuali nella percezione e nell'apprezzamento dei cocktails

Lo scopo della tesi di dottorato ha riguardato lo studio delle differenze individuali dei consumatori nell'apprezzamento di cocktails alcolici con differente profilo sensoriale. I risultati suggeriscono che maggiori informazioni utili all'ottimizzazione di prodotto possono essere ottenute combinando metodologie appartenenti a differenti aree di ricerca come le scienze sensoriali e del consumatore, la psicologia e la semiotica. Un altro aspetto innovativo della ricerca ha riguardato il concetto di complessità percepita e la sua relazione con il gradimento nei cocktails che può essere applicato nello sviluppo di modelli per l'innovazione di prodotto.

Key words: Alcoholic cocktails, consumers, sensory properties, perception, emotions, context, complexity

1. Introduction

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following three activities directed to:

1) characterize consumer clusters based on different liking patterns for cocktail familiarity, attitudes, personality traits and implicit brand perception and to evaluate the effect on consumers liking of product emotions, context appropriateness and sensory properties;

2) cluster consumers based on the description of their preferred context of cocktail consumption and to study cluster differences as a function of individual differences and liking;

3) cluster consumers as a function of the perceived complexity of cocktails and to study the relationships between perceived complexity, liking, familiarity, emotion and contexts within clusters.

2. Understanding consumer individual differences in preferences

Despite the large diffusion of alcoholic beverages, scientific research about consumer individual differences in perception and appreciation of alcoholic cocktails is still very scarce. The study of consumer individual differences could allow to gain competitive advantage in product customization by dividing consumers into homogeneous groups or segments that are characterized by distinct characteristics and

then different behaviors. That could lead to the development of products that closely meet consumer needs. Differences in product perception could be based on familiarity and experience with differing products within the category. Other than that it has been proposed that personality and attitudes may play a large role in determining food preferences. As a measure of individual differences also variations in 6-N-propylthiouracil (PROP) bitter taste responsiveness has been linked to alcohol intake. This measure could be of interest for segmenting consumers as well as characterizing consumer clusters based on preference (Luckow et al., 2006).

Furthermore, the need of a deeper and more comprehensive understanding of the consumer experience with products has led to a growing interest in collecting several measurements. This aspect is of particular relevance for product development and testing as liking alone was not found sufficient to explain product differences in the market (Meiselman, 2013; Thomson, 2007). In particular, several studies have recently shown that emotional responses provide additional information about product experience that may discriminate beyond liking (e.g. Jaeger & Porcherot, 2017; Spinelli et al., 2014). In addition to emotions, it is also relevant to consider the effect of context/situation variables as it is evident that certain products fit better in specific situations of consumption (Piqueras-Fiszman & Jaeger, 2014; Köster, 2003). In this vein, situational cues as context appropriateness (i.e. whether a food product is more or less suitable to a particular consumption context) could be a relevant measure. Finally, the dimension of products complexity has proved to be relevant in the development of food products preferences and acceptability as it is an important property that can contribute to either positive or negative responses (e.g. Mielby et al., 2012). In fact, the complexity of a stimulus, along with its intensity and novelty, determines the extent to which it is liked. Hence, studying this aspect could be relevant to the development of product innovation models.

4. Experimental Procedure

A two-steps approach was applied to study the individual differences in cocktails perception and appreciation among consumers in the selected category of bitter-based alcoholic cocktails, with first step (qualitative) conceived as preliminary to the second step (consumer test).

First Step-Preliminary study: This phase concerned the development of product-specific questionnaires to collect information on emotions, context appropriateness and sensory properties that consumers associate with cocktails and the development of a question to investigate consumer's concepts of cocktail complexity.

Second Step-Consumer test: This phase was dedicated to study the differences in cocktails perception and appreciation between segments of consumers within the considered product category as a function of psychological and physiological individual differences.

5. Materials and Methods

5.1 Subjects

A total of 201 subjects were involved during the PhD project. Subjects were all cocktail consumers, age range 18-40, Italian native speakers, recruited in the Florence area (Tuscany, Italy) using online advertising. Forty-two subjects (20 males and 22 females) were assigned to the preliminary study for product specific questionnaires development, while a total of 159 consumers (85 females, mean age= 25.4) participated in the study on individual differences in cocktails perception and appreciation.

5.2 Cocktail samples

Six different cocktail samples (coded: A, B, C, D, E, F) were used as stimuli in both preliminary study and consumer test. Cocktails samples were prepared following the composition of real cocktails by the use of market-available ingredients (<https://www.campari.com/it>). All samples were prepared with a common bitter ingredient. Sample A was composed by the bitter ingredient only (abv=25 %). Other than the bitter ingredient, orange juice was added to Sample B (abv=6.2 %); sparkling wine and soda water to Sample C (abv=13.8 %); vermouth and soda water to Sample D (abv=13.6 %); gin and vermouth to Sample E (abv=27 %); bourbon and vermouth to Sample F (abv=33.3 %). All ingredients were maintained and mixed at room temperature (18–22 °C) except for soda water and sparkling wine that were kept at 4 °C. Timing for preparation of each sample was standardized as a function of changes in sensory properties among ingredients.

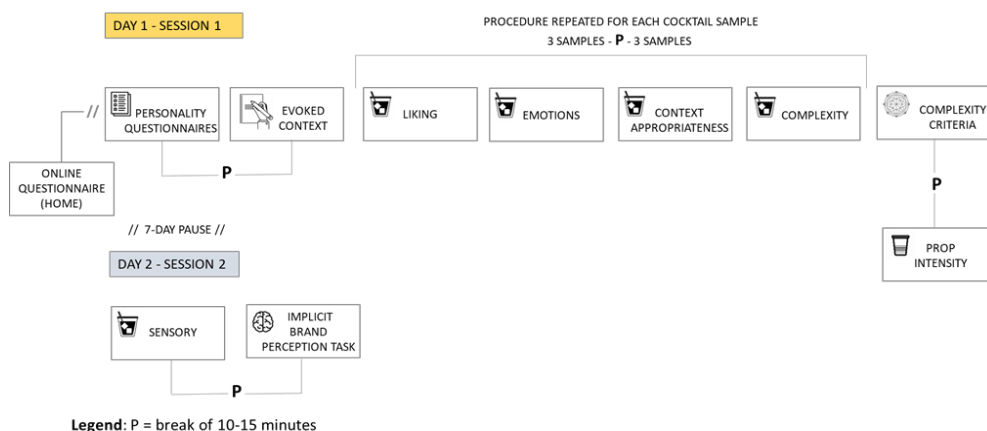
6. General procedures

6.1 Questionnaire development

The first step of the PhD project was dedicated to the development of product specific questionnaires for emotions, context appropriateness, sensory properties and complexity of bitter-based alcoholic cocktails. Data collection and analysis followed the EmoSemio procedure for questionnaire development using individual interviews (Spinelli et al., 2014). The procedure was repeated two times with the two different consumer panels. The first panel of consumers (n=xx) was asked to describe samples for emotions and contexts appropriateness whereas the second panel of consumers was asked to describe the sensory properties of samples.

6.2 Overview of data collection (consumer test).

Participants preliminarily completed some questionnaires at home and came to the laboratory twice in two different days at least 7 days apart from each other (Fig.1) Evaluations were performed in individual booths under white lights. All sessions took place in the afternoon (from 14:30 to 16:30 pm). Data were collected with the software Fizz (ver.2.51 B, Biosystemes, Couternon, France) and with E-Prime 2.0 software (E-Prime 2.0, Psychology Software Tools, Inc., Pittsburgh, PA, USA).



1) Online survey: first, all participants filled out an online questionnaire where they provided information about their demographics and other characteristics of interest. In this phase, cocktails familiarity, consumption frequency of alcoholic beverages and involvement with cocktails were assessed. **2) Personality traits:** Participants completed questionnaires to assess five psychological or personality related traits: behavioral inhibition and behavioral activation; Impulsiveness; seeking of varied, novel, complex, and intense sensations and experiences, and reluctance to eat unfamiliar foods. **3) Evoked contexts:** following the procedure proposed by Spinelli et al. (2017), consumers were asked to answer the open-ended question: "Describe the ideal situation in which you prefer to drink a cocktail". **4) Cocktails evaluation:** The following dimensions of product perception were assessed by: a) liking; b) complexity; c) emotions elicited by the products; d) context appropriateness; e) complexity criteria; f) intensity of sensory descriptors. **5) PROP responsiveness:** PROP taster status was evaluated throughout the rated intensity of a suprathreshold PROP solution evaluated using the gLMS Scale. **6) Implicit brand perception:** an implicit methodology was employed to tap implicit emotional responses to the bitter ingredient producer brand used in the preparation of each sample. A priming task, Affect Misattribution Paradigm (AMP) was employed (Payne et al.,2005).

7. Results and Discussion

7.1 Characterization of clusters based on consumer liking

Hierarchical cluster analysis (HCA) of consumer coordinates for the first three PCA components identified three clusters of consumers who were homogenous in their preference. Cluster 1 was composed by the 50% of consumers; Cluster by 22% and Cluster 3 was composed by 28%. The three clusters differed according to gender, Cluster 3 was composed by a higher number of males (61%) as compared to Cluster 1 (32%) ($\chi^2 = 11.92$ $p = 0.033$). A higher number of consumers with a high positive implicit brand effect was found in Cluster 3 (85%) ($\chi^2 = 5.99$ $p = 0.047$) as compared to Cluster 1 (65%) and 2 (35%). Also differences in alcoholic beverages consumption and attitudes for cocktails were present (Table 1).

Table 1 Consumer clusters characterization.

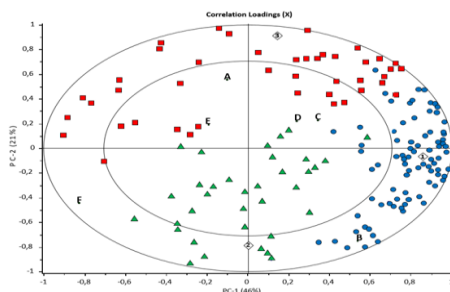


Figure 2 Correlation loadings for PCs 1 and 2 of Principal Component Analysis on liking scores.

Variables	Cluster 1	Cluster 2	Cluster 3	F	p-value
Age	24.75	25.57	26.38	1.42	0.244
Alcohol consumption					
General alcohol consumption	1.96	1.97	2.13	3.23	0.042
Cans of beer (330 ml) for week	1.53	1.35	1.92	4.47	0.013
Glasses of wine (125 ml) for week	1.67	1.72	1.89	0.980	0.377
Shots of liquor (40 ml) for week	1.29	1.43	1.69	3.41	0.035
Glasses of cocktails for week	1.62	1.37	1.64	1.58	0.209
Involvement with cocktails	14.12	11.04	13.78	4.29	0.016

7.2 Preferred context of cocktail consumption

Semiotic Text-Analysis coupled with Principal Divisive Directive Partitioning on open ended questions allowed the identification of three thematic clusters respectively composed by 36,42%, 21,19% and 42,38% of the consumers. Each theme was labelled

according to the focus on the preferred context, as follows: Theme 1: RELAX: focus on an experience of relaxation, comfort and on the social dimension. Individual in this cluster describe their preferred context as a not too crowded context with sofas, soft background music and the possibility to eat something. Theme 2: SOCIALITY: these subjects focus mostly on the social aspects of cocktail consumption. They describe their preferred context as a party or a disco at night where they can seek new experiences. Positive feelings are also related to their preferred context. Theme 3: APERITIF: these subjects focus on a before dinner context. They describe their preferred context as an outdoor public place or a home situation where they meet with friends to recover from work. They focus on the capacity of cocktails in stimulating appetite but they report also beer or wine as an alternative. Fig. 3 shows the result of thematic analysis performed on the lemmas x themes contingency table. Significant effects of sample ($F(5,2) = 23.86$, $p < 0.0001$) and cluster ($F(5,2) = 3.05$, $p = 0.048$) were found on liking ratings, while no significant interaction was observed ($F(5,2) = 0.29$, $p < 0.982$). Post-hoc test (Fisher LSD) indicated that mean liking for the six evaluated cocktails was higher in thematic Cluster 3 (APERITIF) as compared to Cluster 1 (RELAX). Other differences in consumer characteristics between clusters are reported in Table 2.

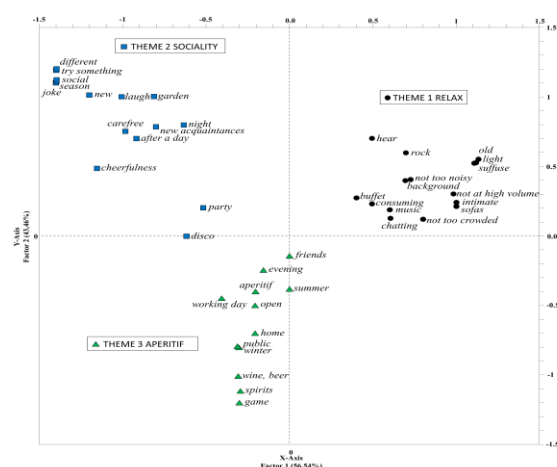


Figure 3 Thematic analysis. Characteristic lemmas of each theme are represented with the corresponding symbol: RELAX (circles); SOCIALITY (squares); APERITIF (triangles).

Table 2. Consumer's characteristics in the three thematic clusters. Different letters indicate significance at 0.05 level by Fisher's LSD post hoc test.

Variables	Thematic cluster 1 (Relax)	Thematic cluster 2 (Sociality)	Thematic cluster 3 (Aperitif)	F	p-value
Age	25,673 *	24,875 *	25,719 *	0,30	0,742
General alcohol consumption	2,005 *	2,070 *	1,964 *	0,82	0,439
Shots of liquor (40 ml) for week	1,467 ^{ab}	1,788 *	1,245 ^b	4,82	0,009
Glasses of cocktails for week	1,613 *	1,670 *	1,554 *	0,28	0,755
PROP intensity	28,809 *	25,906 *	35,492 *	2,19	0,115
Involvement with cocktails	34,95 *	26,00 ^b	34,22 *	4,52	0,013
Sensation seeking	98,564 ^b	107,875 ^a	98,469 ^b	4,359	0,014

7.3 Effect of complexity

The first three dimensions on the Principal Component Analysis (PCA) computed on individual complexity scores explained 76% of the total complexity score variability. Two cluster were identified through K-means cluster analysis computed on consumer coordinates for the first three PCA components: Cluster 1 was composed by 90 consumers (56%), whereas Cluster 2 was composed by 69 consumers (44%). Sample F was perceived as the most complex by almost all respondents in Cluster 1 and part of those belonging to Cluster 2 located on the left of the first dimension. On the contrary, sample B was perceived as the least complex by all respondents in Cluster 1. The participants belonging to Cluster 2 considered sample B as the most complex cocktail in the series. Multiple Factor analysis of product descriptors, complexity and liking showed different results among clusters. In Cluster 1, the first dimension was negatively correlated with descriptors related to negative

emotions (*disgusted, sad*), contexts (such as *after dinner* and *digestive*) and specific flavors (*herbs, spices*), while it was positively correlated to descriptors related to positive emotions (*relaxed, curious*), context (such as *summer* and *holiday*) and sensory (*light, fresh*). The second dimension was positively correlated with the descriptor related to *sparkling mouthfeel* and negatively with *dense*. Complexity and liking were negatively correlated to each other: the first dimension was negatively correlated with complexity and positively correlated to liking. In Cluster 2, the first dimension was positively correlated with positive emotions (such as *curious, surprised, merry, relaxed*), contexts (*cocktail bar, party*) and sensory properties (*light, fresh, sweet, citrus*). The first dimension showed instead a negative correlation with negative emotions (*uncomfortable, sad*), contexts (*winter, digestive*), flavors (*herbs, spices*), *alcoholic* and *medicine*. The second dimension was also positively correlated with the items related to *sparkling mouthfeel* and negatively with *dense, sour taste, intensity of the sample color* and *after dinner* context. Complexity and liking were positively correlated to each other and to the first dimension (Fig.4).

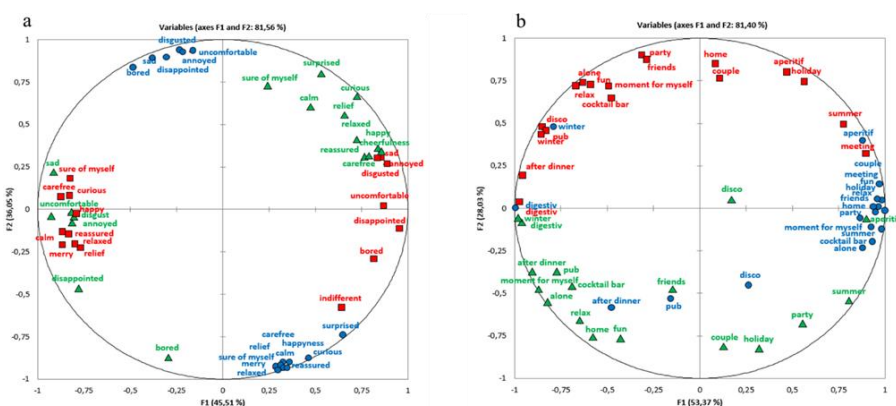


Figure 4 Correlation circle and score plots on the first and second dimension of the MFA for cluster 1 (a) and cluster 2 (b), with complexity and liking (in bold) plotted as supplementary variables.

A significant effect of sample ($F(5,1) = 11.5$, $p < 0.0001$) and a significant interaction (cluster $\times$ sample; $F(5,1) = 23.0$, $p < 0.0001$) were found on complexity rating, while no significant effect of cluster was observed ($F(5,1) = 3.09$, $p < 0.079$). Also, a significant difference in overall liking for the products was found for Cluster 1 ($F(5,89) = 17.63$, $p < 0.001$) and Cluster 2 ($F(5,67) = 25.016$, $p < 0.001$). The index of familiarity for the six cocktails included in the experimental plan showed marginally significant difference between the two clusters ($X^2 = 3.78$, $p = 0.052$). In Cluster 1, fewer consumers with lower familiarity with the samples was found, while, conversely, in Cluster 2 fewer consumers with higher familiarity with the six cocktails samples was found.

8. Conclusions and future perspectives

This exploratory study provides evidences for the use of a multidimensional approach to study consumers preference for alcoholic cocktails. Different clusters were characterized by demographic variables, personality traits, involvement, implicit brand perception and familiarity. The use of thematic text analysis on open-ended questions allowed to observe the preferred contexts of cocktail consumption among different consumers.

Finally, the exploration of the concept of perceived complexity and its relationship with liking in alcoholic cocktails is a promising approach that could be applied in the

development of product innovation models. Even though we examined a broad consumed product, a generalization of results to different typologies of alcoholic cocktails require further analysis. More systematic investigation should be conducted to observe differences in consumer preference for different product categories (i.e. sweet-based cocktails) and also in other cultural contexts.

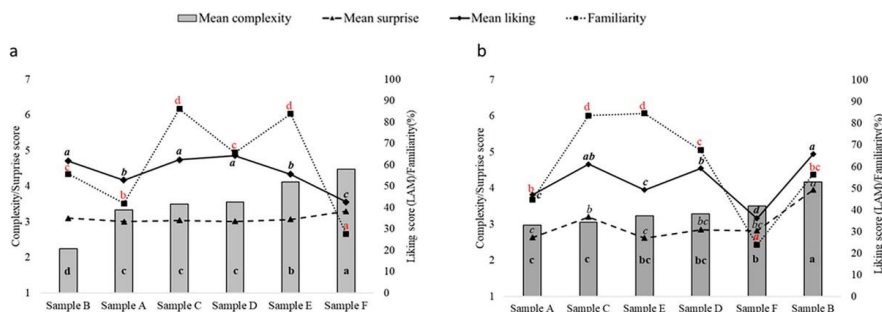


Figure 5 Complexity, surprise and liking mean scores for the six samples for cluster 1 (a) and cluster 2 (b). Different letters (complexity = bold; surprise = italic; liking = bold/italic) indicate significance at 0.05 level (Fisher's LSD post hoc test). Sum of ranks for familiarity is expressed as proportion on theoretical maximum of each sample. Different letters (familiarity = regular in red) represent significant differences between samples at $p < 0.05$ (Dwass-Steel-Critchlow-Fligner post-hoc test).

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Texture and Trigeminal Sensations: New Approaches to Measure the Human Sensitivity

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Individual sensory sensitivity plays a pivotal role on food preference and choice. As with taste and odour perception, individual differences in how texture properties and trigeminal sensations are perceived may contribute to how rejection or preference develop. However, little is known about texture and trigeminal sensitivity. The aim of this PhD thesis research was to develop new approaches to measure the human sensitivities to the texture and trigeminal sensations and verify whether they are influenced by individual variables and may affect the food preferences.

Texture e sensazioni trigeminali: nuovi approcci per misurare la sensibilità umana

La sensibilità sensoriale gioca un ruolo fondamentale nelle preferenze e scelte alimentari. Così come le percezioni relative ai sapori ed agli odori, le differenze individuali relative a come le proprietà di *texture* e le sensazioni trigeminali siano percepite, possono contribuire allo sviluppo di preferenze alimentari. Tuttavia, relativamente poco si conosce sulla sensibilità sensoriale alla *texture* e alle sensazioni trigeminali. Lo scopo di questa tesi di Dottorato è stato di sviluppare nuovi approcci per misurare le sensibilità individuali alla *texture* e alle sensazioni trigeminali e verificare qualora esse fossero influenzate da variabili individuali e potessero influenzare a loro volta le preferenze alimentari.

Key words: Sensory sensitivity; hardness; graininess; sparkling sensation.

1. Introduction

Sensory sensitivity, intended as personal characteristic related to individual differences in detection and reaction to sensory information (Dunn, 1999), plays a pivotal role in food preference and choice. Individual sensitivity to taste, and also to other oral sensations, shows substantial variability between individuals, and many studies showed how these differences are a significant influence on food preference and consumption (Cardello, 1996; Tuorila, 2007). However, besides tastes and odour, texture and trigeminal sensitivities, and how they could affect food rejection or preferences and choice, is poorly investigated.

Texture is a multi-parameter attribute and it involves several senses (Szczesniak, 2002). Also, the texture attributes are unique characteristics, which contribute to the overall enjoyment of the food, for example, attributes such as “crunchiness”, “crispiness” and “thickness” are commonly used terms (Forde et al., 2002). Specifically, some texture attributes can negatively affect the overall perception, e.g. the graininess of a cream, meanwhile, others positively do, e.g. the crunchiness of chips.

On the other hand, the trigeminal sensations trigger a variety of physiological (touch-position and pain-temperature) and behavioral responses, resulting in a subjective experience of pain in humans. As with the texture, whether the trigeminal sensitivity is innate, and whether it could affect food preferences and choice, is not clear.

The reason why little is known about texture and trigeminal sensations is a missing standardized procedure to measure the individual sensitivities. So, with these premises and in accordance with the PhD thesis project previously described (Puleo, 2017), this oral communication reports the main results of three following study cases (A1, A2, A3) directed to develop a new approach to measure the individual sensory sensitivities to specific key attributes, varied in different levels of intensity.

- A1) Measuring of hardness (key texture attribute) sensitivity;
- A2) Measuring of graininess (key texture attribute) sensitivity;
- A3) Measuring of sparkling (key trigeminal sensation) sensitivity.

2. Materials and Methods

A1. Eight formulations of jellies were prepared by mixing orange juice, sugar and agar-agar, in different concentrations, in order to obtain different levels of hardness.

First, ranking test with trained judges ($n=14$), and mechanical measurements by means of a texture analyser, were performed in order to select only four formulations that were significantly different one from each other. Then, 248 consumers (female=112; age average= 33.5 ± 12.96) evaluated first liking by using the LAM scale (Schutz and Cardello, 2001) and secondly the hardness intensity by using the GLM scale (Bartoshuk et al., 2004). The subjects were then clustered based on their hardness sensitivity into three groups: high sensitivity; moderate sensitivity; low sensitivity. Repeated measures ANOVA model was used to analyse the consumer's data (XLSTAT statistical software package version 2016.02, Addinsoft).

A2. Seven samples of cocoa-based cream were obtained by refining in a stirred ball mill, interrupting the process after 15, 20, 30, 35, 45, 70 and 90 minutes, in order to obtain different levels of graininess, key texture attribute chosen for this type of product. Also in this case, in order to reduce the number of samples to be used for measuring the graininess sensitivity, a trained panel was involved to select only significantly different samples in terms of graininess. Furthermore, the selected samples (20, 30, 35, 45 and 70 minutes) were characterized by a particle size analyzer and several indices of the particle size distribution based on the volume of particles were estimated, including the D10, D50, and D90. Finally, 59 subjects (females=33, age average= 32.7 ± 15 years old) evaluated the five creams and scored their liking by using the LAM scale and the graininess intensity by using the GLM scale. Also in this case, subjects were clustered based on their graininess sensitivity into three groups and repeated measures ANOVA model was used to analyse the consumer's data (XLSTAT statistical software package version 2016.02, Addinsoft).

A3. Two different sets of samples of sparkling water were obtained by mixing sparkling and still water in different concentrations. One hundred subjects (58% females, 24.9 ± 3.9 years old) differing in sparkling water consumption behaviour (non-consumers (never), moderate consumers (once a month), frequent consumers ($>1-2$ times a week)) participated in a consumer test consisted of two sessions. Sparkling sensation detection thresholds were measured using the standard ASTM method of best estimate threshold (BET). Seven 3-alternative forced choice tests were performed using the ascending staircase method with the first set of samples (CO_2 concentrations ranging from 0.05 to 1.49 g/L). Intensity of sparkling sensation and liking of the second set of samples, consisted of five sparkling waters (CO_2 ranging from 0.30 to 5.96 g/L) were assessed using LAM and GLM scales, respectively. To characterize consumers, PROP status (Prescott et al., 2004), demographic information, health interest (Roininen et al., 1999), eating behaviour (Strienet al., 1986), private body consciousness (Miller et al., 1981) and sensitivity to punishment and reward (Torrubia et al., 2001) were also determined.

3. Results and Discussion

A1. The aim of this study case was mainly focusing on measuring the hardness sensitivity of the people. By averaging both liking and perceived hardness intensity scored by 248 subjects, some significant differences came out among the sample, as shown in table 1.

Table 1 *Liking and perceived hardness intensity scored by 284 subjects.*

Agar-agar concentration	Liking (LAM scale)	Perceived hardness intensity (gLM scale)
3%	49.73 ^c	8.60 ^a
5%	46.74 ^b	13.77 ^b
7%	43.46 ^a	19.54 ^c
9%	42.54 ^a	22.7 ^c

* In each column values followed by different letters were significantly different ($p \leq 0.05$).

Furthermore, the results of the mechanical analysis showed that the hardness values (N), measured by means of a texturometer, followed a linear trend ($R^2 = 0.97$), as shown in the figure 1a. With this premise and on this base, the intensities of the perceived hardness scored by each subject were modelled by a linear equation as well. The angular coefficients of the linear equations and the R^2 values were analyzed by the percentiles distribution and the consumers were then clustered into three sensitivity groups: lowly sensitive, moderately sensitive and highly sensitive. In particular, the behaviour of the lowly sensitive people ($n=85$) was modelled by linear equations represented by values of the angular coefficient lower than zero and simultaneously with any value of R^2 , or by values of the angular coefficient between 0 and the median and, at the same time, with a value of R^2 lower than the median. On the other hand, the behaviour of the highly sensitive people ($n=77$) was modelled by a linear equation which showed values of the angular coefficient greater than the third quartile and, simultaneously, any value of R^2 , or values of the angular coefficient between the median and the third quartile and at the same time, with a value of R^2 greater than the third quartile. Figure 1b shows, by way of example, the trend of the perceived hardness for one subject belonging to each group.

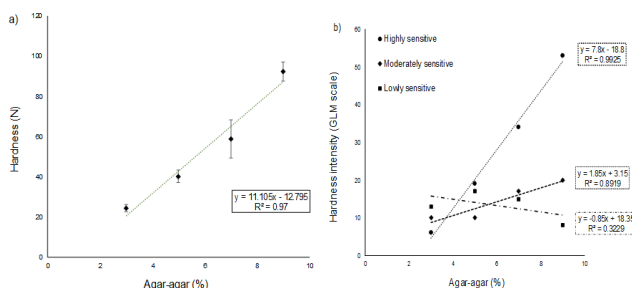


Figure 1 *Hardness measured by texture analyzer (a) and perceived hardness intensity (b) scored by three groups of sensitivity.*

Moreover, table 2 synthesizes the results of repeated measures ANOVA within the groups, demonstrating how the used criteria were valid for clustering the subjects in three different groups. Indeed, the lowly sensitive people were not able to discriminate any sample; the moderately sensitive people did not discriminate the last two samples; the highly sensitive people were absolutely able to discriminate among all the samples.

Table 2 *Perceived hardness intensities scored by each group of sensitivity.*

Agar-agar concentration	Lowly sensitive	Moderately sensitive	Highly sensitive
3%	11.53	7.33 ^a	6.81 ^a
5%	14.05	12.11 ^b	15.34 ^b
7%	14.41	18.77 ^c	26.05 ^c
9%	13.53	21.26 ^c	34.84 ^d

Different hardness sensitivity did not seem to affect liking scores; in general, the consumer's liking decreased as jellies hardness increased.

A significant effect was found among hardness sensitivity and gender, in particular males were more sensitive than females ($p=0.027$), whereas no significant effect was observed between hardness sensitivity and age. However, age significantly affected the liking ($p=0.001$), as well as the interaction between age and gender ($p=0.01$). In particular, both old male and female consumers averagely liked more the samples compared to the other two groups of age. These results are consistent with the study conducted by Kälviäinen and colleagues (2000), who showed how age and gender affected the hedonic evaluations of different strawberry candies.

A2 The aim of this second study case was mainly focused on measuring the graininess sensitivity of the people. By averaging both perceived graininess intensity and the liking scores, some significant differences came out among the sample, as shown in table 3. There were no significant differences between the first two samples, neither for graininess intensity nor for liking. However, as expected, with the refining time increasing the average graininess intensity decreased, while the liking increased. These results are expected and absolutely consistent with the literature. Indeed, for products like chocolate creams, particle size should be in a range between 15 and 25 μm to ensure the right palatability. Products presenting particle size higher than 25 μm are perceived as grainy and rough, resulting in lower acceptability (Birkett, 2009).

Table 3 *Perceived graininess and liking scored by 59 consumers.*

Refining time (min)	Liking (LAM scale)	Graininess (gLM scale)
20	62.41 ^a	18.54 ^{ab}
30	61.80 ^a	22.61 ^b
35	64.93 ^{ab}	17.08 ^{ab}
45	67.10 ^{ab}	14.98 ^a
70	68.86 ^b	13.98 ^a

Furthermore, the results of the instrumental analysis showed that the D_{90} values (μm), measured by means of a particle size analyser, followed a power model with an R^2 equal to 0.9636, as shown in the figure 2a. So, by following the same approach used for the study case 1, on the base of the power model trend observed from the instrumental data, the perceived graininess scores were modelled by a power equation as a function of the refining time, for each consumer. After that, the power equations coefficients and the R^2 values were estimated as well. The exponents and the R^2 coefficients obtained for each consumer were categorized using the characteristic values of the percentile distribution. Again, the consumers were then clustered based on both the percentile distribution of the exponents and the R^2 values into three groups: lowly sensitive, moderately sensitive and highly sensitive. In particular, people who showed an exponent lower than the first quartile,

and at the same time, a R^2 value higher than the third quartile were clustered in the high sensitivity group ($n=17$). On the other hand, people who showed an exponent between the median and the third quartile, and at the same time, a R^2 value lower than the median, were clustered in the low sensitivity group ($n=26$). All the other subjects were split in the moderate sensitivity group. Figure 2b shows, by way of example, the trend of the perceived graininess vs refining time for one subject belonging to each group.

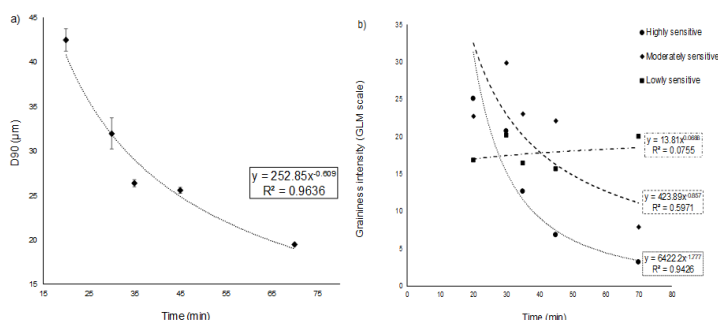


Figure 2 D_{90} values measured by particle size analyser (a) and perceived graininess intensity (b) scored by three groups of sensitivity.

Furthermore, table 4 synthesizes the results of repeated measures ANOVA within the groups, demonstrating how the used criteria were valid for clustering in three different groups the subjects. Indeed, by looking within the groups, the lowly sensitive people did not perceive any difference between the samples, in terms of graininess. The moderately sensitive people were able to find a difference for the most refined sample that was perceived as the least grainy one, whereas all the other samples were perceived similar one to each other. Finally, the highly sensitive people well discriminated the samples, showing a good ability to perceive the differences.

Table 4 Perceived graininess intensities scored by each group of sensitivity.

Refining time (mins)	Lowly sensitive	Moderately sensitive	Highly sensitive
20	20.12	29.88 ^b	25 ^d
30	19.96	23 ^b	20.71 ^{cd}
35	16.85	22.69 ^b	12.65 ^{bc}
45	16.42	22.1 ^b	6.77 ^{ab}
70	15.62	7.88 ^a	3.12 ^a

Finally, no significant relationships were found with age and gender, even though a little trend was observed with gender. In particular, in this case females seemed to be more sensitive than males. In fact, 54% of males were included in the low sensitivity group.

A3 The aim of this study case was mainly focused on understanding whether the sparkling sensitivity is innate or may be influenced by food behaviour and individual characteristics (i.e. taste sensitivity and psychological variables).

First, average detection threshold of sparkling sensation (BET), measured between 100 subjects, was 0.26 g/L, consisted result with the study conducted by Le Calvé and colleagues (2008). According to this value, subjects were clustered in two groups: highly sensitive ($n=48$), individuals who had a lower threshold than the geometric mean; lowly sensitive ($n=52$), individuals above it. Moreover, subjects were also clustered in different groups according to their frequency of consumption of sparkling water: non-consumers

(n=26), moderate consumers (n=51), frequent consumers (n=23). Finally, three different groups were also obtained according to the PROP status. In particular, by looking at the percentile distribution of ratings, the upper limit of the first quartile and lower limit of the third quartile were 26 and 61 on gLMS, respectively. These were the cut-offs to categorize individuals in Non-Tasters (gLMS<26; n=48), Moderate-Tasters (n=26) and Super-Tasters (gLMS>61; n=26). These values were comparable with the cut-offs used by Fischer (2013) and Hayes (2008) to categorize subjects according to their taste sensitivity. Finally, concerning the data from psychological questionnaires, the internal consistency calculated for each questionnaire and explained by Cronbach's alpha, was always greater than 0.7, except for the Private Body Consciousness ($\alpha=0.56$). After that, the personality traits ratings were first categorized using the characteristic values of the percentile distribution (first and third quartiles), then, 3 segments or levels of interest/sensitive (low, moderate and high) were considered.

The interactions between individual sparkling detection thresholds and all the individual factors were tested by means of chi-square tests, as showed in table 5.

Table 5 Effect of individual factors on sparkling detection thresholds - χ^2 analysis on frequencies.

Individual factors	χ^2 observed	χ^2 critical	p-value
Gender	0.003	3.841	0.955
Nationality	0.927	7.815	0.819
Frequency of consumption	3.968	5.991	0.138
PROP status	0.403	5.991	0.818
General Health Interest	0.003	5.991	0.999
Light Product Interest	0.232	5.991	0.89
Natural Product Interest	0.926	5.991	0.629
Craving for sweet foods	1.715	5.991	0.424
Using food as reward	4.855	5.991	0.088
Sensitivity to Punishment	0.417	5.991	0.812
Sensitivity to Reward	0.646	5.991	0.724
Restrained eating	3.483	5.991	0.175
Emotional eating	0.305	5.991	0.859
External eating	2.818	5.991	0.244

Detection thresholds were not influenced by any measured individual factor, suggesting that the sparkling sensitivity might be innate.

Secondly, we also investigated whether there were significant differences in liking and in the perception of sparkling sensation. By averaging both liking and perceived sparkling intensity scores, some significant differences came out among the sample, as shown in table 6. In particular, there were no significant differences in liking between the samples, except for the first sample which resulted as the most liked one. On the other hand, concerning the perceived sparkling intensity, by looking at the scores of the perceived sparkling intensity, all the samples were perceived as different ($p<0.0001$) and, as expected, the scores followed an upward trend, increasing when the concentrations increased as well.

Table 6 Liking and perceived sparkling intensity scored by 100 consumers.

CO2 concentration (g/L)	Liking (LAM scale)	Sparkling intensity (gLM scale)
0.21	58.83 ^b	2.44 ^a
1.05	49.56 ^a	7.98 ^b
1.68	53.26 ^a	15.95 ^c
2.94	53.15 ^a	36.05 ^d
4.2	53.18 ^a	42.6 ^e

*In each column values followed by different letters were significantly different ($p \leq 0.05$).

No relationships were found between liking and perception of sparkling sensation with gender, PROP status or psychological traits. Liking and the perception of sparkling sensation were only affected by the frequency of sparkling water. In particular, non-consumers liked sparkling water significantly more than moderate or frequent consumers, probably because mainly for cost reasons they did not consume sparkling water. Sparkling sensation was perceived significantly more intensive by non-consumers compared to moderate- and frequent consumers. This result is probably due to the expectations: people who are used to consume sparkling water were more conscious of the sensation compared to people who are not used to.

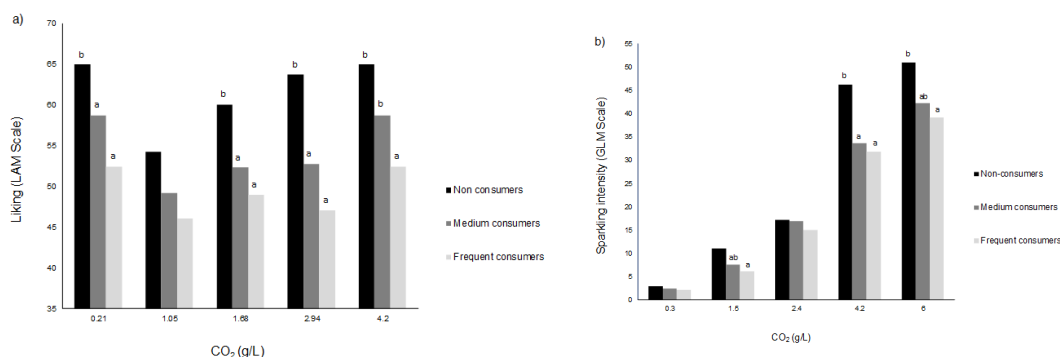


Figure 3 Liking (a) and perceived sparkling intensity (b) scored by three groups of frequency of consumption.

4. Conclusions and future prospects

Considering the lack in the literature, these studies were conducted with the purpose to develop a method to measure the sensitivity to key texture attributes and key trigeminal sensations. Our results suggest, first, that differences in texture and trigeminal perception exist and the approach we used was valid to measure them. Also, individual characteristics, such as gender, age and food behaviour play an important role on both sensitivity and food liking.

In conclusion, the present approaches can be used to investigate other key attributes and other individual variables that can be related to individual perception, also with a view to validate the proposed methods. Indeed, our future prospects are oriented both on investigating whether the sensitivities to different texture attributes and trigeminal sensations are related to each other.

This knowledge is crucial to highlight or avoid specific textural attributes for a specific target of consumers, consequently, it will be useful for the food industry to develop tailored foods.

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Use of Tensiometric Techniques to Design and Develop Food and Nutraceutical Formulations

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This PhD thesis dealt with the development of new nutritional formulations based on the sugar compounds of natural honey by surface tensiometry approach, and their *in vitro* release properties related to a bioactive compound having healthy properties.

Uso di tecniche tensiometriche per progettare e sviluppare formulazioni alimentari e nutraceutiche

Questa tesi di dottorato ha riguardato lo sviluppo di nuove formulazioni nutrizionali basate sulla composizione zuccherina base del miele mediante l'impiego di tecniche tensiometriche e la valutazione *in vitro* delle loro proprietà di rilascio di un composto avente proprietà salutistiche.

Key words: Contact Angle; food, sugars; Surface Tensiometry; Nuclear Magnetic Resonance.

1. Introduction

In accordance with the PhD thesis project previously described (Rossi, 2019), this oral communication reports the main results of the following four activities directed to:

A1) characterization of binary solutions of sugars present in honey by surface tensiometry methodologies;

A2) formulation and development of ternary sugar solutions composed by mixtures of binary sugar solutions previously characterized using the surface tensiometry methodology;

A3) analyses of the correlations between chemical, rheological and surface properties of the binary and ternary sugar solutions;

A4) Formulation of “honey like” model based on the ternary sugar solutions selected based on the results of the surface tensiometry methodology, and evaluation of their *in vitro* release property.

2. The surface tensiometry

The knowledge of surface phenomena occurring at the interface between different components of the same matrix (food, pharmaceutical) is important to understand the influence of the formulation and process variables on the characteristics of the final product. The surface tensiometry is a non-invasive analytical approach to study these phenomena, which allows to determine the surface free energy of solid substrates (SFE; mJ/m^2) and surface tension of liquid systems (ST; mN/m^2). The SFE of solid substrates and the ST of liquids systems are composed of polar (CP) and dispersion (CD) components depending on their nature and composition. The surface tensiometry analysis is applied in the food sector to characterize the surface tension of liquid compounds (e.g. emulsifiers, proteins) in simple model systems (e.g. binary or ternary solutions) as well as to characterize the surface free energy and functionalities of solid materials (e.g. wettability of packaging films)

(Karbowski et al. 2006, Rossi et al. 2018). The surface tension and interfacial free energy of food systems may affect quality, stability and functionality of the final product, and the main objective of the surface tensiometry characterization of complex food systems is to unravel the causes of negative effects derived by the interaction between the single compounds of the food with, for instance, reduced bioavailability or decreased systemic effects (Bailey 2010, Singh 1999, Winstanley and Orme 1989). The knowledge of the influence of specific foods components on the bioavailability of bio-active lipophilic agents in complex food matrices can also allow the development of new food matrices that could improve the biological activity of nutraceuticals and drugs (McClements and Xiao 2014).

3. Experimental Procedure

In this PhD thesis, typical binary and ternary aqueous solutions of sugars some of the naturally present in honey, were respectively prepared and characterized using the contact angle (CA) method. Binary and ternary and sugar aqueous solutions such as glucose, fructose and sucrose 23% (w/w) were characterized measuring the CAs over time on the surface of test solids having different surface free energy (SFE; mJ/m^2) including glass, considered as a reference solid test, aluminium, and hydrophobic Fomblin HC/18 Perfluoropolyether (PFPE). The number of determinations (plots) of CAs over time were three for each sugar aqueous solutions and only one of them were selected in relation to the minor variation of drop volume during the kinetic CA analysis. The first objective was to define the wettability of glass with different kind of binary sugar solutions and evaluate the discriminating capability of the surface tensiometry technique to distinguish them. The correlations between chemical and physical property of sugars and the comparison between chemical and surface tensiometry data of different fructose binary aqueous solutions were performed too. Hence, ternary solutions containing other sugar aqueous solutions also were developed from the binary sugar aqueous solutions on the base of the highest and lowest affinity degree in terms of wettability of glass, Al, and PFPE test solids and correlated with rheologic data. The ternary sugar aqueous solutions developed by surface tensiometry constitute the base for the production of different nutritional formulations “honey like”.

4. Materials and Methods

Binary aqueous solutions of glucose ($M=1.28 \text{ mol/L}$), fructose ($M=1.28 \text{ mol/L}$), and sucrose ($M=0.67 \text{ mol/L}$) having concentration of 23% (w/w) were formulated taking into account their percentages in a standard honey. The ternary sugar aqueous solutions (Table 1) developed from the binary sugar aqueous solutions are reported in Table 1 and contained maltose and threulose also.

Table 1 Ternary sugar aqueous solutions.

Solid test glass (sg)	
Solution 1 <i>Higher tensiometric affinity</i> 23% fructose+23% threalose	Solution 4 <i>Less tensiometric affinity</i> 23% glucose+23% sucrose
Solid test Aluminium (Al)	
Solution 1b <i>Higher tensiometric affinity</i> 23% fructose+23% sucrose	Solution 2b <i>Less tensiometric affinity</i> 23% maltose+23% threalose
Solid test Fomblin HC/18 Perfluoropolyether (PFPE)	
Solution 1a <i>Higher tensiometric affinity</i> 23%threalose+23% sucrose	Solution 2a <i>Less tensiometric affinity</i> 23%fructose+23% maltose

The measurements of CAs was performed using a Drop Shape Analyzer 30 tensiometer (Krüss GmbH, Krüss, Hamburg, Germany) together with accessories and software. The CA analysis over time was performed using the Circle Fitting (CIR) calculation method (Hong et al. 2009), considering a period of 60s corresponding to 1500 frames (fr) overall and using a resolution of 2 frames per second (fr/s). The reference test solid used for the characterization of binary sugar aqueous solutions was glass for its hard and flat surface, while for the characterization of ternary sugar aqueous solutions were used aluminium (Al) and Fomblin HC/18 Perfluoropolyether (PFPE) also. The Nuclear Magnetic Resonance (NMR) analysis were performed on binary and ternary sugar aqueous solution, and in particular on fructose binary aqueous solution having different concentration (12% w/w and 23% w/w). NMR analyses were performed using a 400 Mhz NMR apparatus (Bruker Corp., Billerica, MA, USA) in order to study the variation of the intensity, area and width of water peak (deuterated water and water solution) in relation to the different kind of sugar aqueous solutions and concentration. For the determination of the rheological characteristics of the ternary sugar aqueous solutions, a Rotovisco RV 20 with a RC 20 reocontroller equipped by NV, MVP1, and MV sensors (Haake, Karlsruhe, Germany) was used, a shear rate range 0.1-1000 1/s in 10 minutes was adopted at the temperature of T=20°C. The surface tensiometry data of the sugar aqueous solutions were correlated and compared with reological and chemical results.

5. Results and Discussion

The use of CIR method in situations in which the solid test used hasn't a superhydrophobic nature, has allowed to reduce significantly the distortions observed in the CA measurements after the application of the Y-L method. For this reason the CIR method was used for the calculation of CAs during the spreading of the drops of binary aqueous solutions such as glucose, fructose, sucrose, and ternary sugar aqueous solution using PFPE and Al test solids also. In Figure 1a and 1b were reported the drop spreadings of binary sugar solutions and the correlation between CA and water solubility at 25°C.

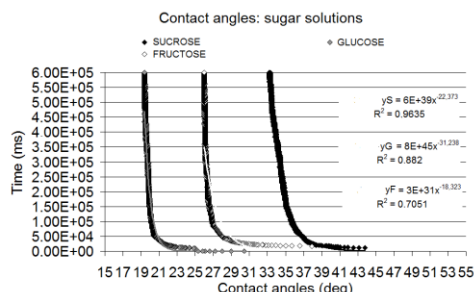


Figure 1a Behaviour of CA in the time for sucrose, glucose and fructose solutions.

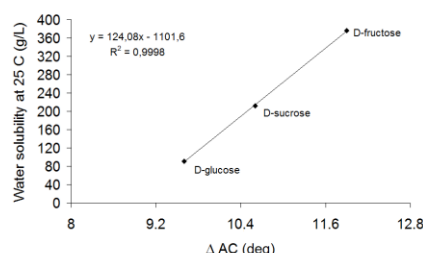


Figure 1b. Accuracy and precision of CA measurements by CIR mode.

Figure 1a shows a satisfactory discriminating capability of the surface tensiometry technique in distinguishing the three binary sugary aqueous solutions, and Figure 1b confirm the excellent capability of the surface tensiometry technique to characterize the physical properties of the sugar solutions with high precision and accuracy.

Figures 2a and 2b show an example of link between the variation of the intensity of NMR signals of water and the different spreading behaviours of drops over time of fructose aqueous solutions in relation to their different concentrations (12% w/w and 23% w/w).

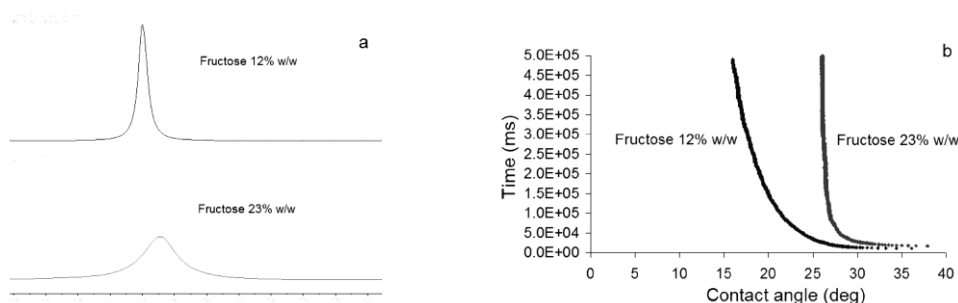


Figure 2 Comparison between (a) NMR and (b) CA data of Fructose aqueous solutions with different concentrations.

The comparison between NMR and CA data shown in Figure 2 clearly demonstrated the influence of the free water on the wettability characteristic of glass with aqueous solutions having different concentration of Fructose. In Figure 3a and 3b are reported an example of correlation between rheological and CA data of two kind of ternary sugar aqueous solutions having opposite surface tensiometry characteristic and determined using glass such as a test solid. (solutions 1 and 4) .

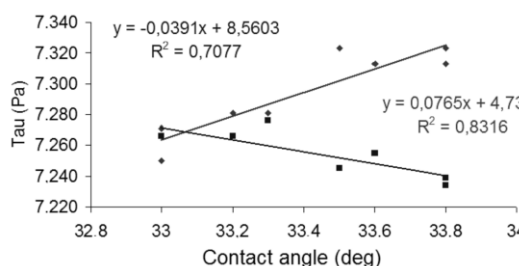


Figure 3a Correlation between theologic and CA data of ternary sugar aqueous solution 4.

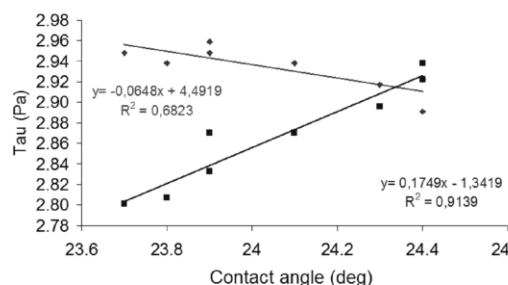


Figure 3b Correlation between theologic and CA data of ternary sugar aqueous solution 1.

7. Conclusions and Future Perspectives

This study allowed the development of six ternary sugar aqueous solutions which components are typical of the honey. The good correlations between chemistry (NMR, water solubility), structure (rheology), and surface (CA) validated the method that led to the formulation of ternary sugar aqueous solutions having complementary features from binary sugar aqueous solutions by the surface tensiometry approach. These results are fundamental for the preparation of new "honey-like" formulation having different nutritional characteristics and *in vitro* release property of a compound having nutritional activity and the experiments of A4 are currently ongoing.

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Artificial Insulin Secretory Granules' Membranes: Thermodynamic Stability and Interaction with Food Fatty Acids

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This PhD thesis dealt with the preparation and both the thermodynamic and spectroscopic characterization of model cell membranes which highly simulate the phospholipid bilayer of Langerhans β -cells' Insulin Secretory Granules (ISGs) in order to understand the effects of the lipid composition and the action of foodborne stress (high FFAs concentrations) on membrane stability in the frame of T2DM. Furthermore, the interaction between the ISG-like membrane and a pore-forming peptide was studied in order to highlight the role of cell membrane stability on the pathogenesis of T2DM.

Membrane artificiali di granuli secretori di insulina: stabilità termodinamica e interazione con acidi grassi alimentari

Questa tesi di dottorato ha riguardato la preparazione e la caratterizzazione termodinamica e spettroscopica di membrane cellulari modello che simulano ampiamente il doppio strato fosfolipidico dei granuli secretori di insulina (ISGs) delle cellule β di Langerhans al fine di comprendere gli effetti della composizione lipidica e dello stress di origine alimentare (alte concentrazioni di FFAs) sulla stabilità della membrana nell'ambito del T2DM. Inoltre, l'interazione tra la membrana simil-ISG e un peptide pore-forming è stata studiata al fine di evidenziare il ruolo della stabilità della membrana cellulare sulla patogenesi del T2DM.

Key words: Model membranes; Free fatty acids; Differential scanning calorimetry; Insulin secretory granules.

1. Introduction

In accordance with the PhD thesis project previously described (Saitta, 2017), this oral communication reports the main results of the following three activities directed to:

A1) prepare and characterize different model cell membranes through calorimetric and spectroscopic techniques by varying their composition and their morphology;

A2) assess the influence of the environment on membrane thermodynamics by including different FFAs at various concentrations into the model membranes preparations;

A3) study the interaction between membrane and a pore-forming peptide in order to highlight the role played by the lipid composition of the membrane and by the presence of FFAs in this process.

2. Model Cell Membranes and the Calorimetric Approach

The structure of lipid vesicles makes them exemplary model systems able to provide simulations of either cytoplasmic membrane or cell compartments, such as organelles and vesicles involved in endo- and exocytosis. It is clear that it is hard to perfectly mime a real biological membrane. However, despite the numerous studies on model membranes carried out mainly through calorimetric, spectroscopic and imaging techniques, such model

systems were characterized by a simple composition which does not keep into consideration the complexity and the asymmetry of the real bilayers (Rothman & Lenard, 1977). Besides that, some physical features were also not always considered in an appropriate manner, such as membrane curvature. In this frame, simulating peculiarities of a real cell membrane might be a key point for the correct interpretation of membrane-based mechanisms in cells since each factor might produce modifications in membrane thermodynamics (Saitta et al., 2019) (Table 1), which in turn might lead to functionality modifications.

Table 1 *Main factors that may influence cell membrane thermodynamics and functionality.*

Factor	Example
Morphology	Multilamellarity Curvature
Composition	Phospholipid tails length and presence of unsaturations Phospholipid headgroups Presence of non-phospholipid constituents (e.g. cholesterol, etc.)
Inner and/or outer environment	pH Cations' charge Presence of "perturbing" agents (e.g. FFAs, membrane's proteins, peptide oligomers or fibrils, etc.)

A key technique for the investigation of cell membrane thermodynamic stability is represented by the Differential Scanning Calorimetry (DSC). Briefly, the sample and the reference cells undergo the same temperature scan program, at constant pressure, and the heat flow difference (in watts) between the two cells is recorded. In the resulting thermogram, the apparent molar heat capacity trace, $C_p^{app}(T)$, is scaled with respect to the pre-transition trace to obtain the excess molar heat capacity, $C_p^{exc}(T)$, where the heat capacity at constant pressure, C_p , is defined as:

$$C_p \equiv \left(\frac{\partial H}{\partial T} \right)_{p,n} \quad (1)$$

and H is enthalpy, T is the temperature, p is the pressure and n is the mole number. At $p=\text{const}$ and $n=\text{const}$, the heat Q involved in a process corresponds to the enthalpy variation that, after the integration of Eq. 1, becomes:

$$Q = \Delta H = \int_{T_1}^{T_2} C_p \cdot dT \quad (2)$$

Since the thermogram reports C_p^{exc} versus T , the peak area represents the transition ΔH .

Providing heat to phospholipid bilayers leads to a gel-to-liquid crystalline phase transition that affects the van der Waals interactions between hydrocarbon chains, increasing their mobility. Variations in the transition temperature and enthalpy can reveal important information related to the membrane physicochemical properties.

3. FFAs on Cell Membranes

Low levels of Free Fatty Acids (FFAs) are naturally present in biological membranes (around 0.3-10% of total lipids) (O'Connor et al., 1999) as well as in plasma. They have been shown to be involved in several membrane-mediated cellular processes as membrane-bound enzyme activity, lipid-assisted protein transport across the bilayer, fusion of lipid vesicles and cells and/or alteration of the microdomains of cell phospholipid bilayers

as well as their physical properties (Ibarguren et al., 2014). Moreover, they also act as signalling molecules for several cell mechanisms, for instance insulin secretion (Itoh et al., 2003). In particular, insulin is co-stored with another peptide, amylin, within Langerhans β -cells' Insulin Secretory Granules (ISGs). These hormones are co-secreted in blood stream in response of nutrients intake.

However, altered FFAs levels are generally associated to pathological states. For instance, they are recurrent in diabetic and/or obese subjects and plasma FFAs concentration is generally high in both with levels that tend to be increasingly higher with the increase of weight (Björntorp et al., 1969). Specifically, for Type 2 Diabetes Mellitus (T2DM), studies regarding the possible involvement of FFAs in the onset and/or progression of the disease are widely reported in the literature, referring both to their possible involvement in altered metabolic pathways and to the direct action of FFAs on membranes. Moreover, the action of FFAs has also been hypothesized to play a role in the interaction of amylin, an amyloidogenic protein, with cell membranes likely leading to the pancreatic β -cells failure by apoptosis (Westermarck et al., 2011).

Several studies are reported to highlight the FFAs-membrane interaction mainly using spectroscopic, imaging, molecular dynamics and/or theoretical approaches, but only few works are devoted to a thermodynamic characterization of the role of the FFAs on the overall membrane stability, in any case by considering highly simplified systems (Ortiz & Gómez-Fernández, 1987).

4. Experimental Procedure

In this PhD thesis I performed a stepwise thermodynamic study of several unilamellar phospholipid vesicles at physiological pH by mainly using High Sensitivity DSC with the purpose of simulating as best as possible the ISG phospholipid bilayer. Such vesicles were prepared with different size and increasing compositional complexity, discriminating the phospholipid tails and headgroups contribution to the membrane stability and achieving the preparation of a fourteen-components final model membrane which represents the 80% of the phospholipids' tails and the 80% of the headgroups in real ISGs (MacDonald et al., 2015). Moreover, the influence of cholesterol and several FFAs was investigated highlighting the magnitude of the effects on such a detailed membrane.

5. Materials and Methods

Small, Large and Giant Unilamellar Vesicles (SUVs, LUVs and GUVs, respectively) were prepared through different evaporation methods depending on vesicle size. LUVs and SUVs were then extruded through polycarbonate filters (pore size of 400nm and 100nm, respectively) mounted on a heated mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA) fitted with two 1mL gastight syringes (Hamilton, Reno, NV, USA).

A Setaram micro DSCIII (Setaram Instrumentation, Caluire, France) operating with 1mL hermetically closed pans was used for SUVs, LUVs and GUVs at 0.5°C/min scanning rate. Vesicles size was detected at 25°C in a light-scattering instrument (Litesizer™ 500, Anton Paar, Graz, Austria) in side-scatter mode for SUVs and LUVs and in back-scatter mode for GUVs. Measurements on membrane fluidity were also carried out through fluorescence anisotropy measurement by using a PerkinElmer LS55 Fluorescence Spectrometer (PerkinElmer, Waltham, MA, USA) equipped for fluorescence polarization measurement under controlled temperature both in isothermal conditions and by applying heating ramps.

Since the obtained calorimetric profiles were often complex and not well only described by the peak's maximum temperature, T_{max} , in this thesis two new parameters were additionally proposed (Saitta et al., 2019): the *Transition Average Temperature*, $\bar{T}$, as

$$\bar{T} = \int_{T_0}^{T_f} T \cdot f(T) dT \quad (3)$$

where the frequency function, $f(T)$, is just the normalized calorimetric peak distribution

$$f(T) = \frac{C_p^{exc}(T)}{\Delta H^o}, \quad (4)$$

and the *Average Cooperativity Index*, ACI , defined as

$$ACI = \sqrt{\int_{T_0}^{T_f} (T - \bar{T})^2 \cdot f(T) dT} \quad (5)$$

in order to better estimate the cooperativity and the peak width in multicomponent and/or multiphasic samples (e.g. the higher the ACI , the higher the peak width and cooperativity). This approach is a statistical distribution approach applied to calorimetric signals.

6. Results and Discussion

6.1 Contribution to membrane thermodynamic stability

The DSC profiles for unilamellar vesicles with different size revealed that curvature effects are relevant for one-component vesicles, but become of minor importance in more complex membranes, also including those with binary compositions (Fig. 1).

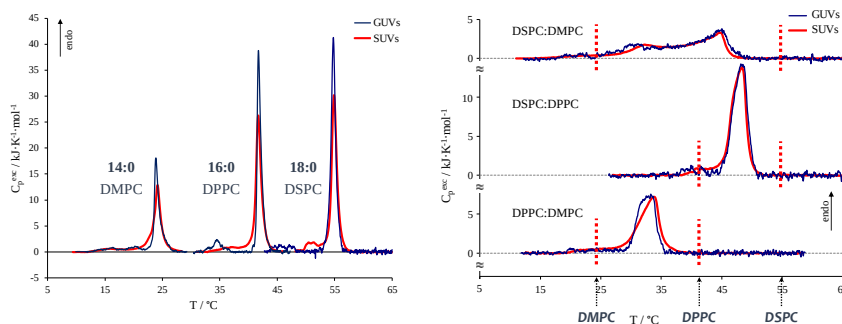


Figure 1 Micro-DSC thermograms for SUVs and GUVs prepared as one-component (left) and binary mixtures (right). In these samples, acyl chains with different length were considered by keeping the phospholipid headgroup the same.

Vesicles with binary and ternary compositions were studied and the effects of different phospholipid acyl chains and headgroups were determined by considering phospholipid with the same headgroup and acyl chain, respectively. The overall stability of mixed membranes depended on the phospholipid's molar ratio and the constituents' contribution was somehow "additive" from both an entropic ($\bar{T}$ in Fig. 2a) and an enthalpic point of view. However, such an additivity rule was only followed for completely saturated membranes. Indeed, the presence of unsaturated phospholipids, even in small amounts, had a relevant overall destabilizing effect and enhanced the homogeneousness of the phase distribution. As for the magnitude of the effects in terms of ACI , i.e. transition

cooperativity and peaks width, the trends highlighted in Fig. 2b revealed that the differences in tails length are able to produce a more severe dispersion of the membrane's thermodynamic phases than the differences in headgroups.

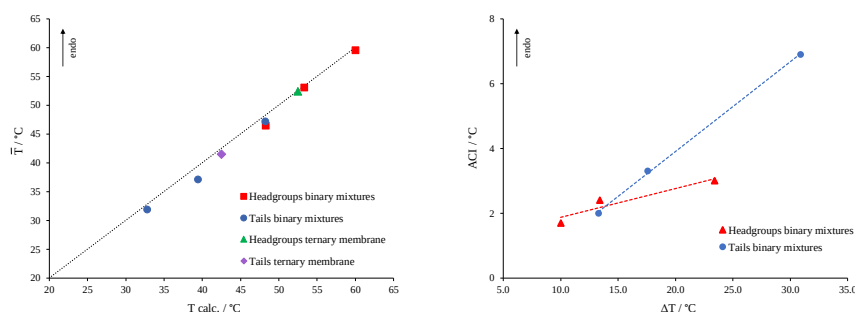


Figure 2 On the left, trend of the transition average temperature, $\bar{T}$, of several representative model membranes versus the arithmetic average of the related pure systems' temperature, T_{calc} . On the right, representation of the influence of the gap between the T_{max} values of the related pure systems, ΔT_{max} , on ACI values when generated by differences in headgroups or acyl chain length.

6.2 Influence of cholesterol and FFAs

Cholesterol addition at different percentages (5%, 10% and 20%) produced phase rearrangements and separation beside a progressive enthalpy depletion until the achievement of saturation (Table 2).

Table 2 Thermodynamic parameters for some membranes. The heating curves were used to obtain ΔH° , T_{max} , $\bar{T}$ and ACI. The ISG-like membrane consists in the fourteen-components membrane with 10% of cholesterol.

System	ΔH° kJ·mol ⁻¹	T_{max} °C	$\bar{T}$ °C	ACI °C
Fourteen-components membrane	35 ± 2	50.4 ± 0.5	45.5 ± 0.3	6.3 ± 0.2
+5% CHOL	26 ± 2	45.5 ± 0.3	45.4 ± 0.3	6.7 ± 0.2
+10% CHOL	23 ± 2	44.8 ± 0.3	43.2 ± 0.3	5.7 ± 0.2
+20% CHOL	18 ± 2	46.4 ± 0.3	43.7 ± 0.3	6.0 ± 0.2
ISG-like membrane				
+20% SA	34 ± 2	52.1 ± 0.3	48.4 ± 0.3	6.6 ± 0.2
+20% OA	15 ± 2	42.6 ± 0.3	38.0 ± 0.3	5.8 ± 0.2
+20% EA	29 ± 2	45.7 ± 0.3	43.5 ± 0.3	5.2 ± 0.2

The fifteen-components membrane containing 10% of cholesterol was selected as reference final ISG-like membrane. Considering three FFAs as example, a stabilizing effect for the saturated FFA (stearic acid, SA) and a destabilizing effect for the *cis*-unsaturated FFA (oleic acid, OA) were observed in terms of enthalpic and entropic contributions (Fig. 3 and Table 2), revealing a similar scenario obtained for less complex membranes (Saitta et al., 2019). The *trans*-unsaturated FFA (elaidic acid, EA) showed a small enthalpic stabilization without consistent entropic effects. Indeed, though we observed a slight shift

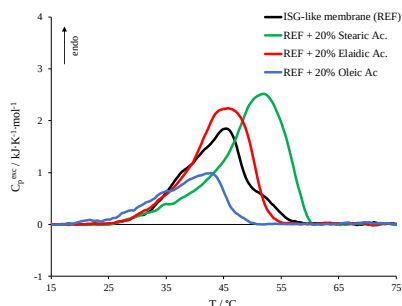


Figure 3 Micro-DSC profiles for an ISG-like vesicle (black curve) and vesicles with the addition of 20% FFAs. Thermograms are reported for membranes including stearic acid (green curve), elaidic acid (red curve) and oleic acid (blue curve).

of the T_{max} towards higher temperatures, the $\bar{T}$ did not undergo any variation, revealing that elaidic acid only led to a moderate phase reorganization.

Given all the obtained thermodynamic data, I was able to predict and prepare an *ad hoc* simpler membrane resembling the ISG-like one's thermodynamics. Such a redirecting to a simpler membrane was allowed since the investigated FFAs revealed the same thermodynamic behaviour regardless of the membrane compositional complexity. Moreover, the simplification of membrane composition would have allowed a better understanding of the membrane-peptide interaction in FFAs-added vesicles.

Beside the micro-DSC data, membrane fluidity measurements were also performed for six incorporated FFAs and are reported in Fig. 4. Saturated and *trans*-unsaturated FFAs decreased the fluidity of the lipid bilayers, whereas it became higher when *cis*-unsaturated FFAs were added.

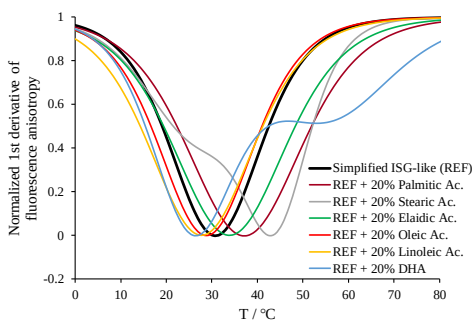


Figure 4 Normalized 1st derivative of fluorescence anisotropy of DPH probe in vesicles with 20% of FFAs compared to the FFA-free simplified ISG-like membrane. Lipid:probe molar ratio was 500:1. The tested FFAs were: stearic and palmitic acids as saturated FFAs; elaidic acid as *trans*-monounsaturated FFA; oleic acid as *cis*-monounsaturated FFA; linoleic (ω -6) and docosahexaenoic (ω -3) acids as *cis*-polyunsaturated FFAs.

6.3 Membrane-peptide interaction

Nisin, a pore-forming peptide, was selected for the study of membrane-peptide interaction in order to further investigate the effects of different FFAs. Fluorescence anisotropy measurements at different nisin concentrations showed an increase of anisotropy values (more ordered structure with higher peptide concentration). Last

measurements with different techniques (e.g. micro-DSC) are still in progress and are crucial for the interpretation of the fluorescence anisotropy results.

7. Conclusions and Future Perspectives

The present work allowed the comprehension of the effect of several cell membranes' properties on their physicochemical behaviour, which is known to influence their functionalities in biological environments. Therefore, such a stepwise preparation and characterization of a highly complex model membrane (an ISG-like vesicles with fifteen components) represented strong thermodynamic foundations for any following discussion in the frame of food-health relationship from a molecular point of view, as well as it will for other biological aspects. Moreover, the deep analysis of the influence of lipid composition also held data on the behaviour of cell membranes of subjects who preferentially consume food rich in saturated or unsaturated fatty acids.

Several FFAs that differ for their chemical structure were selected, taking into consideration two saturated FFAs, two monounsaturated FFAs (*cis*- and *trans*-stereoisomers) and two polyunsaturated ones (an ω -3 and an ω -6). For instance, their impact at high concentration was tested on model membranes with different complexity levels and revealed that bent molecules as those containing *cis*-unsaturations have moderate destabilizing effects (moderate increase of bilayer fluidity) if compared to the severe stabilizing effects of straight-chain molecules. As for the last case, such stabilization would represent a wrong condition from a biological point of view since cell membranes appear too stiff.

Finally, the study of nisin-membrane interaction in presence of FFAs paved the way for the thermodynamic and kinetic investigation of the interaction with more complex structures as amyloidogenic peptides (e.g. amylin) allowing the expansion of this approach not only to T2DM but also to other similar conditions (as for the molecular mechanisms involved) as Alzheimer's and Parkinson's diseases. Moreover, the thermodynamic information achieved through this work will also allow the investigation of other cell membranes trying to highlight possible molecular mechanisms hidden behind the relationship between nutrition and health.

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Cereal Milling By-Products Valorisation: from Unexploited Residues to Valuable Resources

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This PhD thesis dealt with the recovery and valorization of industry-scale cereal milling by-products starting from the safety assessment, passing to the application of solid-state fermentation, air classification and micronization as innovative techniques and finally the evaluation of nutritional and sensory improvements of the novel high-value raw materials obtained.

Valorizzazione dei sottoprodotti della lavorazione dei cereali: da residui a preziose risorse

Questa tesi di dottorato ha riguardato il recupero e la valorizzazione dei sottoprodotti della lavorazione dei cereali includendo gli aspetti relativi alla sicurezza, l'applicazione di fermentazione a stato solido, turboseparazione e micronizzazione come tecniche innovative ed infine la valutazione sensoriale e nutrizionale dei nuovi prodotti ottenuti.

Key words: cereal milling by-products, solid state fermentation, bioactive compounds, air-classification, micronization, rice bran lipid profile.

1. Introduction

In accordance with the PhD thesis project previously described (Spaggiari, 2017), this oral communication reports the main results of the following tasks directed to:

A1) the assessment of the mycotoxins contamination of the milling by-products and the concomitant profiling in terms of bioactive compounds underling the impact of the processing on such residues;

A2) study and apply lactic acid fermentation in solid state (SD) to wheat bran using a single lactic acid bacteria isolated from a dairy product (LAB), thus to evaluate the nutritional, sensorial and overall quality changes of treated wheat bran;

A3) investigate the air-classification and micronization techniques as sustainable and improving techniques for the concentration of lipid compounds with emulsifying properties present in rice bran;

A4) assess the nutritional improvements of bread enriched with pre-fermented wheat bran in different cell line cultures (IEC-6, rat small intestine epithelial; CCD-18, human colon myofibroblast; CaCo-2, human epithelial colorectal adenocarcinoma), the effects on the glucose transporters and the bioavailability of bioactive compounds (carried out during the exchange study period performed at CIAL-CSIC of Madrid, Spain, from November 2018 to July 2019).

2. Cereal milling by-products

Cereal grains are considered as staple food in many countries. Among them, maize, rice and wheat yield approximately 87% (FAOSTAT; 2017) of worldwide cereal production. Nevertheless, these products are rarely consumed as whole grain, in fact they undergo to

a several processes that are commonly known as milling (selection, sieving, grinding, etc.). The final result is the separation of the outermost layers of the seed from the endosperm which is mainly used for the most spread baked goods production (breads, pasta, etc.). These residue fractions are generally composed by bran (aleurone, pericarp, testa) and germ (caryopsis embryo) and constitutes about 21-24% of milling plant outputs (Serna-Saldivar, 2010), translatable to a massive hardly manageable material production. The composition of these by-products can vary among cereal species, but in general is where vitamins, lipids, proteins and bioactive compounds are mostly concentrated (Prückler et al., 2014). Despite their good nutritional properties, these by-products are mainly directed to the feed formulation manufacturing, losing their potential as food ingredients.

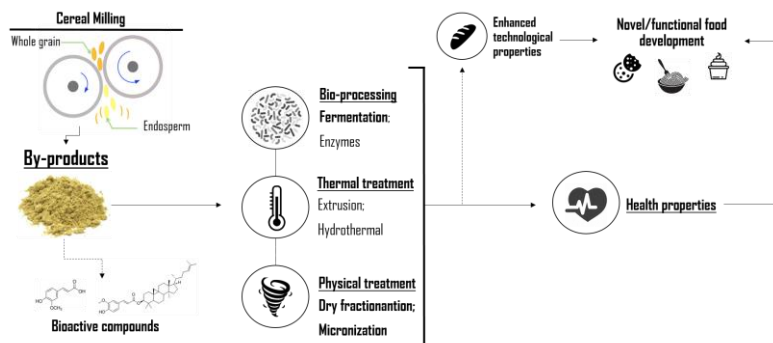


Figure 1 Technologies used for the valorization of cereal milling by-products. In bold are depicted the techniques evaluated in present study.

3. Innovative agro-food by-products valorization techniques

During last years the agro-food industry has placed particular interest on the recovery and valorisation of industrial processing by-products in order to lower the potential impact on the environment while achieving a competitiveness improvement on the food market. The most challenging aspect for the cereal by-products recovery is their detrimental effects on food preparation and the resulting low sensory profile of the final product (Heiniö et al., 2016). In this context, many innovative technologies have been studied (**Figure 1**) for the cereal by-products exploitation. In particular, lactic acid fermentation (LAF) and physical treatment (PT) are considered promising techniques (Rizzello, Coda, Mazzacane, Minervini, & Gobetti, 2012; Verni, Rizzello, & Coda, 2019) for the overall quality improvement of cereal processing residues. LAF, for example, can improve the nutrient profile of by-products through the release of bound and non-absorbable phenolic compounds enhancing their bioavailability or more in general produce novel bioactive metabolites. Nevertheless, several aspects must be studied and clarified for the complete knowledge of these methodologies.

4. Experimental Procedure

In this PhD thesis a bottom-up approach, divided in three interconnected macro areas was followed. First (I), mycotoxins and their metabolites, phenolic acids (PAS), betaine and choline were identified and quantified in wheat, rice and maize whole grain and corresponding milling by-products in order to select the most valuable matrices for the next valorisation step. After that (II), durum wheat bran was submitted to solid state lactic acid fermentation and its effect on phenolic compounds, arabinoxylans (WEAX), phytates (PAC), *in vitro* bioactivity (antioxidant capacity, AOC), *ex-novo* formation of novel metabolites and volatile profile were studied. Meanwhile, rice bran (RB) was treated using air classification

and micronization technology and mono- (MAG), di- (DAG) and triacylglycerols (TAG) were profiled in the resulting fractions. Lastly (III), the fermented pre-treated wheat bran was included in a lab-scale high-fiber bread formulation, consequently digested under *in vitro* gastrointestinal simulated conditions and the samples obtained were studied for their cell viability, intra-cellular reactive oxygen species (ROS) counteraction, anti-inflammatory activity (nitric oxide, NO, production) under fisiological and inflamated cell conditions.

5. Materials and Methods

According to the studies conducted during the project development, advanced analytical techniques (liquid and gas chromatography, coupled to high- and low-resolution mass spectrometry) were used for the identification of compounds of interest. Furthermore, for the SD fermentation of wheat bran, *Lactobacillus rhamonsus* 1473 (isolated from Parmigiano-Reggiano) was used and microbiological counting methodologies (total bacteria and spore-forming bacteria) were carried out. Antioxidant capacity (AOC) and total phenolic content (TPC) were assessed using spctrophotmetrical assays, likewise phytic acid and WEAX. The air-classification and micronization processing were performed at SeparMicroSystem sas (Brescia, Italy). Finally, the development of the cell cultures experiments was done under sterile and controlled conditions and performend in three independent times.

6. Results and Discussion

6.1 Impact of milling processing on mycotoxins and bioactive compounds

The monitoring of contaminants during cereal grains processing is essential for the safety assessment of the entire food chain. In fact, tricothecenes and their modified forms are the most studied class of mycotoxins found in cereal crops. In our study the quantification of 15-Acetyl-Deoxynivalenol (15-Ac-DON), 3-Acetyl-Deoxynivalenol (3-Ac-DON) deoxynivalenol (DON), deoxynivalenol-3-Glucoside (DON-3-Glc) nivalenol (NIV), zearalenone (ZEN), T2 and HT2 toxins was assessed on five different cereal species (2 cultivars of *Tritordeum*, bread and durum wheat and barley) whole grains and corresponding pearled fractions (from 0-5%, outer layers, to 40-100%, inner part). As an example, the DON and DON-3-Glc content found in infection-sensitive durum wheat cv. Saragolla and resistant cv. bread wheat Illico whole grain and pearled fractions are depicted in **Figure 2**. Arguably, a decreasing trend on mycotoxins content were found from the outermost layers (0-5%) to the inner fractions corresponding to the endosperm (40-100%). Nevertheless, the most relevant result to point out was the firstly reported identification of oligoglicoside DON forms in natural contaminated durum wheat (Spaggiari et al., 2019).

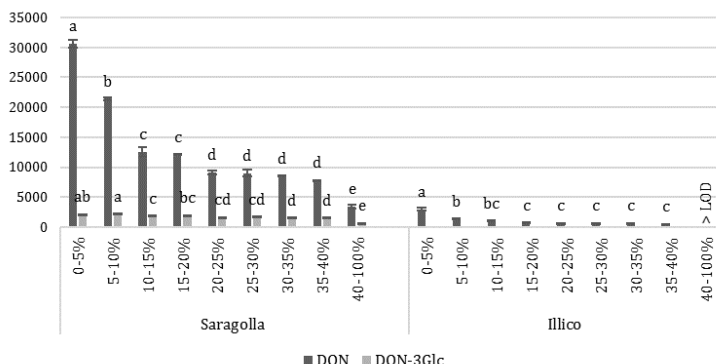


Figure 2 DON and DON-3-Glc concentration ($\mu\text{g kg}^{-1}$ d.w.) among pearled fractions durum wheat (cv. Saragolla) and bread wheat (cv. Illico). Different letters on top of each bar indicate a significant difference ($p < 0.05$) using Tukey-b's post-hoc test.

Table 1 Free and bound phenolic acids content ($\mu\text{g g}^{-1}$ d.w.) of *T. aestivum* L, *T. turgidum* subsp. *turanicum* and corresponding milling by-products.

Wheat species	Milling product	4-HB		p-C		Caff		Sin		t-Fer	
		Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Bread wheat	BWG	0.11±0.05b	2.30±0.07b	0.07±0.01	5.02±0.44b	0.31±0.06b	1.22±0.03b	1.19±0.08c	40.29±3.24ba	1.94±0.65b	564.15±0.88c
	F	<LOQ	0.30±0.04b	<LOQ	<LOQ	0.14±0.01b	<LOQ	<LOQ	2.38±0.25b	<LOQ	19.00±1.53d
	BB	0.48±0.19a	11.73±3.69a	0.08±0.02	16.91±5.34a	0.78±0.14a	4.92±1.4a	7.93±1.42a	123.77±49.82a	9.52±1.56a	1330.79±7.93a
	BM	0.19±0.04b	5.02±0.12b	0.09±0.01	9.12±0.35b	0.39±0.00b	0.87±0.10b	3.35±0.37b	84.96±1.94ba	3.07±0.74b	1148.41±63.76b
	TWG	0.10±0.02b	1.48±0.29c	0.08±0.01	4.44±0.06c	0.12±0.00	0.53±0.05b	3.71±0.92b	13.42±1.16b	1.02±0.29c	208.33±15.05c
Turanicum wheat	TF	<LOQ	0.63±0.00c	<LOQ	1.15±0.27d	0.12±0.00	0.16±0.01c	<LOQ	4.78±0.86b	<LOQ	66.40±3.03d
	TB	0.63±0.25a	10.87±0.39b	0.19±0.09	20.78±0.34b	0.14±0.01	3.16±0.05a	6.08±1.23a	59.70±1.57a	6.80±1.82a	1571.14±59.63a
	TM	0.41±0.10b	12.70±0.46a	0.16±0.05	25.71±1.39a	0.14±0.00	3.07±0.13a	2.99±0.41b	68.36±6.65a	3.84±0.60b	1331.04±74.22b

4-HB: 4-hydroxybenzoic acid; p-C: p-Coumaric acid; Caff: caffeic acid; Sin: sinapic acid; t-Fer, trans-ferulic acid. Different letters in the same column indicate a significant difference ($p < 0.05$) using Tukey-b's post-hoc test. <LOQ: 0.05 $\mu\text{g g}^{-1}$ d.w.

Annually, about 300 million tons of cereal milling by-products are produced by the agro-food sector and their final use is mainly as animal feed. Since this approach has a lower valorization potential than applications in food, either directly or after tailored processing, the milling industry is increasingly interested in finding new strategies of residues re-utilization.

6.2 Lactic acid SD fermentation nutritional and sensory improvements

The *T. turgidum* subsp. *turanicum* bran was fermented during 48h after a sterilization step (121°C 20 min). The mainly changes of total phenolic content (TPC), antioxidant capacity (evaluated using DPPH, ABTS and FRAP tests) in their bound and free form, PAC and WEAX of treated wheat bran compared to non-fermented wheat bran are reported in **Table 2**. Fermented wheat bran showed a higher overall soluble *in vitro* bioactivity. In addition, phytic acid content decreased almost 37 % in respect to raw wheat bran, while WEAX increased three times after 48 hours of fermentation. Moreover, ten high-value amino acid and lipid degradation metabolites were identified in fermented wheat bran (data not presented).

Table 2 Changes of chemical features in wheat bran samples.

Sample	TPC mg GAE Kg ⁻¹		DPPH		ABTS mm TEAC g ⁻¹		FRAP		PAC g 100 gr ⁻¹	WEAX mg g ⁻¹
	Free	Bound	Free	Bound	Free	Bound	Free	Bound		
WB	1174.9±18 4.7b	2451.2±10 9.4b	3.6±0.1 b	3.6±0.1 b	3.6±0.1 b	3.6±0.1 b	3.6±0.1 b	3.6±0.1 b	2.7±0. 2a	12.6±0. 1d
AWB	1043.5±0.7 c	3203.5±0.0 a	17.7±0. 2a	17.7±0. 2a	17.7±0. 2a	17.7±0. 2a	17.7±0. 2a	17.7±0. 2a	2.6±0. 0a	14.7±0. 2c
FWB 24	1447.2±17 8.1a	2343.8±31 5.0b	2.2±0.0 c	2.2±0.0 c	2.2±0.0 c	2.2±0.0 c	2.2±0.0 c	2.2±0.0 c	2.3±0. 2b	22.7±2. 9b
FWB 48	1553.3±70. 4a	2271.1±37 4.1b	17.9±1. 0a	17.9±1. 0a	17.9±1. 0a	17.9±1. 0a	17.9±1. 0a	17.9±1. 0a	1.7±0. 1c	32.4±2. 8a

Results are represented as mean of three biological replicates (n=3) and three experiment replicates (n=3) ± standard deviation. Data with different letters are significantly different (p<0.05), following the Tukey b's post-hoc test. WB, raw wheat bran, AWB, autoclaved wheat bran, FWB24, fermented wheat bran after 24h, FWB48, fermented wheat bran after 48h, PAC: phytic acid. WEAX: water-extractable arabinoxylans.

Finally, the fermented wheat bran aroma profile was characterised by a strong floral/fruity note, while woody/oxidized flavours were found in non-fermented bran (**Figure 3**).

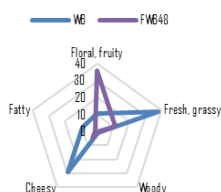


Figure 3 Overall odor profile of raw wheat bran (WB) and fermented wheat bran (FWB48). Results are the sum of the compounds identified according the volatile profile analysis with the same odor note

6.3 Concentration of MAG, DAG and TAG in air-classified and micronized rice bran

Rice bran has a not negligible amount of lipid content (15-21%, Sohail, Rakha, Butt, Iqbal, & Rashid, 2017). Among lipid compounds, mono- and diacylglycerols are receiving more attention for their emulsifying properties (Suman et al., 2009). In **Table 3** are reported the results regarding the impact of air-classification and micronization on the lipid components of rice bran. Air-classification significantly concentrated the lipid content in fine fraction, however the micronization did not further increased this parameter. In addition, the saturated MAG also increased significantly in this fraction. Nevertheless, the predominant lipid class of rice bran oil were TAG.

6.4 Evaluation of bioactive properties of pre-fermented wheat bran enriched bread

A wheat bread was formulated using pre-fermented wheat bran (BFB) in order to increase its overall nutrient profile; thus, a white bread (WhB) and non-treated bran bread (BrB) were used as control. After the *in vitro* simulated gastrointestinal digestion, the soluble fraction was recovered and used as treatment in cell culture lines at different concentration (0.01-2 mg mL⁻¹). Concentrations lower than 1 mg mL⁻¹ did not affect the cell mitochondrial activity (cell viability > 80%). Moreover, the BFB and BrB digesta presented a significant activity to contrast the ROS production, under physiological conditions but not under induced inflamed status. In addition, NO production was evaluated as anti-inflammatory measure and BFB presented a significant difference in respect to the control.

Table 3 Yield, crude fat content and characteristic lipid attributes of RB samples samples.

Sample	Yield (%)	Crude fat content mg/100g	%MAG*	%DAG*	%TAG*	%Saturated MAG**	Unsaturated/Saturated TAG
RB	100	15,8±1,1cd	15.9±0.8	18.2±0.9	65.9±3.3	18.5±0.9	50.1±2.5
RBc	82	14,4±1,8bcd	15.3±0.8	18.3±0.7	66.4±3.3	17.0±0.8	51.2±3.3
RBf	16	18,7±0,3ef	19.5±1	17.7±0.4	62.8±3.2	21.4±0.1	45.5±2.6
RBFf	2	18,7±1,5ef	18.7±0.9	17.7±0.9	63.5±3.1	21.7±0.3	46.7±2.3
MRB	100	14,8±1,3bca	15.3±0.8	17.9±0.7	66.8±3.3	16.3±0.8	54.9±2.7
MRBc	78,2	13,3±0,4bc	14.9±0.7	17.1±0.9	67.9±3.4	16.7±0.6	62.1±3.1
MRBF	18,8	19,6±2,0f	22.5±1.1	20.2±0.8	57.3±2.9	22.3±1.3	37.1±2.9
MRBFf	3	18,7±1,2ef	18.9±0.9	18.6±0.9	62.5±3.1	21.5±1.0	45.7±2.3

Results represent a mean of three independent analyses. a-f Different superscript letters in the same row differ significantly ($p<0.05$) using Tukey's *b* post-hoc test. * calculated in function of the total MAG, DAG and TAG content; ** in respect to the total amount of MAG. N.F., not found.

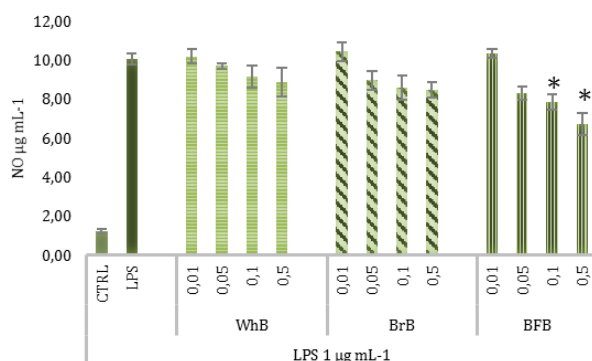


Figure 4 NO production estimated by LPS (lipopolysaccharide) on RAW 264.7 macrophage of breads digests at different concentration. * means significant difference ($p<0.001$) to control (CTRL).

7. Conclusions and Future Perspectives

Annually, about 300 million tons of cereal milling by-products are produced by the agro-food sector and their final use is mainly as animal feed. Since this approach has a lower valorization potential than applications in food, either directly or after tailored processing, the milling industry is increasingly interested in finding new strategies of residues re-utilization.

It is well-known that the health benefits of whole grain products are mainly due to several components present in the outer layers of the seed, bran and germ. However, these matrices pose several disadvantages, which complicate its application in food systems. Among these, negative sensory attributes like the bitter taste are an important issue contributing to low consumer acceptance. Thus, further research on the applicability of cereal by-products in the food manufacturing is needed. Moreover, the economic feasibility is decisive from the scale-up point of view. For this purpose, each individual pre-treatment or processing steps need to be evaluated regarding, for example, energy and resources input, possibly with strong collaboration with the local industries.

8. Nomenclature

15-Ac-DON 15-Acetyl-deoxynivalenol; 3-Ac-DON, 3-Acetyl-deoxynivalenol; 4-HB, 4-hydroxybenzoic acid; AOC, antioxidant capacity; AWB, autoclaved wheat bran; BB, bread wheat bran; BFB, bread with fermented bran; BM, bread wheat middlings; BrB, bread with non-treated bran, BWG, bread wheat whole grain; CaCo-2, human epithelial colorectal adenocarcinoma; Caff, caffeic acid; CCD-18, human colon myofibroblast; CTRL, control; DAG, diacylglycerols; DON, deoxynivalenol; DON-3-Glc, deoxynivalenol-3-Glucoside; F, bread wheat flour; FRAP, Ferric Reducing antioxidant power assay; FWB24, fermented wheat bran for 24h; FWB48, fermented wheat bran for 48h; IEC-6, rat small intestine

epithelial; LAB, lactic acid bacteria; MAG, monoacylglycerols; MRB, micronized and air-classified rice bran; MRBC, coarse fraction of micronized and air-separated rice; MRBF, fine fraction of micronized and air-separated rice bran; MRBFf, ultra fine fraction of micronized and air-separated rice bran; NIV, nivalenol; NO, nitric oxide; PAC, phytic acid; PAs, phenolic acids; p-C, p-Coumaric acid; RB, raw rice bran; RBc, coarse fraction of air-separated rice bran; RBF, fine fraction of air-separated rice bran; RBFf, ultra-fine fraction of air-separated rice bran with different operating conditions; ROS, reactive oxygen species; SD, solid state; Sin, sinapic acid; TAG, triacylglycerols; TB, turanicum wheat bran; TF, turanicum wheat flour; t-Fer, tran-ferulic acid; TM, turanicum wheat middlings; TPC, total phenolic content; TWG, turanicum wheat whole grain; WB, raw wheat bran; WEAX, water extractable arabinoxylans; WhB, white bread; ZEN, zearalenone.

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Exploitation of Energy Metabolism in Obligate Heterofermentative Lactic Acid Bacteria

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This PhD thesis is focused on the study of energy metabolism of heterofermentative lactic acid bacteria (LAB) and potential applications of respiratory phenotypes for biotechnology purposes. The production of γ -aminobutyric acid (GABA) from anaerobic, aerobic and respirative cultures has been also evaluated. Furthermore, a polyphasic approach for the identification of *Leuconostoc mesenteroides* strains at species and subspecies level was optimized.

Sfruttamento del metabolismo energetico in batteri lattici eterofermentanti obbligati

Questa tesi di dottorato ha riguardato lo studio del metabolismo energetico di batteri lattici eterofermentanti (LAB) e delle potenziali applicazioni di fenotipi respirativi per scopi biotecnologici. La produzione di acido γ -aminobutirrico (GABA) è stata, inoltre, valutata nelle colture anaerobiche, aerobiche e respirative. Infine, è stato ottimizzato un approccio polifasico per l'identificazione di ceppi di *Leuconostoc mesenteroides* a livello di specie e sottospecie.

Key words: heterofermentative LAB; aerobic and respiratory metabolism; γ -aminobutyric acid (GABA) production; phenotypic and molecular identification.

1. Introduction

In accordance with the PhD thesis project (Storti, 2017), this oral communication reports the main results concerning:

A1) the effect of atmosphere of incubation (oxygen availability) and supplementation with respiratory cofactors (hemin and menaquinone) on the growth kinetics, oxidative stress response and technological properties of heterofermentative LAB strains;

A2) the effect of carbon sources, pH, atmosphere of incubation (oxygen availability) and hemin and menaquinone supplementation on the growth kinetics, biomass yield, metabolites production and activities of enzymes related to oxygen metabolism of *Leuconostoc mesenteroides*;

A3) the effect of atmosphere of incubation (oxygen availability) and supplementation of respiratory cofactors on the GABA production from *Lactobacillus brevis* strains;

A4) identification of *Leuc. mesenteroides* strains at species and subspecies level.

2. State of the Art

LAB are a heterogeneous group of microorganisms with a wide ecological distribution. Several species have great industrial interest and are used as starter and/or adjunct cultures in the production of fermented foods; some strains with confirmed healthy

properties are exploited also as probiotics. LAB are traditionally classified as anaerobic oxygen (O_2) tolerant microorganisms. Recently, some authors have demonstrated that some strains are able to grow in aerobic conditions and to synthesize a minimal respiratory chain (i.e. electron donor NADH dehydrogenase, menaquinone e^- shuttle, terminal cytochrome *bd* oxidase, O_2 final e^- acceptor) in presence of hemin and menaquinone (Zotta et al., 2017). The shift from anaerobic to aerobic and respiratory cultivation leads to energy boost and improvements in growth, biomass production, cell robustness, oxidative stress tolerance and production of aroma compounds. Studies on aerobic and respiratory metabolism were mainly carried out in *Lactococcus lactis* (Lechardeur et al., 2011; Pedersen et al., 2012) and in other homofermentative strains (*Lactobacillus casei* and *Lb. plantarum* groups, Zotta et al., 2017), whereas scant information is available about heterofermentative ones. Physiological advantages of aerobic lifestyle have been confirmed in *Leuc. mesenteroides* (Plihon et al., 1995; 1996), *Lb. sanfranciscensis* (Jänsch et al., 2011), *Lb. panis* (Kang et al., 2013) and *Lb. buchneri* (Eikmeyer et al., 2015). Further benefits due to respiratory growth have been observed in *Leuc. mesenteroides* (Sijpesteijn, 1970), *Lb. brevis* (Brooijmans et al., 2009), *Leuc. gasicomitatum* (now reclassified as *Leuc. gelidum* subsp. *gasicomitatum*, Jääskeläinen et al., 2013), *Lb. reuteri* and *Lb. spicheri* (Ianniello et al., 2015). Since the role of heterofermentative LAB in many fermentative processes in which O_2 , heme and menaquinone are naturally present, the understanding of aerobic and respiratory metabolism could open new perspectives for exploitation of heterofermentative respiratory phenotypes in food applications. Therefore, the aim of PhD thesis was to evaluate in heterofermentative strains the effect of aerobic and respiratory conditions on the growth performance, biomass production, oxidative stress response, technological properties, changes of metabolic pattern, activities of enzymes implicated in O_2 utilization, and production of foods with high added value, such as GABA-enriched foods. It is known, in fact, that some heterofermentative LAB, such as *Lb. brevis* (Li et al., 2010) and *Lb. buchneri* (Cho et al., 2007), are GABA-producing microorganisms. Hence, the exploitation of GABA-producing respirative phenotypes could be a natural strategy for the production of GABA in food products. Moreover, since some strains used in this study had been identified by phenotypic methods, a polyphasic approach based on the analysis of RNA polymerase subunit beta (*rpoB*) polymorphism, Multiplex-PCR assay and phenotypic tests was optimized.

3. Materials and Methods

3.1 Effect of oxygen, hemin and menaquinone on the kinetics of growth and technological properties of heterofermentative strains

3.1.1 Eighteen strains belonging to the species *Lactobacillus brevis*, *Lb. fermentum*, *Leuc. citreum*, *Leuc. mesenteroides*, *Leuc. pseudomesenteroides*, *Weissella confusa*, *W. cibaria*, *W. minor* and *W. viridescens* were cultivated for 24 h at 30°C in mWMB (Ianniello et al., 2016), pH 6.8, in anaerobiosis (AN) and aerobiosis (AE) with or without supplementation of hemin (H, 2.5 µg/ml), menaquinone (M, 1 µg/ml) or both (AN, AN+H, AN+M, AN+HM, AE, AE+H, AE+M, AE+HM). Absorbance at 650 nm (A_{650}) and pH were measured at 1 h intervals for the first 8 h and at the end of incubation (24 h). Kinetics of growth were estimated with primary dynamic model (Baranyi & Roberts, 1994). O_2 uptake (Ricciardi et al., 2014) and response to hydrogen peroxide (H_2O_2) and pyrogallol (Ianniello et al., 2016) were determined in exponential and stationary growth phases.

3.1.2 The technological characterization has been performed on the strains cultivated in AN, AE and AE+HM (i.e. respiration, RS). The decrease in pH, dough leavening, exopolysaccharides (EPS) production and antimicrobial activity were evaluated according to Zotta et al. (2008). Moreover, general aminopeptidases (PepNC), proline

iminopeptidase (Pepl), glutamyl aminopeptidase (PepA), X-prolyl dipeptidyl aminopeptidase (PepX), phytase, phosphatase and β -glucosidase activities were estimated as described in Zotta et al. (2007), while catalase activity was measured according to Ianniello et al. (2016).

3.2 Factors affecting energy, aerobic and respiratory metabolism in *Leuconostoc mesenteroides*

3.2.1 *Leuc. mesenteroides* E30 was cultivated in batch fermentation in mWMB (20 g/l glucose), with or without sodium citrate, under AN and RS (15% dissolved oxygen concentration, dO₂%, supplementation with 1.25 μ g/ml H and 1 μ g/ml M) at pH 5.5 or 6.5, for 24 h at 30°C. A₆₅₀ was measured and growth kinetics were estimated (Baranyi & Roberts, 1994). O₂ uptake was determined in AN according to Ricciardi et al. (2014), while in RS by using polarographic electrode for the detection of dO₂%. The consumption or the production of metabolites (enzymatic kits), H₂O₂, pyruvate oxidase (POX), NADH oxidase (NOX), NADH peroxidase (NPR) and catalase (CAT) activities were measured as described in Ianniello et al. (2016).

3.3 GABA production from *Lactobacillus brevis* strains

3.3.1 Eleven *Lb. brevis* strains cultivated at 30 °C in MRS and MRSG (MRS supplemented of 1% w/v monosodium glutamate, MSG), pH 6.8, were screened for GABA production. After 24 h and 48 h of incubation, the A₆₅₀ and pH values were measured, and surnatants were recovered. GABA production in surnatants was qualitatively measured with a TLC (thin-layer chromatography) assay (Lim et al., 2017).

3.3.2 The biomass recovered from MRS and MRSG cultures at both 24 h and at 48 h (section 3.3.1) was resuspended and incubated (up to 24 h, 37 °C) in buffer containing 50 mM glutamic acid and 0.1 mM Tween 80. At the end of incubation, the complex pH indicator methyl red/methylene blue was added and the change of colour from violet (pH < 5.4) to green (pH > 5.4) was annotated (modified from Yang et al., 2006).

3.3.3 Five selected strains were cultivated in mWMB (Ianniello et al., 2016) and mWMB supplemented of 1% w/v MSG (mWMBG), pH 6.8, in AN (static bottles), AE (O₂, shaken flasks) and RS (O₂ and supplementation of H, 2.5 μ g/ml, and M, 1 μ g/ml, shaken flasks) conditions for 24 h at 30 °C. A₆₅₀ and pH values were measured at 1 h intervals for the first 9 h and at 24 h, and growth kinetics were estimated (Baranyi & Roberts, 1994). GABA production was measured in the surnatants collected during the growth as described in section 3.3.1.

3.4 Identification of *Leuconostoc mesenteroides* at the species and subspecies level with a polyphasic approach

3.4.1 A collection of 92 strains belonging to different species of the genera *Lactobacillus*, *Leuconostoc* and *Weissella* was used in a polyphasic approach. A *Leuc. mesenteroides* species-specific PCR assay was developed to amplify the variable region of RNA polymerase subunit beta (*rpoB*) gene and the amplicons purified, sequenced and aligned were used for phylogenetic analyses.

3.4.2 *rpoB*, L-arabinose isomerase (*araA*), dextransucrase (*dsr*) and PTS-sorbose transporter subunit IIC (*sorA*) were selected and used in a Multiplex-PCR assay. *rpoB* was used as taxonomic marker for species identification, while *araA*, *dsr* and *sorA* as subspecies-discriminant genes.

3.4.3 The strains were phenotypically tested for the evaluation of colony morphology (size, colour) on mMRS (Ricciardi et al., 2015), acidification (yellow colonies) on aMRS (MRS agar containing L-arabinose as carbon source), production of dextran (translucent slimy colonies) on sMRS (MRS agar containing sucrose as carbon source), acid production from carbohydrates (L-arabinose, cellobiose, fructose, galactose, D-glucose, D-xylose, lactose, maltose, ribose, sucrose, trehalose) and ability to grow at 37 °C. Statistical analyses were performed using R 3.4.2 (R Core Team, 2017).

4. Results and Discussion

4.1 Effect of oxygen, hemin and menaquinone on the kinetics of growth and technological properties of heterofermentative strains

4.1.1 The presence of O₂ positively affected the growth rate of many strains; otherwise, the effect of hemin and/or menaquinone was strain specific. Generally, hemin supplementation significantly improved the growth rate both in AN and when used alone in AE, whereas a positive effect due to menaquinone on the growth was found in some aerobic cultures. The ability to consume O₂ and the growth rate were not correlated, whereas pH was inversely related to growth. The response to oxidative stress varied among strains. Aerobic conditions improved the resistance to H₂O₂ and to pyrogallol of some strains. Aerobic growth supplemented with hemin clearly boosted the response to H₂O₂ in few strains. The tolerance to pyrogallol was satisfactorily high in all growth conditions. According to Zotta et al. (2017), these results confirmed that the activation of aerobic and respiratory metabolism improved growth, robustness and stress tolerance of heterofermentative strains.

4.1.2 AE and AE+HM (i.e. RS) enhanced the leavening and acidifying activities of some *Lb. brevis* strains. Contrarily, the EPS production and the antimicrobial activity were not affected by aerobic incubation and supplementation of respiratory cofactors. In some cases, higher PepNC, PepI, PepA and PepX activities were observed in aerobic and respiratory cultures. The degradation of phytate and the β -glucosidase activity were also increased in some strains by aerobic and/or respiratory conditions. Catalase activity of *Leuc. mesenteroides* E30 and E39 in all growth conditions suggested both manganese- and heme-dependent catalases, while the degradation of H₂O₂ was observed in *Lb. brevis* E06 and E31 and in *W. minor* E14 only in presence of hemin.

4.2 Factors affecting energy, aerobic and respiratory metabolism in *Leuconostoc mesenteroides*

4.2.1 *Leuc. mesenteroides* E30 (selected for growth performance, stress response and technological properties) gained advantages from aerobic and respiratory growth in terms of growth rate and increase in biomass. The presence of O₂ and the supplementation of hemin and menaquinone at pH 6.5 resulted in the highest growth rate. The effect of citrate on the specific growth rate varied with the incubation condition and it impaired the growth rate in RS. O₂ availability positively affected the biomass yield. Anaerobic cultures did not consume O₂, whereas a similar trend in O₂ uptake was observed in respiratory ones. The aerobic incubation caused remarkable changes in the metabolic pattern, reducing the production of lactic acid and ethanol and increasing the concentration of acetic acid. On the other hand, citrate did not affect the yields in acetic acid and ethanol. In all conditions glucose was consumed at the end of the incubation, unlike the citrate already exhausted in exponential phase. POX activity was not found in all tested conditions. NOX activity increased in the presence of O₂, while NPR activity was affected by atmosphere of incubation, supplementation and their interactions. These results confirmed the important

role of NOX-NPR coupled system in response to oxidative stress and the maintenance of the redox balance in LAB (Zotta et al., 2017). The catalase activity detected in absence of hemin suggested the potential presence of the Mn-dependent isoform. In all growth conditions, H₂O₂ was not detected and its degradation was probably due by the activity of NPR and catalase.

4.3 GABA production from *Lactobacillus brevis* strains

4.3.1 The supplementation of MSG increased the growth of few *Lb. brevis* strains after both 24 and 48 h of incubation compared to the cultivation in unsupplemented MRS. The presence of MSG also resulted in a substantial increase in pH at 24 h. After 48 h of incubation no further increase in pH was observed for all *Lb. brevis* cultivated in MRS, contrarily to those grown in MRSG.

4.3.2 The color change of pH indicator after 24 h of incubation was observed only for five strains in MRS-growing cultures and for the most of the strains cultivated in MRSG. After 48 h, instead, the color change of the indicator due to glutamic acid decarboxylase activity was observed only for MRSG cultures. Most of *Lb. brevis* strains cultivated in MRSG showed a good GABA production (significant TLC spot volume) after both 24 and 48 h of incubation, while only few strains grown in MRS had a weak GABA production (small TLC spots)

4.3.3 Aerobic and respiratory conditions increased the growth of all strains compared to anaerobic cultivation. MSG supplementation conferred an advantage only for few strains in anaerobic and respiratory conditions. TLC assay demonstrated that GABA production depended on the biomass concentration and the spots were observed only at 24 h of incubation. The presence of O₂ (aerobic and respiratory conditions), however, inhibited the GABA system in three strains.

4.4 Identification of *Leuconostoc mesenteroides* at the species and subspecies level with a polyphasic approach

4.4.1 The analysis of *rpoB* gene, selected as species-level taxonomic marker, was effective in distinguishing *Leuc. mesenteroides* from other related species. However, the polymorphic region of *rpoB* was useful only to discriminate the subsp. *cremoris* and subsp. *mesenteroides*.

4.4.2 Multiplex-PCR assay concurrently discriminated the strains at species (*rpoB*-band) and subspecies level. Six different profiles were observed among the 57 *Leuc. mesenteroides* members, but no amplification band was observed for the other *Leuconostoc* and *Weissella* strains, confirming the specificity of primers and the effectiveness of the assay. With exceptions of subsp. *mesenteroides* and subsp. *cremoris* clearly separated, the results of Multiplex-PCR did not fully reflect the *rpoB* clustering and the discrimination of some strains remained ambiguous.

4.4.3 The results of phenotypic tests were not supportive for subspecies identification since, in most cases, the metabolic capabilities of the strains were not consistent with their genotypic classification. Among the phenotypic features, the capability to produce acid from arabinose and dextran from sucrose, as well as the growth at 37 °C, were the only traits able to discriminate at subspecies level. Subsp. *mesenteroides* and subsp. *cremoris* had different phenotypic properties, while subsp. *mesenteroides* and subsp. *jonggajibkimchii* were similar, and the metabolic diversity of some strains did not allow to distinguish subsp. *mesenteroides* and subsp. *dextranicum*.

5. Conclusions and Future Perspectives

This PhD thesis demonstrated the benefits of aerobic and respiratory metabolism on the growth, cell physiology, survival and production of bioactive compounds of heterofermentative LAB. Respiratory cultivation of heterofermentative strains could be successfully exploited to develop improved starter or adjunct cultures able to improve the qualitative, nutritional and sensory properties of foods and beverages. Moreover, further studies are needed to understand the molecular mechanisms underlying aerobic and respiratory metabolism of heterofermentative LAB.

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***In Vitro* Probiotic Properties of Newly Isolated *Lactobacillus* Strains from Infant Feces**

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As a member of LAB, lactobacilli identified as GRAS and used as commercial starter in fermented dairy products or as probiotics related to human health. Given this point, the aim of this study was to identify and select some new potential probiotic strains of *L. paracasei* and *L. rhamnosus* which recently were isolated from human infant feces. All strains were evaluated for different *in vitro* probiotic properties such as antibiotic susceptibility, hemolytic activity, resistance to simulated gastrointestinal conditions, bile salts hydrolysis activity, and adhesion to HT-29 human epithelial cell. Based on results from *in vitro* characterization, some strains were chosen for anti-cancer activity against colorectal cancer cells (HT-29) and biofilm inhibitory activity against *E. coli* and *Listeria innocua*. The outcome of this study revealed that the strain *L. paracasei* DTA81 found to possess very interesting probiotic properties alongside with anti-cancer activity and biofilm inhibitory activity.

Proprietà probiotiche in vitro di nuovi ceppi di *Lactobacillus* isolati da feci di neonati

I lactobacilli sono dei batteri lattici, i lactobacilli sono considerati GRAS e sono comunemente usati come starter nelle fermentazioni lattiero-casearie o come probiotici. Sulla base di queste premesse lo scopo di questo studio è quello di identificare e selezionare nuovi ceppi probiotici di *L. paracasei* e *L. rhamnosus* isolati da feci di neonati. Tutti i ceppi sono stati analizzati in vitro ricercando particolari attività probiotiche come antibiotico resistenza, attività emolitica, resistenza all'ambiente gastrointestinale, capacità di idrolizzare i sali di biliari ed infine la loro capacità di legarsi alle cellule epiteliali umane HT-29. Sulla base dei risultati ottenuti dalla caratterizzazione in vitro sono stati selezionati alcuni ceppi per la loro spiccate proprietà anticancerogene contro le cellule cancerogene (HT-29) e la loro capacità di produrre biofilm per inibire l'attività di *E. coli* e *Listeria innocua*. Lo studio ha permesso di individuare il ceppo DTA81 di *L. paracasei* che ha mostrato eccezionali proprietà probiotiche tra cui spiccate proprietà anticancerogena e di produzione di biofilm con attività inibitoria.

Keywords: *L. paracasei*, Probiotic properties, anti-cancer properties, biofilm, gastrointestinal resistance.

1. Introduction

In accordance with the PhD thesis project this oral communication reports the main results of the following two activities directed to:

- A1) *In vitro* probiotic properties of different newly isolated lactobacilli.
- A2) Anti-cancer activity.
- A3) Biofilm inhibitory activity of potential probiotics.

The word “probiotic” is derived from the Greek word meaning “for life”. It is usually referred to microorganisms that confer beneficial effects to human and animals by interacting with their gut microbiota (De Vrese, 2008). Lactic Acid Bacteria (LAB) are presently the most studied and characterized bacterial group containing probiotic strains, particularly in the genus *Lactobacillus*. This genus contains more than 170 species and it is considered a taxonomically complex group. Lactobacilli are very widespread in nature and are commonly isolated from several different matrices, such as fermented foods, plant material, soil and human gut. The increase in knowledge on probiotics has led to the development of food products that can provide health benefits beyond basic nutrition. Probiotic foods represent a huge segment of functional food available on the market worldwide, projected to reach a value of US \$46.55 billion by 2020 (N. Singh, 2018). According to the WHO/FAO definition, probiotics can be considered live microbes which confer a health benefit to the host when ingested in adequate amounts. It has been demonstrated that probiotic effects are strains-specific, thus strain identification and evaluation of its safety aspects must be performed prior to connect a specific strain to its own health benefit. Among LAB, the *Lactobacillus* genus includes the highest number of GRAS (Generally Recognized As Safe) species (Klaenhammer, 2011). Besides the safety aspects such as resistance to antibiotics and blood hemolytic activity, other important criteria to define a strain as probiotic include survival to the human gastro-intestinal conditions, ability to adhere to the intestinal epithelial surface, which indicates possible antimicrobial activity against pathogens, prevention of colon cancer. In this study, we identified and selected some potential *Lactobacillus* probiotic strains isolated from infant feces. We studied the capability to withstand the transit through the gastro-intestinal tract and to hydrolyze bile salts and utilize prebiotic molecules. The absence of hemolytic activity and of transmissible antibiotic resistance were also studied. Finally, we looked for health-related traits, namely ability to attach and contrast the development of HT-29 colorectal cancer cells.

2. Materials and Methods

All strains were evaluated for different probiotic potential characteristics such as antibiotic susceptibility tests (Charteris, 1998), hemolytic activity (Pieniz, 2014), resistance to simulated gastrointestinal conditions (Favarin, 2015), bile salts hydrolysis activity (Jeronymo, 2014), adhesion to HT-29 human epithelial cell (Jacobsen, 1999). Based on results from *in vitro* characterization, some strains were chosen for anti-cancer activity against colorectal cancer cells (HT-29) and biofilm inhibitory activity against *E. coli* and *Listeria innocua*.

6. Results and Discussion

6.1 Safty tests

None of the tested strains indicated β -hemolytic activity when grown on MRS agar containing 5%(w/v) sheep blood. All the strains were γ -hemolytic (without hemolysis activity) whereas *Staphylococcus aureus* ATCC 6538 used as positive control showed β hemolytic activity. The result of antibiotic susceptibility test in this study shows that all the resistance to different antibiotics are considered as natural (intrinsic resistance) and all strains could be used as probiotic regarding antibiotic resistance (table 1).

6.2 Probiotic properties

All the strains in this study showed strong resistance to gastric juice with less than 1log viability reduction (fig1). Regarding intestinal condition resistance, among *Lactobacillus* strains, strains, *L. paracasei* DTA79, *L. paracasei* DTA81, *L. paracasei* DTA93 indicated the best resistance respectively. The quantitative binding of *Lactobacillus* strains to HT-29 colorectal cancer cell was determined according to Piatec et al 2012. Among lactobacilli, strains DTA79, DTA81, and DTA93, strongly adhered to HT-29 cells whilst strains DTA76 and DTA96 indicated adhesive characteristic and strains DTA72, DTA83 and DTA105 were non-adhesive. *L. paracasei* DTA81 showed the best adherence ability among all *Lactobacillus* strains, about ten times higher and stronger than commercial strain *L. rhamnosus* GG, thus indicating it as a very interesting probiotic candidate (fig 2).

Table 1 Antimicrobial susceptibility of *Lactobacillus* strains (R, resistant; MS, moderately susceptible; S, susceptible).

Antibiotic	Amount	strains								GG
	(µg)	DTA72	DTA76	DTA79	DTA81	DTA83	DTA93	DTA96	DTA105	
Amoxicillin	10	S	S	MS	S	S	S	MS	S	MS
Ampicillin	10	S	S	S	S	S	S	S	S	S
Cephalexin	30	R	R	R	R	R	R	R	R	R
Chloramphenicol	30	S	S	S	S	S	S	S	S	S
Ciprofloxacin	5	R	R	R	R	R	R	R	R	R
Cloxacillin	5	R	R	MS	MS	MS	MS	MS	R	MS
Erythromycin	15	S	S	S	S	S	S	S	S	S
Gentamycin	10	R	R	R	R	R	R	R	R	R
Kanamycin	30	R	R	R	R	R	R	R	R	R
Penicillin G	10 IU	MS	MS	MS	MS	S	MS	S	S	S
Streptomycin	10	R	R	R	R	R	R	R	R	R
Tetracycline	30	S	S	S	S	S	S	S	S	S
Trimethoprim	5	R	R	R	R	R	R	R	R	R
Vancomycin	30	R	R	R	R	R	R	R	R	R

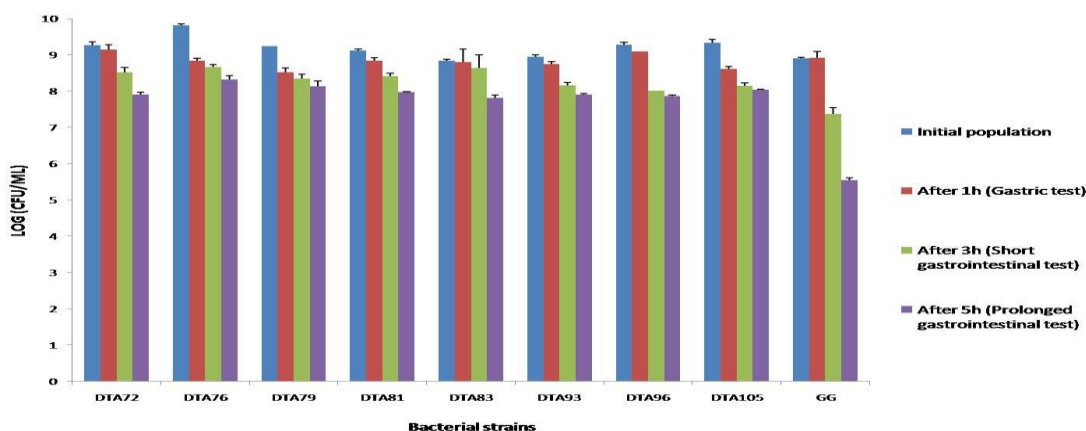


Figure 1 Survival of strains of *S. thermophiles*, *L. paracasei*, and *L. rhamnosus* GG during exposure to in vitro simulated gastrointestinal conditions. Results are expressed as the mean $\pm$ SD (n=3).

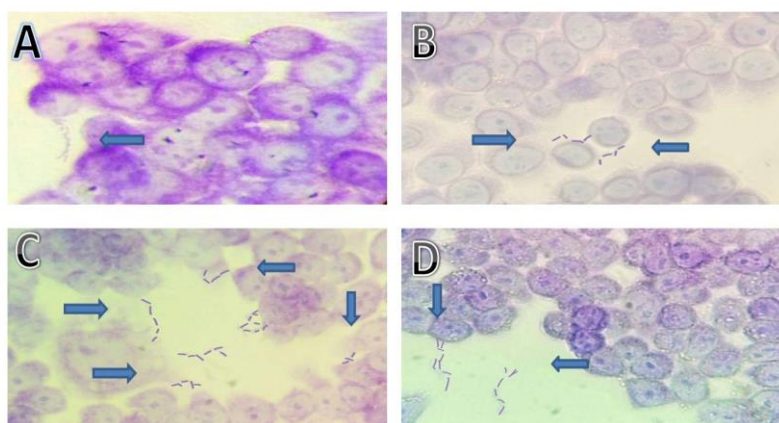


Figure 2 Right figure, adhesion of *Lactobacillus* strains to HT-29 cells observed under the optical microscope (1000X). Strains: A) GG, B) DTA79, C) DTA81, D) DTA93.

6.3 Anti-cancer activity against colorectal cancer cells (HT-29)

Colorectal cancer is a disease mostly has been studied by Caco-2 and HT-29 cells that can even cause death widely in the world. Strains *L. rhamnosus* DTA79, *L. paracasei* DTA93, DTA96, DTA81, and *S. thermophilus* MTH17CL396, M17PTZA496, TH982, which had indicated good adhesion activity to HT-29 cells and revealed good probiotic potential were examined for anti-cancer activity. According to the results reported in table 2-8, some lactobacilli supernatants indicated strong anti-cancer activity against HT-29 cancer cells. In comparison with commercial probiotic strain, no significant difference ($P < 0.05$) was found between *L. paracasei* DTA93 and *L. rhamnosus* GG when tested with multiple comparison tests (Tukey's test). The *L. paracasei* DTA96 was the only strain that did not show any anti-proliferative activity. *L. paracasei* DTA81 showed the strongest anti-cancer activity with $39.4\% \pm 0.05$, $34.1\% \pm 0.03$, $21.5\% \pm 0.02$ at concentrations of 2000, 4000, 8000 $\mu\text{g/mL}$ respectively (table 2).

Table 2 Anti-cancer effect of lyophilized supernatants collected from *Lactobacillus* cultures on HT-29 cancer cells after 48 h.

Supernatant concentration ($\mu\text{g/mL}$)	HT-29 cells viability (%)									
	DTA79	DTA81	DTA93	DTA96	GG	MRS medium				
125	97.4 8 $\pm$ 8	97.8 1 $\pm$ 3	70.2 0 $\pm$ 1	98.2 0 $\pm$ 5	65.8 0 $\pm$ 1	90.2 7 $\pm$ 0	0.2 0.1			
250	74.9 4 $\pm$ 2	97.5 1 $\pm$ 5	63.0 0 $\pm$ 3	86.9 0 $\pm$ 6	63.9 0 $\pm$ 9	91.6 7 $\pm$ 3	0.0 0.1			
500	65.5 6 $\pm$ 7	93.1 0 $\pm$ 1	59.9 8 $\pm$ 7	88.3 0 $\pm$ 1	58.9 0 $\pm$ 2	90.0 3 $\pm$ 5	0.0 0.2			
1000	57.3 3 $\pm$ 5	53.2 7 $\pm$ 7	47.4 2 $\pm$ 1	84.7 0 $\pm$ 6	54.1 0 $\pm$ 2	89.4 7 $\pm$ 8	0.1 0.2			
2000	46.6 4 $\pm$ 3	39.4 2 $\pm$ 5	44.7 9 $\pm$ 0	89.2 0 $\pm$ 5	48.2 0 $\pm$ 2	88.6 1 $\pm$ 9	0.1 0.2			
4000	37.7 8 $\pm$ 0	34.0 9 $\pm$ 3	40.4 8 $\pm$ 1	90.5 0 $\pm$ 5	41.1 0 $\pm$ 3	82.6 3 $\pm$ 0	0.2 0.0			
8000	31.2 7 $\pm$ 7	21.4 8 $\pm$ 2	28.5 6 $\pm$ 2	88.8 0 $\pm$ 4	29.1 0 $\pm$ 2	89.4 4 $\pm$ 6	0.0 0.1			

6.4 Biofilm inhibitory activity of potential probiotics.

Biofilm inhibitory activity of lactobacilli strains against *E. coli* and *L. innocua* was evaluated using two different approaches. Figure 3 indicates the outcome of the biofilm inhibitory activity achieved by inoculating a *Lactobacillus* strain first and subsequently either *E. coli* (Fig. 3A) or *L. innocua* (Fig. 3B) (exclusion test) and inoculating simultaneously one *Lactobacillus* strain together with either *E. coli* (Fig. 3A) or *L. innocua* (Fig. 3B) (competition test). In exclusion method, all *Lactobacillus* strains showed inhibitory activity by reducing the number of adhered *E. coli* and *L. innocua* cells to a different extent; however *L. paracasei* DTA81 and DTA93 indicated the highest inhibitory effects by 1.05 and 0.80 log cell reduction, respectively on *E. coli* and 0.58 and 0.60 log, respectively on *L. innocua*. On the other side, similar results were seen in competition method where strains DTA81 and DTA93 still were the best biofilm inhibitors by 0.78 and 0.65 log cells reduction, respectively on *E. coli* and 0.29 and 0.42 log cells reduction, respectively on *L. innocua*.

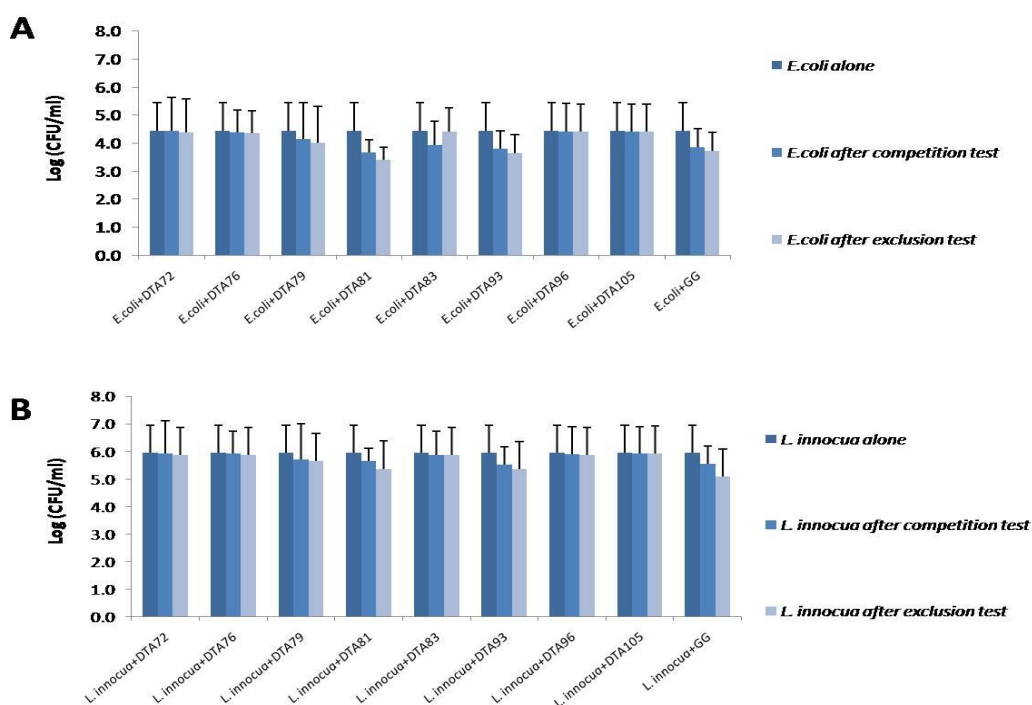


Figure 3 Biofilm inhibitory activity of *Lactobacillus* strains against *E. coli* (A) and *L. innocua* (B) in competition and exclusion tests. Results are express as mean $\pm$ SD ($n = 3$) of *E. coli* and *L. innocua* viable cells.

7. Conclusions and Future perspective

The results of the present study indicate that the two newly isolated strains *L. paracasei* DTA81 and DTA93 were found to possess *in-vitro* probiotic properties and anti-cancer activity. Some traits resulted very close to and in some cases superior to those of the widespread commercial probiotic strains *L. rhamnosus* GG that we used as reference.

Strain *L. paracasei* DTA81 showed an amazingly high adherence ability to HT-29 cells line which resulted about ten times stronger than that of the commercial strain *L. rhamnosus* GG and, to our knowledge, represents the highest level reported to date for this type of cells. Besides, both *L. paracasei* DTA81 and *L. paracasei* DTA93 were able to effectively inhibit biofilm formation by other bacteria. Therefore, further investigation, including *in-vivo* studies, is strongly advisable to evaluate the potential health benefits in the real host. Overall, our study confirms what is demonstrated in many other studies regarding the potential of the genus *Lactobacillus* as a very interesting source for the discovery of new health beneficial microbes.

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Use of Selected Lactic Acid Bacteria for Fermentation of Non-Conventional Vegetable Matrices: Nutritional and Functional Implications

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This PhD thesis research project dealt with the set-up of biotechnological protocols, through lactic acid bacteria fermentation, to improve the nutritional and functional properties of vegetable matrices. Firstly, lactic acid bacteria strains isolated from faba bean flour and sourdough were characterized and their fermentative aptitude was evaluated, investigating the ability of reducing antinutritional factors and synthesizing exopolysaccharides. The second part of the project concerned food industry waste and by-products (bread and brewers' spent grain), and their reutilization within the food chain.

Impiego di batteri lattici selezionati per la fermentazione di matrici vegetali alternative: implicazioni nutrizionali e funzionali

Questo progetto di tesi ha riguardato la messa a punto di protocolli biotecnologici, mediante fermentazione con batteri lattici selezionati, atti a migliorare gli aspetti nutrizionali e funzionali di matrici vegetali. Ceppi di batteri lattici isolati da farina e lievito naturale di fava, sono stati caratterizzati e ne è stata valutata l'attitudine fermentativa, ponendo particolare attenzione alla loro capacità di ridurre il contenuto in composti anti-nutrizionali e sintetizzare esopolisaccaridi. Successivamente sono stati messi a punto protocolli per il riutilizzo, nell'ambito della catena alimentare, di scarti e sottoprodotti dell'industria alimentare (pane e trebbie di birra).

Key words: Bioprocessing, faba bean, brewer's spent grain, wasted bread, bioactive compounds.

1. Introduction

In accordance with the PhD thesis project previously described (Verni, 2017), this oral communication reports the main results of the two activities:

A2) Study of the main metabolic traits of lactic acid bacteria isolated from faba bean and characterization of their performances during faba bean fermentation;

A3) Set-up of biotechnological protocols to exploit food industry by-products such as wasted bread and brewers' spent grain (BSG).

2. Fermentation biotechnology

Legumes and cereals constitute a significant portion of human diet. Among legumes, faba bean plays an important role in the agriculture and diet of many developing countries and is a major source of proteins, dietary fibres and other compounds beneficial for health. However, its role in human diet is limited by the presence of antinutritional factors interfering with nutrient absorption and sometimes causing pathologic conditions (Multari et al., 2015). Cereal industry by-products, on the other end, are commonly used as feed or compost. The extraction of functional compounds, whether for food, cosmetics, or pharmaceutical has been exploited, yet, this approach implies that more by-products will

be generated and does not consider the possibility of re-utilizing them in the food chain (Ravindran & Jaiswal, 2016). Fermentation with selected lactic acid bacteria (LAB) proved to be a very feasible option to enhance the technological, sensory, and especially nutritional and functional features of faba bean and cereal industry by-products. Increased minerals and vitamins bioavailability, protein content and digestibility, fiber and phenolic compounds solubility are the most common aspects fermentation acts on. In some cases, more compelling benefits have been discovered, such as the synthesis of bioactive compounds acting as antimicrobial, antitumoral, antioxidant agents. (Verni et al., 2019a, b).

3. Faba Bean Bioprocessing

3.1 Materials and methods

Twenty-seven LAB strains, isolated from faba bean flour and sourdough, were characterized for several nutritional, technological and functional properties (Verni et al. 2017). Their metabolic profiles were determined by Biolog System using 95 different carbon sources. β -Glucosidase and phytase activities were also determined. Aiming at evaluating the potential probiotic effect, strains were tested for the ability to resist to the gastrointestinal tract using simulated gastric and intestinal fluids, while *Staphylococcus aureus* DSM20231, *Listeria monocytogenes* ATCC19115, and *Escherichia coli* DSM30083, were used to assess their antibacterial activity (Verni et al. 2017).

Weissella cibaria E3485, *Weissella confusa* E3403, *Pediococcus pentosaceus* E3483 and *Leuconostoc kimchi* E3484, selected for the ability to synthesize exopolysaccharides (EPS), were singly inoculated in faba bean doughs and incubated at 30°C for 24 and 48h. Mono-, di- and oligosaccharides were analyzed by high performance anion exchange chromatography. Whereas, the amount of EPS was determined by an enzyme-assisted method as reported in Rizzello et al. (2018).

Aiming at evaluating their nutritional profile and pro-technological aptitude, the flour obtained from thirteen Mediterranean faba bean accessions was fermented, at 30°C for 48h, with *Lactobacillus plantarum* DPPMAB24W, a strain previously selected for the high β -galactosidase activity. Before and after fermentation the main biochemical features of the doughs were evaluated. Protein content and digestibility, α -galactosides, condensed tannins, vicine and convicine concentration were determined as reported in Verni et al. (2019c).

3.2. Results and discussion

3.2.1 Study of LAB metabolic traits

All the 27 strains were able to metabolize α -D-glucose, D-fructose, D-cellobiose, mannose, maltose, maltotriose and dextrin, while only 7 strains metabolized D-raffinose. *Leuconostoc mesenteroides* I01 and I57 showed the highest raffinose consumption. This α -galactoside is not degraded in the upper gastrointestinal tract due to the lack of α -galactosidase activity, and, at high concentrations, it can be fermented by the intestinal microbiota, causing symptoms such as abdominal discomfort, flatulence and diarrhoea (Teixeira et al., 2012). Faba bean is rich in vicine and convicine, responsible, in susceptible individuals, for the hemolytic disease favism. Hydrolysis of such molecules and their derivatives can occur during fermentation, as consequence of microbial β -glucosidases activity. All the strains belonging to the genus *Pediococcus* and *Weissella koreensis* I149 exhibited β -glucosidase activity higher than the median value (0.015 U). Despite the differences, phytase activity was largely distributed among the strains and ranged from 0 to 0.958 ± 0.013 U. Twelve strains showed the ability to produce EPS, especially those

belonging to the *Weissella* genus. Strains of *Leuc. mesenteroides* (I01, I57, I211, I21) were also able to produce them, but in lower amount. Only 4 strains (*P. pentosaceus* I56, I76, I47, I214), survived the simulated gastrointestinal tract, showing a viability of 8-9 log CFU/ml at the end of the treatment. Overall, *L. monocytogenes* was inhibited by almost all the strains tested, except for *W. koreensis* I148, I149, *Leuc. mesenteroides* I211, *Enterococcus* spp. F09 and *Lactobacillus sakei* F71. No antimicrobial activity was found towards *S. aureus*, while 8 strains (*P. pentosaceus* I02, I014, I76, I147, F01, *W. koreensis* I19, F113 and *E. casseliflavus*) exhibited a strong inhibitory activity towards *E. coli*. Data collected from the characterization of faba bean LAB and related fermented doughs were subjected to permutation analysis (Fig. 1) which allowed to group the strains into clusters, based on their peculiarity.

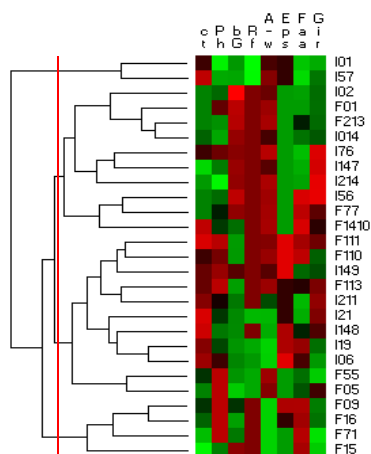


Figure 1 Permutation analysis of condensed tannins concentration (cT), phytase activity (Ph), β -glucosidase activity (bG), raffinose concentration (Rf), antioxidant activity, (A-w), EPS synthesis (Eps); free amino acids concentration (Faa), peptides concentration (Pt), in vitro resistance to gastro-intestinal conditions (Gir). Colors correspond to normalized mean data levels from low (green) to high (red).

3.2.2 Faba bean fermentation

Sucrose was the most abundant disaccharide in faba doughs enabling the formation of EPS. As consequence of the oligosaccharides hydrolysis, galactose, was detected after the first 24 h of fermentation, whereas verbascose and stachyose progressively decreased. The EPS producing strains formed slime on MRS agar supplemented with sucrose, but none in presence of raffinose. Being raffinose a substrate for levansucrase, but not for glucansucrase, dextrans synthesis was hypothesized (Teixeira et al., 2012). After 24 h of fermentation, the amount of dextrans in the doughs ranged from 0.24 to 1.47% of dry weight. The highest amount was found in the doughs inoculated with *W. cibaria* E3485 and *W. confusa* E3403. Despite the relative similarity in dextran content, a very different rheological behaviour was found, since the latter showed a markedly higher viscosity. The different thickening ability of EPS might be due to the structure and molecular weight of the dextrans produced by the two strains and, possibly, by the different interactions between the other faba bean components (Xu et al., 2017).

Before fermentation faba bean accessions had a more marked identity, showing wide variability in protein content (that ranged from 24.83% to 30.03%) and α -galactosides concentration. The heterogeneity observed for the raw flours was flattened after fermentation, however the different composition of the raw matrix affected the starter performances, especially the production of organic acids. All the faba bean accessions benefitted from fermentation, gaining an improved nutritional profile, richer in more bioavailable peptides and amino acids (Fig. 2), and showing higher *in vitro* protein digestibility. Fermentation also enabled the partial or complete reduction of α -galactosides.

As for condensed tannins content, slight differences were found among accessions for vicine, which drastically decreased during fermentation, whereas convicine was not detected in any of the raw flours.

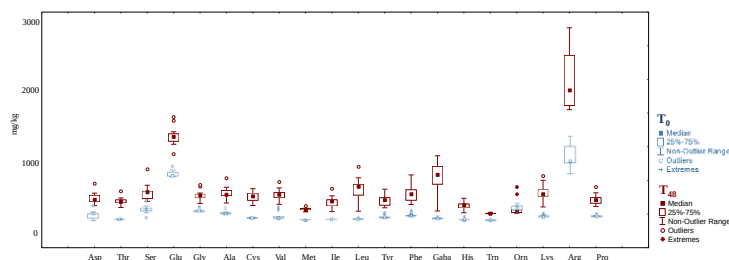


Figure 2 Boxplot showing the distribution of single free amino acids of faba bean doughs before (blue) and after (red) 48h of fermentation at 30°C with *Lb. plantarum* DPPMAB24W.

4. Improvement of Brewers' Spent Grain Antioxidant Activity

4.1 Materials and methods

BSG, originated from Peroni beer production, was fermented, at 30°C for 24h, with 33 LAB strains belonging to the Collection of the Department of Soil, Plant and Food Sciences. Based on the ability to improve the content of total phenolic compound (TPC) and the antioxidant activity, measured on DPPH and ABTS radicals (Rizzello et al., 2010), three strains were selected for further investigation. The fermentation protocol was optimized adding commercial enzyme preparation (Depol 761P, Biocatalysts, 200 nkat/g) having xylanase activity. The free and bound phenolic compounds were extracted from BSG as described by Bonoli et al. (2004) and the obtained extracts were chemically characterized by liquid chromatography (UPLC-PDA-ESI-QTOF, ACQUITY Ultra Performance LC™ Systems, Waters, USA). The separation was performed using an ACQUITY UPLC BEH Shield RP18 column with acetic acid (1% v/v) and acetonitrile as eluents. Ultimately, the protective effect of BSG water/salt-soluble extracts against oxidative stress was tested on human keratinocyte cell lines.

4.2 Results and discussion

The content of TPC in BSG prior fermentation was 1.99 ± 0.12 mmol/100 g (d.m.). Except for the not inoculated control, after 24h of incubation, for all fermented samples was observed an increase of TPC varying from 10 to 35%. BSG fermented with *Lb. brevis* H46 and *Lb. plantarum* PU1 showed an antioxidant activity on DPPH higher ($P < 0.05$) than the control, whereas fermentation with *Lb. plantarum* PRO17, *P. pentosaceus* I76 and I214 significantly increase the antioxidant activity on ABTS, exceeding 0.2 mM of trolox equivalents. Since phenolic acids in BSG are esterified to arabinoxylan residues (Mandalari et al., 2005), xylanases were used to facilitate their release. BSG treated enzymatically before fermentation enabled the highest improvement in the antioxidant activity (Fig 3), therefore, to address this activity, raw and bioprocessed BSG were characterized for the phenolic profile.

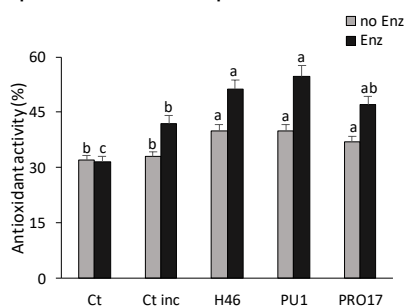


Figure 3 Antioxidant activity on the radical DPPH of raw (Ct), incubated (Ct inc) and fermented BSG (H46, PU1, PRO17), with and without the pretreatment with xylanases.

Among the free and bound phenolics, 39 compounds were identified. Bound profile was characterized mostly by phenolic acids (vanillic, caffeic, *p*- and *o*-coumaric, ferulic, isoferulic and sinapic) and their oligomers (dimers, trimers and tetramers). Catechin and epicatechin, were also identified. Free phenolics profile of raw BSG was rather meager. Catechin and two compounds deriving from hop, chrysoeriol and xanthohumol, (Kao, 2018) were the major phenolics in unprocessed BSG. Bioprocessed samples, especially those fermented, had high content of dihydrocaffeic and phloretic acid, product of the microbial reduction of caffeic and coumaric acid, respectively (Filannino et al., 2018). Six ferulic acid derivatives were tentatively identified in bioprocessed BSG considering the MS² signal.

To further investigate the ability of BSG to act as radical scavenging, human keratinocyte cell lines were grown in the presence of raw and bioprocessed BSG extracts. Except for the untreated BSG, none of the bioprocessed samples showed cytotoxic activity at the concentration of 1mg/mL. After exposure to hydrogen peroxide, all bioprocessed BSG, especially those fermented, exhibited protection against induced oxidative stress, comparable to that of α -tocopherol. Fermented BSG, also showed anti-inflammatory activity. The highest effect was observed for BSG fermented with *Lb. plantarum* PRO17, still high after 24 h after the inflammation was induced. Since both methanolic and water/salt-soluble extracts displayed antioxidant properties, a combined effect of phenolic compound and bioactive peptides, could be hypothesized. Further investigations, involving the characterization of the peptide profile, are necessary to better address these activities.

5. Wasted Bread as Substrate for Starter Cultivation

5.1 Materials and methods

Aiming at the valorization and recycle of bread waste, a protocol to obtain a growth medium for the cultivation of starters was set-up. White bread cutting, wasted from sandwiches production, were grinded and added to distilled water in a percentage ranging from 10 to 50%. Proteolytic and amylolytic enzymes, singly or as a mixture, were then added and the suspensions were incubated at 30-55°C for 6-48h depending on the enzymes optimal conditions provided by the manufacturers. After the incubation, the mixtures were centrifugated (14000 rpm for 20 min) to separate not hydrolysate solid residues and supernatants were collected. Aiming at maximizing microbial growth, to the medium were added glucose, yeast extract, peptone, tryptone, meat extract, whey protein isolates (WPI) and concentrate (WPC). Supplements were added singly or in mixture at concentrations ranging from 5 to 30 g/L. The solution was adjusted to pH 5.6 and sterilized at 121°C for 15 min. To avoid interferences due to different turbidimetry during the OD measurement, the mediums were further filtered using sterile membrane size of 0.2 μ m cut-off (Millipore, USA).

Lactobacillus plantarum LB1 and *Saccharomyces cerevisiae* E10, belonging to the Culture Collection of the Department of Soil, Plant and Food Sciences (University of Bari, Italy), were chosen as indicator microorganisms for the set-up and the optimization of the protocol for WBM production. To establish a correlation between the chemical composition of the media and microbial growth, glucose, peptides and amino acids concentrations were determined for each experimental condition (Rizzello et al., 2010). The suitability of the Wasted Bread Medium (WBM) for the growth of other starters (LAB, yeasts, fungi and acetic acid bacteria) routinely employed in food industry were grown in WBM to assess the suitability of the medium to be used as substrate for biomass production. The effect of chemical preservatives commonly added to bread to prevent spoilage on the growth of *Penicillium roqueforti* was also evaluated.

5.2 Results and discussion

The optimization of the medium followed the classical approach of the one-factor-at-a-time experiments, removing, supplementing or replacing carbon and nitrogen sources, whether they were actual supplements or enzymes or conditions able to modify their content. All the data resulting from the media chemical characterization and LAB and yeasts growth were factored into three-dimensional surface plots (Fig. 4). Carbon is the most important component in a medium, acting as energy source and playing an important role in the growth (Singh et al., 2017), therefore, amylolytic enzymes were used to release glucose from starch. Sugar content higher than 20 g/L inhibited LAB growth, probably because of the osmotic stress, on the contrary, yeasts, which can tolerate up to 40% of glucose, were not affected by it (Kurtzman et al., 2011).

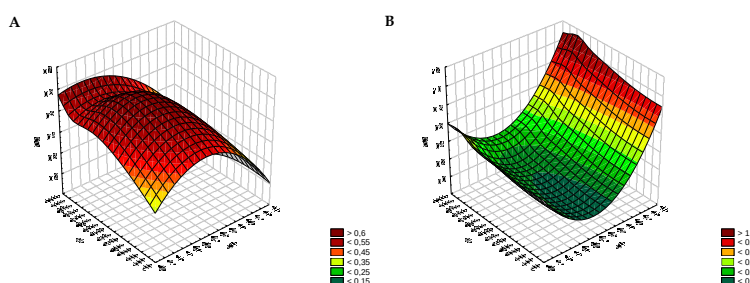


Figure 4 Three-dimensional surface plot of the interaction of glucose and organic nitrogen (peptides and amino acids) concentrations (g/L) on the growth of *Lactobacillus plantarum* LB1 (A) and *Saccharomyces cerevisiae* E10 (B).

Like carbon, the selection of nitrogen source and its concentration in the media also play a crucial role in microbial metabolisms (Singh et al., 2017), which is why, a great deal of effort was put into find the best combination enzyme/substrates that supported microbial growth. While yeasts can utilize a wide variety of nitrogen sources, both organic and inorganic (Kurtzman et al., 2011), LAB have more complex requirement, needing both proteins and their breakdown products. For this reason, media having more than 30 g/L of nitrogen, especially if a combination of different sources, were those that best allowed bacterial growth, even better than MRS.

The optimized WBM was used to propagate other LAB and yeasts, including those used in yogurt and wine production. Some differences were noticed among LAB strains probably because of an inadequate content of micronutrients, which may have influenced their ability to adapt to the medium. *P. roqueforti*, one of the starter cultures used in dairy industry, was also tested and WBM resulted a great medium for its cultivation. Nevertheless, since food preservatives are part of the ingredients in industrial breads, their effect on fungal growth was assessed. Even though inhibition was found at the highest concentrations, the growth always exceeded that in the reference medium.

6. Conclusions and Future Perspective

One of the reasons of the rising demand for healthier plant-based food is the increasing awareness of the adverse risks associated with the consumption of animal proteins. Legumes as well as cereal by-products have a great potential as functional food and fermentation could be considered a promising technology to reduce undesired compounds and synthesize those of interest. Therefore, the incorporation of bioprocessed matrices in cereal-based food formulas, which has been explored over the last few years,

could be a valuable option to *i*) revitalize stall markets, such as bread's and pasta's, *ii*) fulfill consumers need for healthier food, and *iii*) valorize side streams and waste from food processing industry, contemplating their reutilization within the food chain.

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Role of Functional Food Supplements in Patients with Gastrointestinal Disorders and Food Intolerances (Lactose and/or Fructose)

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This PhD thesis dealt with the study of role of different nutraceutical food supplements (mainly probiotics supplements) in patients with gastrointestinal disorders (mainly Irritable Bowel Syndrome, IBS) and food Intolerances (lactose and/or fructose) on gut and salivary microbiota. Nutraceutical mixtures were tested in Italian patients and will be tested in Romanian ones, and those that will give the best results in terms of amelioration of symptomatology, gut and salivary microbiota and related metabolism improvement, will be assembled in the company involved in the project.

Ruolo di integratori alimentari in pazienti con disordini gastrointestinali ed intolleranze alimentari

Questa tesi di dottorato ha riguardato lo studio di differenti miscele nutraceutiche (soprattutto integratori probiotici) in pazienti con disordini gastrointestinali (principalmente Sindrome del Colon Irritabile, IBS) ed intolleranze alimentari (lattosio e/o fruttosio) sul microbiota intestinale e salivare. Le sostanze nutraceutiche sono state testate nei pazienti Italiani e saranno testate sui pazienti Rumeni, e quelle che daranno i risultati migliori in termini di miglioramento dei sintomi, del microbiota intestinale ed orale e relativo metabolismo, saranno assemblate nell'azienda coinvolta nel progetto.

Key words: microbiota, metabome, IBS, food intolerances, probiotics

1. Introduction

In accordance with the industrial PhD proposal project (Vitellio et al., 2017), this oral communication reports the main results of the following four activities directed to:

- A1) The selection of a novel nutraceutical mixture to be tested;
- A2) The recruitment of at least 150 patients with gastrointestinal disorders and food intolerances (lactose and/or fructose), the somministration of alimentary questionnaires;
- A3) The collections of samples (faeces, urine and saliva) before and after the treatment with the selected nutraceutical mixture;
- A4) The analysis of dietary intake, gut microbiota and related metabolome analysis, and symptomatology evaluation before and after the treatment.

2. Gut microbiota and metabolome modulation in gastrointestinal disorders and food intolerances

The gut microbiota is an ecological community of symbiotic microorganisms (including microbes and other unicellular organisms) that literally inhabits our bodies, outnumbering human cells by 10 to 1 (De Palma et al., 2014). Research underlines the correlation between a loose in the balance in the composition of salivary and gut microbiota, and the incidence of gastrointestinal disorders and food intolerances. Thus, the role of probiotics is strongly emerging in the management of these disorders. Probiotics are defined as live bacteria or yeasts that positively supplement the gut microbiota (Wang et al., 2017). Hence, probiotics can be administered for treatment of gastrointestinal disorders, as well as carbohydrates malabsorption and sugar intolerance (e.g. lactose).

Moreover, the beneficial use of LAB and probiotics in general aids in the extraction of energy and nutrients, such as short-chain fatty acids (SCFAs) (acetate, propionate, and butyrate) and amino acids from food. Furthermore, the production of SCFAs stimulates ileal propulsive contractions, releases neuroendocrine factors, acidifies the intracolonic pH, and relaxes intestinal gut tissues (den Besten et al., 2013). Thus, the improvement of the gut and salivary microbiota and related metabolome through some selected probiotics represents an innovative way for the management of these disorders (Vitellio et al., 2019). Moreover this PhD thesis represents the first study that can provide informations about the correlation between probiotics, microbiota, metabolome and diet, with a specific outcome in IBS patients and in food intolerant (lactose and/or fructose) patients.

3. Materials and Methods

A double blind placebo-controlled challenge in patients with IBS and in patients with lactose intolerance in lactose free diet and persisting symptoms. Patients with lactose intolerances were diagnosed according to previous guidelines (Gasbarrini et al., 2009), meanwhile IBS patients were diagnosed according to Rome IV criteria (Mearin et al., 2016). Patients were randomized to receive either probiotics: 3 g as packets with *Bifidobacterium longum* BB536 four billion CFU, *Lactobacillus rhamnosus* HN001 one billion CFU with B6 vitamin 1.4 mg) or placebo (PL: similar packets containing maltodextrins, corn starch, silicon dioxide, and no probiotic). The study included a first period of one month of treatment followed by 15 days of wash-out, and a second period of one month of treatment after cross-over. Analyses were performed at days 0 (T0), 30 (T30), 45 (T45), and 75 (T75), and included symptoms, anthropometric and diet evaluations, fecal microbiota, and related metabolome measurements.

4. Results and Discussion

4.1 Lactose intolerant patients

The initial group consisted of 135 adult symptomatic patients of both genders (54 males and 81 females) (Figure 1). Exclusion criteria were organic diseases with a diagnosis of structural abnormality of the gastrointestinal tract (i.e., inflammatory bowel diseases such as Crohn's disease, ulcerative colitis), pregnancy, abdominal surgery within the previous six months, infective diseases, drug or alcohol abuse, metabolic disturbances, mental illness, concomitant immunological, haematological or neoplastic disease, severe hepatic insufficiency (i.e., Child–Pugh class A–C), severe heart failure (NYHA class III–IV), and inability to provide the informed consent. The 99 remaining patients underwent the hydrogen (H₂) breath test to detect LI intolerance and 75 patients (19 males and 56 females, mean age 46 ± 3.1 SE years, range 20–67) were positive. The patients were

encouraged to introduce lactose-free dairy products to undergo a lactose-free diet for at least six months. Patients ($N = 34$) were ultimately enrolled if symptoms (i.e., altered bowel habits such as constipation or diarrhea, bloating, and abdominal pain) persisted after six months. Because of 11 drop-out patients, the final cohort consisted of 23 subjects.

The baseline characteristics appear in Table 1 and did not change over the time. The amounts of daily micro/macronutrients at baseline appear in Table 2 and did not change over the time. The adherence score to Mediterranean diet for 96% patients was in the range of 10–15 points (sufficiently adherent), while 4% of patients were included in the range of 5–9 points (low adherence).

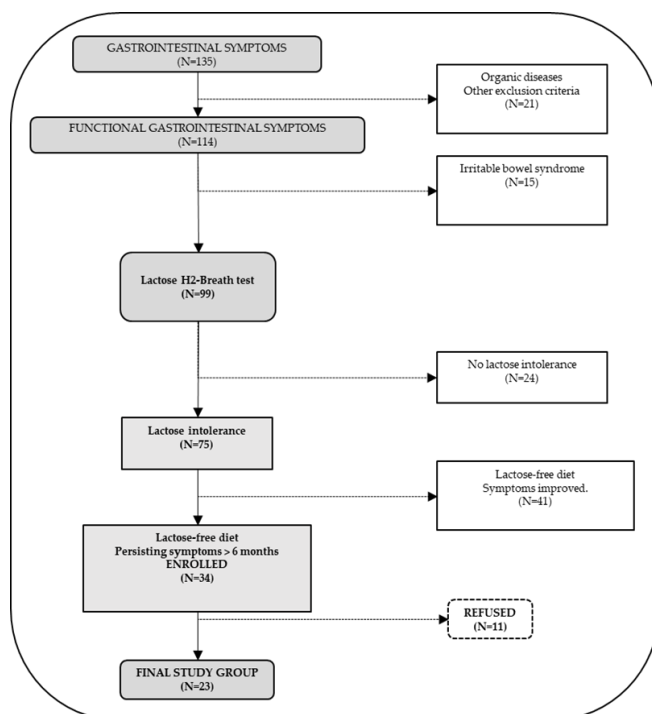


Figure 1 Flow-chart of enrolled lactose-intolerant patients in the study.

Table 1 Baseline characteristics of patients ($N=23$). Data are mean $\pm$ SE.

Baseline Characteristics	
Age, years	48 $\pm$ 3.1 (48)
Females, <i>n.</i> (%)	19 (83%)
BMI (Kg/m ²)	23.2 $\pm$ 0.68 (22.3)
Symptoms	
Bloating (VAS, mm)	69 $\pm$ 5.4 (80)
Abdominal pain (VAS, mm)	61 $\pm$ 4.6 (62)
Bowel habits	
Bristol Score (range 1–7)	3 $\pm$ 0.38 (2)

Table 2 Amounts of daily micro/macronutrients at baseline Data are mean $\pm$ SE.

Nutrients	Intake
Fiber (g)	9.1 $\pm$ 0.73
Carbohydrates (%)	41.64 $\pm$ 1.29
Protein, total (%)	18.49 $\pm$ 0.71
Animal protein (%)	62.7 $\pm$ 2.9
Plant protein (%)	37.3 $\pm$ 2.9
Lipids (%)	39.9 $\pm$ 0.88
Saturated fatty acids (%)	34.9 $\pm$ 1.18
Olive oil (g)	23 $\pm$ 2.79
Vitamin B6 (mg)	0.84 $\pm$ 0.06

4.2 Faecal microbiome and metabolome

Compared with 16S-rRNA (total bacteria), the highest significant differences between the fecal microbiota of the two groups (probiotic and placebo) were found using 16S-rRNA data (metabolically active bacteria). Eleven phyla (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Chlorobi, Synergistetes, Spirochaetes, Tenericutes, Chloroflexi, Cyanobacteria, and Fibrobacteres) were identified. Proteobacteria relative amount was higher ($p < 0.05$) in probiotic samples compared with placebo samples. The relative abundance of genus of *Bifidobacterium* was higher ($p = 0.002$) in probiotic (0.46%) compared with Placebo group (0.22%). Compared with Placebo, probiotic drove several genus to a higher amount, including *Slackia*, *Enterococcus*, and *Thricoccus* ($p < 0.05$). *Lactobacillus* genus did not differ between probiotic and placebo. A decreased relative abundance of *Klesbiella*, *Serratia*, and *Enterobacter* was detected in treated patients compared with PL ($p < 0.05$). The content of the metabolites within the same group largely varied; nevertheless, some significant differences were figured out between the two conditions according to previous work (Table 3) (Khailova et al., 2014).

Table 3 Volatile organic compounds (VOCs). Concentration (min, max) of VOCs (ppm) headspace fecal samples of treated (probiotic) and placebo patients.

Compounds	Probiotic	Placebo	p-value
1-Butanol	0 (0, 0)	11.22 (0, 12.58)	0.037
5-Hepten-2-ol, 6-methyl-	51.74 (5.98, 72.3)	30.81 (0.79, 39.28)	0.029
Phenol	4.12 (0, 8.56)	19.78 (3.48, 28.56)	0.048
1-Hexadecanol	4.03 (0, 6.29)	8.17 (0, 13.94)	0.043
Acetic Acid	44.53 (26.62, 63.82)	23.34 (7.72, 37.47)	0.042
Propanoic Acid, 2-methyl-	29.78 (13.4, 45.04)	11.26 (0.71, 17.35)	0.009
Hexanal	5.81 (0, 13.22)	11.3 (2, 19.38)	0.009
2-Nonenal	5.38 (2.24, 8.9)	3.35 (0, 4.34)	0.045
Indolizine, 3-methyl-	248.96 (87.94, 372.62)	99.53 (31, 93.91)	0.045
5-Hepten-2-One, 6-methyl-	310.76 (55.31, 396.75)	124.03 (19.91, 210.84)	0.048
D-Limonene	87.43 (11.19, 187.9)	483.68 (55.46, 804.44)	0.042

4.3 Clinical Scores during the Double Blind, Cross-Over Challenge

The treatment with probiotic caused a significant ($p = 0.028$) decrease of bloating, compared with placebo. However, no effect was evident in the case of abdominal pain. Abdominal pain covers not the totality of lactose intolerant patients (Yuce et al., 2016). The globally assessed Bristol score did not differ between placebo and probiotics. However, at sub-analysis, the percent of patients with a score within the “normal” range of 3–4 increased from 30.4% to 73.9% after probiotic ($p = 0.0072$, chi-squared). In particular, Bristol scores suggestive of constipation (i.e., 1–2) reached a normal range in 7/8 patients (87.5%) after treatment, while in patients with less formed stools (i.e., 5), 3/8 (37.5%) scored normal after probiotics ($p = 0.045$)

4.5 IBS patients

The cohort consisted of 30 IBS patients (9 males and 21 females) aged 48 ± 11 years, including 62% of diarrhoea predominant IBS (IBS-D) subjects and 38% of constipation predominant IBS (IBS-C) subjects. The prevalence of IBS was higher in women than in men. IBS-SSS and interference with quality of life were moderate. According to subtype of IBS, the prevalence of IBS-D was greater than that of IBS-C. Table 4 showed the effect of the treatment on symptoms.

Table 4 Levels of parameters according to sequence (1 = Placebo/Probiotic and 2 = Probiotic/Placebo) and globally. Data are mean $\pm$ SE.

Parameter	Treatment	Sequence 1 N=10	Sequence 2 N=15	Global N=25	P-value
Abdominal pain	Placebo	65 ± 22	52 ± 22	58 ± 23	<0.0001
	Probiotic	37 ± 25	25 ± 22	30 ± 23	
	Placebo-probiotic	-30 ± 22	-27 ± 31	-28 ± 28	
Bloating	Placebo	58 ± 28	70 ±16	65 ± 22	<0.0001
	Probiotic	45 ± 29	30 ± 21	36 ± 25	
	Placebo-probiotics	-14 ± 28	-40 ± 27	-29 ± 28	
Relief following defecation	Placebo	39 ± 12	40 ± 12	39 ± 12	<0.0001
	Probiotic	57 ± 17	65 ± 15	62 ± 16	
	Placebo-probiotic	18 ± 20	25 ± 11	22 ± 19	
Interference with quality of life	Placebo	65 ± 13	62 ± 15	63 ± 14	<0.0001
	probiotic	39 ± 18	34 ± 20	36 ± 19	
	Placebo-probiotic	-26 ± 24	-27 ± 28	-27 ± 23	
IBS-SSS	Placebo	271 ± 64	253 ± 46	260 ± 54	<0.0001
	probiotic	196 ± 57	172 ± 50	181 ± 53	
	Placebo-probiotic	-75 ± 79	-82 ± 81	-81 ± 63	

4.6 Fecal cultivable microbiota and metabolome

No statistical difference existed between probiotic and placebo according to heterotrophic aerobic and anaerobic bacteria, presumptive *Staphylococcus*, *Bacteroides*, *Porphyromonas* and *Prevotella*, *Enterobacteria*, *Aeromonas* and *Pseudomonas*, and

enterococci for total microbes. ($p=NS$). Compared to T0, the treatment drove the increases of presumptive lactic acid bacteria ($p=0.035$). A positive trend was also found for *Bifidobacteria* in treated patients compared to the T0 ($p=0.041$) and placebo ($p=0.048$). A similar result was previously described (Verdanelli et al., 2011). The relative abundance trend of VOCs showed a significantly ($p<0.05$) increase of propanoic, butanoic, and pentanoic acids and hydrocarbons during probiotic compared to T0 and a decrease of phenol, considered a toxic metabolite (Ahmed et al., 2013). Butanoic acids, whose anti-inflammatory proprieties are well known, was more abundant during probiotic period compared to placebo ($p<0.05$) (Falony et al., 2006).

5. Conclusions and Future Perspectives

IBS and lactose intolerance represent the most common cause of gastrointestinal disturbance. These disorders represent a social and an economic burden. For instance, lactose intolerance represents the most common food intolerance affecting up to the 75% of global population. Thus, the idea of the modulation of gut microbiota with selected probiotics is strongly emerging in these diseases management. However, the majority of the studies lacked in details and they are mainly conducted on a limited scale of patients. We delineated the effect of a selected nutraceutical mixture on our group of patients: IBS patients and lactose intolerant patients. Thus, this work aimed also to define the specific microbial signature in these disorders. More specifically, the assessment of the effectiveness of the treatment, as, appears to be of paramount importance if the data of Italian patients will be compared to that ones that will be obtained with the Romanian ones. In particular, the activity in Romania, also aims to assess the different micro/macro nutrients dietary intake, the faecal and salivary microbiota composition, and the estimation of the prevalence of the type of food intolerance (lactose or fructose). The nutraceutical mixture that will provide the best results in terms of amelioration of symptomatology and microbiota, will be assembled in Farmalabor, Canosa (BAT), Italy.

6. Nomenclature

IBS, Irritable Bowel Syndrome; SFCA, Short Chain Fatty Acids, IBS-D, Irritable Bowel Syndrome-Diarrhea; IBS-C, Irritable Bowel Syndrome-Constipation; IBS-SSS, Irritable Bowel Syndrome Severity Score, VOCs, Volatile Organic Compounds.

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Development of an Electrochemical Biosensor for Food Safety: Detection of Food Pathogens

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This PhD thesis deals with the development and application of two electrochemical biosensors DNA-label free based on Organic Electrochemical Transistor (OECT) and on voltammetry. The purpose is the detection of *Listeria monocytogenes* and *Campylobacter* spp. in foodstuffs to improve routine food analysis.

Sviluppo di un biosensore elettrochimico per la sicurezza alimentare: rilevamento di patogeni alimentari

Questa tesi di dottorato riguarda lo sviluppo e l'applicazione di due biosensori elettrochimici a DNA-label free, uno basato su un transistor elettrochimico organico e uno sulla voltametria. Lo scopo è il rilevamento di *Listeria monocytogenes* e *Campylobacter* spp. negli alimenti per migliorare l'analisi routinaria.

Key words: Electrochemical biosensor, OECT, voltammetry, DNA bioreceptor, label-free probe, pathogens.

1.Introduction

Listeria monocytogenes and *Campylobacter* spp. are two important foodborne pathogens (EFSA, 2018). They can be acquired by ingestion of contaminated food mainly ready to eat (RTE), undercooked chicken, and milk and dairy products respectively. The symptoms are gastrointestinal disorder that can switch in a serious disease like listeriosis and campylobacteriosis in weak individuals, such as immuno-compromised, elderly and newborns and pregnant women. The recommended ISO standard methods (ISO 11290 and ISO 10272) are time consuming and laborious. Therefore, food industry needs a rapid and sensitive analytical method able to detect pathogens in order to comply the legislative rules defined by food authorities, ensuring food safety and consumers health.

The biosensors respond to this need, indeed show several advantages compared to classical and molecular methods in use.

A biosensor is a device composed by two elements: i) a bioreceptor (DNA probe, aptamer, antibody etc.) that can detect a target and ii) a transducer which converts the biological interaction in an electric signal. Currently the biosensors are being developed for both screening and quantitative (CarmenMorant-Miñana & Elizalde, 2015; Gaffar et al., 2017) detection, as they are sensitive, specific, simple to use, and cheap. Their utilization can help real-time monitoring. Moreover, the possibility of miniaturization and portability can allow analysis along food chain production or at various market sites (Lin P et al., 2011; Kuo et al., 2018). In particular, the electrochemical biosensor is suitable for this purpose thanks to the simplicity of the instrumentation and the possibility to miniaturize the system without reduction in the analysis performance. Other advantages are high sensitivity (Gabig-Ciminska et al., 2004; Tao et al., 2017), low cost and the possibility to utilize low voltage values (Gentile et al., 2014).

This work is focused on the development and testing of two electrochemical biosensors that use label-free DNA probes and in the combination between bioreceptor and transducer to improve the selectivity and sensitivity. An Organic Electrochemical transistor (OECT) biosensor was developed to detect *L. monocytogenes*. Instead, a voltammetric biosensor (VB) was developed to detect *Campylobacter jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*, considered the major cause of gastrointestinal disorders. The OECT biosensor consists of two interfaces: the gold (Au) gate electrode and the polymer channel surface. The gate electrode is modified with the addition of the bio-receptor (DNA probe), thus after the binding of the analytes to the gold surface of the gate a change in the potential is detected at polymer channel. Instead, the VB is composed by an Au work electrode that is functionalized with the DNA probe, and after binding to the analyte a change in the current signal is detected.

In accordance with the PhD thesis project this oral communication reports the main results of the following activities directed to:

- A1) Design of primer and probe (bioreceptor) and optimization of the system
- A2) Comparison between classical microbiology and molecular biology methods used for the detection of *L. monocytogenes* and *Campylobacter spp* in foods
- A3) Development and Testing of Electrochemical Biosensors: based on OECT and Voltammetry
- A4) Validation of the method using real-life food samples

2. Materials and Methods

2.1 *L. monocytogenes* detection

In silico analyses of aligned *Listeria* spp sequences (accession numbers: DQ054587.1, AF532293.1, M80352.1, M80349.1, M80353.1, M80354.1 and M80350.1) for the design of a new reverse primer to work with the previously developed primer Mar1 (Manzano et al., 1997) to produce an amplicon useful for PCR assay were performed. Design of a specific DNA probe to be used as a capture probe in the OECT biosensor.

Optimization of primer and probe using DNA extracted from pure cultures of the reference strains listed in Table.1.

Eight samples of smoked salmon bought at market level were analyzed: i) using the *Listeria* Precis™ methods validated AFNOR, ii) PCR method and iii) the OECT biosensor. Fifteen raw ham, fifteen cured ham and fifteen swab samples from surfaces of the working area of the factory were subjected to the same analysis methods for *Listeria* detection.

Table 1 List of the reference strains from collections. ATCC: American Type Culture Collection (Manassas, VA, USA). DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), DI4A: Department of Agricultural, Food, Environmental and Animal Sciences.

	MICROORGANISM	COLLECTION CODE
POSITIVE CONTROLS	<i>Listeria monocytogenes</i>	ATCC 7644
	<i>Campylobacter jejuni</i>	DSM 4688
	<i>C. coli</i>	DSM 24155
	<i>C. lari</i>	DSM 11375
NEGATIVE CONTROLS	<i>C. upsaliensis</i>	DSM5365
	<i>Listeria innocua</i>	DSM 20649
	<i>L. seeligeri</i>	DSM 20751
	<i>L. marthii</i>	DSM 22813
	<i>L. welshimeri</i>	DSM 15452
	<i>L. ivanovii</i>	52491
	<i>Campylobacter fetus</i>	DSM 5361
	<i>C. cryaerophila</i>	DSM 7289
	<i>Helicobacter pylori</i>	DSM 7492
	<i>H. suis</i>	DSM 19735
	<i>Arcobacter butzleri</i>	DSM 8739
	<i>Salmonella enterica</i>	DSM 9145
	<i>Escherichia coli</i>	DI4A
	<i>Bacillus cereus</i>	DSM 2301
	<i>B. subtilis</i>	DSM 4181
	<i>Lactobacillus plantarum</i>	ATCC BAA-793
	<i>Lb. rhamnosus</i>	ATCC 53103
	<i>Lb. paracasei</i>	DSM 5622
	<i>Lb. brevis</i>	DSM 20054
	<i>Staphylococcus aureus</i>	DI4A
	<i>Saccharomyces cerevisiae</i>	ATCC 36024

2.2 Campylobacter spp. detection

In silico analyses of *Campylobacter* spp sequences (accession numbers: GQ167702.1, GQ167720.1, AB644222.1 and DQ871249.1) were performed to design a new couple of primers for qPCR assay.

Design of a new DNA probe for the utilization with the voltammetric biosensor.

Twenty samples of chicken meat were bought at various butcher shops, and analyzed according to: i) 10272:2006 ISO method for *Campylobacter* spp, ii) qPCR and iii) Voltammetric biosensor.

The DNA used for the molecular analysis was extracted as reported by Manzano et al., (2003) from homogenization saline solutions (t_0), and enrichment broths [24 (t_{24}) or 48 (t_{48}) h].

Reference strains of *Listeria* and *Campylobacter* from DSMZ, ATCC international collections and DI4A collections were extracted and used as positive and negative controls.

3. Results and Discussion

3.1 Optimization of the molecular approach for *L. monocytogenes* detection

The specie-specific primers and probe for *L. monocytogenes* were designed into *iap* (Invasion Associated Protein) gene, selected because it has an internal region with high variability between pathogenic *L. monocytogenes* and the other non-pathogenic *Listeria* spp. Therefore, it can ensure a high specie-specificity. The designed new oligonucleotides (MarB reverse primer and ListE probe) were selected by in silico testing before proceeding with the optimization protocol for the techniques selected.

The primers [Mar1 of 19 nucleotides (nt) and MarB 17 nt] were tested using various PCR conditions such as T° annealing and $MgCl_2$ concentrations. The primers were applied in qPCR assay producing a LOD (Limit of Detection) of 10 fg/ μ L, after testing for specificity using reference strain.

The designed probe (List-Capt of 34 nt) was first tested for specificity and sensitivity by dot blot. The specificity was confirmed on reference strains listed in Table 1, and the sensitivity produced a LOD of 1 ng/ μ L. Thus, the probe was labeled with a thiol group at 5'-

end to allow the immobilization on the Au electrode surface. The functionalized Au coated coverslips electrode were used in the OECT biosensor. The functionalization was validated using topography and the Volta potential map analysis (Fig. 1) conducted by Atomic Force Microscopy (AFM).

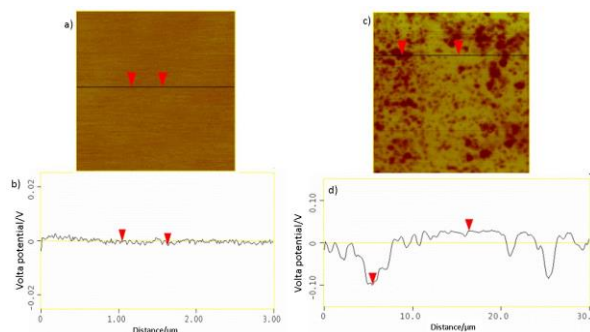


Figure 1 Characterization of Au electrode surface by AFM. a) Volta potential map and b) the topographic line scan of bare Au surface; c) The Volta potential map and in d) the topographic line scan of the functionalized surface with probe and DNA.

The optimization of the OECT biosensor showed in Fig. 2 was focused on the shape of the electrode (plate), electrolyte concentration of phosphate buffer saline (PBS), and functionalization with the designed DNA probe.

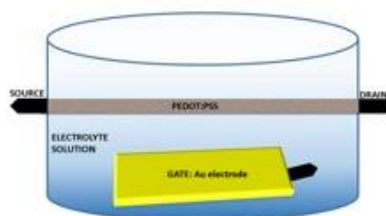


Figure 2 OECT biosensor.

After optimization, the optimized protocols of PCR and the protocol of the biosensor, were applied to analyze the recovered food samples. Three analysis were conducted in parallel with the plate count based, molecular approach and the novel approach based on Electrochemical Biosensor.

3.2 Detection of *L. monocytogenes* in cold smoked samples

Eight samples of cold smoked salmon were analyzed in duplicate, once immediately after purchasing and the other one close to expiring date for a total of 16 samples. To detect *L. monocytogenes* were used: the traditional plate count method, to evaluate the natural microbial contamination, the *Listeria* PreciTM methods, and the OECT. The presence of *L. monocytogenes* was observed in one sample at the end of the shelf life. The positive result obtained with the AFNOR method (Fig. 3) was confirmed with qPCR and OECT.

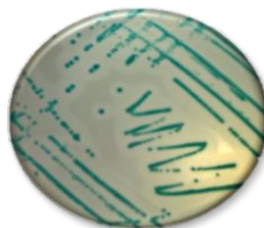


Figure 3 The positive salmon smoked cold sample on the selective medium, Brilliance Listeria Agar.

3.3 Detection of *L. monocytogenes* in ham and ham factories

The presence of *L. monocytogenes* was detected by microbial plate count in three raw ham samples and in three environmental samples. As expected, *Listeria monocytogenes* was absent in cured ham. The data were confirmed by PCR (Fig.4) and OECT biosensor.

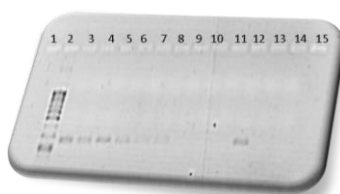


Figure 4 PCR results of samples. Line1-15:; line2: 100pb ladder; line3: n.1 fresh ham; line4: n. 2 fresh ham; line5: n. 1 swab sample; line6: n. 2 swab sample; line7:n.3 swab sample; line8:n. 3 fresh ham; line9: n. 4 fresh ham; line10: n.4 swab sample; line 11: n.5 cured ham; line 12: *L. monocytogenes* ATCC 7644; line 13: *L. innocua* DSM 20649; line 14: PCR negative control.

3.4 Optimization of the molecular approach for *Campylobacter* spp detection

The specie-specific primers and probe for *Campylobacter* spp. were designed into 16-23S gene encoding for rRNA and 16S rRNA respectively. This gene was selected to detect the four species *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*, responsible for campylobacteriosis. A conserved nucleotide region present in all the four species was necessary to allow the pathogen detection still maintaining a high specie-specificity. The designed primers (CampyPfw 19 nt and CampyPrv 20 nt) and the specific probe CampyP3 (36 nt) were analyzed by silico testing before proceeding with the optimization protocol for the qPCR and biosensor techniques.

PCR conditions (T° annealling and $MgCl_2$ concentration) were optimized using PCR, giving high specificity (Fig. 5) after been tested on reference strains (Table 1), and the PCR sensitivity of 1 pg/ μ L was obtained. Subsequently, they were applied in qPCR to improve sensitivity obtaining a LOD of 100 fg/ μ L.

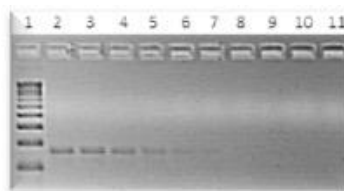


Figure 5 Sensitivity test of CampyP fw-rv conducted with *C. jejuni* at several concentration DNA. Line1:100pb ladder; Line2: 25ng/μL; Line3: 10ng/μL; Line4: 1ng/μL; Line5: 100pg/μL; Line6: 10pg/μL; Line7: 1pg/μL, Line8: 100fg/μL; Line9: 10fg/μL; Line10: 1fg/μL; Line11: PCR negative control.

The selected probe (CampyP3) was tested first using dot blot to verify specificity, obtained on the reference strains listed in Table 1, and sensitivity, reaching a LOD of 1 ng/μL. Thus, the probe was labeled with a thiol group at 5'-end to allow the functionalization of the screen-printed gold electrode used with the biosensor based on voltammetry. All the following parameters were optimized before the application of the biosensor analysis: i) buffer concentration, ii) blocking solution, iii) bio-receptor (DNA probe concentration), iv) solution composition and number of washing steps, v) temperature and time for functionalization.

The optimized protocols were applied to analyze the 20 chicken meat samples. Three analysis were conducted in parallel with the plate count based method, molecular approach with PCR, qPCR and the novel approach based on cycle voltammetry.

3.5 Detection of *Campylobacter* spp. in chicken meat samples

Five samples were positive for *Campylobacter* spp. using the ISO 10272: 2006, qPCR and with the electrochemical biosensor. Instead, five samples resulted positive only using the qPCR molecular method, indeed they gave a negative result with the ISO 10272: 2006. They are not yet tested with the biosensor. The incongruity results can be explained with the enrichment broth used. The Bolton broth is considered able to resuscitation of the *Campylobacter* spp, but last years several studies have shown that it is not selective (Moran et al., 2011). This enrichment broth contains several antibiotics such as Cefoperazone. The extended spectrum beta-lactamase (ESBL) are resistant to this antibiotic. Therefore, the isolation of *Campylobacter* spp. is a problem involving an underestimation due to growth competition (Hazeleger et al., 2016; Habib et al., 2011). Moreover the ISO standard method recommended a selective medium, the mCCDA, and also in this case the antibiotics component are cefoperazone thus the selectivity problem persists, leading to false negative results.

4. Conclusion and Future Perspectives

Taking into account the detection techniques developed, optimized and applied for the analysis of real food samples, several considerations can be done.

The plate count based approach ISO 10272:2006 for *Campylobacter* and the *Listeria* Precis method need a long analysis time from 5 to 8 days. It is considered too long for food companies releasing short shelf-life foods. Moreover, the detection of living cells can be difficult due to food industries treatments used for food stabilization, that can stress cells that enter in a VBNC (viable but not culturable) state and are unable to grow on agar medium.

The procedures are also laborious.

The molecular approach based on PCR/qPCR is specific, but to get high sensitivity an enrichment step is still needed for the detection of DNA, as demonstrated by the negative results obtained from homogenization samples (t_0). These techniques although

more expensive, can reduce the time for analysis to 2-3 days.

The novelty approach with the electrochemical biosensors described can match with the needs of rapidity, and sensitivity in the detection of food pathogens. The sensitivity obtained is not enough to detect the pathogen without an enrichment step like for the molecular approach.

Thus, in order to improve the sensing performance the modification of the gate electrode with nanoparticles or changing the nanostructure need further study.

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^1H -NMR Spectroscopy to Investigate the Connection Between Probiotics and Health

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As a part of my PhD thesis, the works here described attempted to investigate the connection between probiotics and health from a metabolomics perspective, by proton nuclear magnetic resonance (^1H -NMR) spectroscopy. In order to obtain reliable and reproducible concentrations of the molecules constituting the metabolome of various biofluids, the first fundamental step was to set up standard operating procedures (SOP) for common biofluids (i.e. feces and urine), specific for ^1H -NMR.

Spettroscopia ^1H -NMR per Studiare la Connessione tra Probiotici e Salute

I lavori descritti nella presente comunicazione hanno occupato una parte della mia attività di dottorato, e hanno mirato a studiare la connessione tra i probiotici e la salute dal punto di vista metabolomico, mediante la spettroscopia di risonanza magnetica nucleare (^1H -NMR). Al fine di ottenere concentrazioni affidabili e riproducibili delle molecole che costituiscono il metaboloma di vari biofluidi, il primo passo fondamentale è stato quello di istituire procedure operative standard (SOP) per biofluidi comuni (vale a dire feci e urina), specifici per ^1H -NMR.

Key words: ^1H -NMR spectroscopy; urine; feces; metabolome; probiotics; health.

1. Introduction

Probiotics are classified as functional foods, which may help to reduce the risk of disease or promote better health when administered in appropriate amounts. Nowadays, more and more works focusing on probiotics and gut microbiota try to investigate the connection between their molecular features and their impacts on the health status of individuals. These efforts accelerate our understanding of the molecular connections established between probiotics and their beneficial effects in the human body. Among all the “omics” technics that could be applied for the task, the metabolome is considered as the best representation of the phenotype, being downstream of genome, transcriptome and proteome (Laghi et al., 2014).

Metabolomics can give an access to the simultaneous characterization of hundreds of low molecular weight metabolites (<900 Da), in a biofluid or tissue. Through metabolomics it is possible to analyze the diversities of metabolic response of a tissue to biological stimuli. As one of the main platforms for metabolome research, high-resolution proton nuclear magnetic resonance (^1H -NMR) spectroscopy can match the requirement perfectly. Firstly, NMR profiles contain qualitative and quantitative information on hundreds of different small molecules present in a tissue (Elmi et al., 2019). Furthermore, through NMR-based metabolomics, molecules whose concentrations significantly altered due to different treatments are directly obtained through uni/multi variate statistical analysis (Parolin et al., 2018).

Two key questions are raised in the current communications. First, the extrapolation of molecules' concentrations from NMR spectra can be severely hindered by shifts of the

signals caused by factors like pH and ionic strength, especially for urine. Therefore, how to obtain useful pieces of information from the metabolome of biofluids? Second, does the same probiotic strain exert the same effect/s in all individuals or are the effects specific and host-dependent?

For the first question, in order to create robust databases, a first key step should be the design of convenient SOP for samples manipulation. To shed lights on the second question, a trial we set up to investigate the possibility to use a high concentration multispecies probiotic formulation (a mix of 8 bacterial strains of live lactic acid bacteria and bifidobacterial) typically employed for humans, to improve the training performance of Standardbred horses. In parallel, the performance of probiotics was also assessed typically for the treatment of infantile colic disease.

2. Experimental Procedure

In accordance with the PhD thesis project, this oral communication reports the main results of the following four activities directed to:

A1) set up ^1H -NMR based SOP;

A2) investigate the variances of horse urine metabolome relating to physiology features (i.e. genders and ages), meanwhile, as a baseline for next investigation;

A3) investigate the connection between probiotics and horse athletic performance on the basis of A2;

A4) in parallel, try to assess performance of probiotics on human health, especially for children colonic disease.

3. Materials and Methods

3.1 set up ^1H -NMR based SOP (A1)

In order to set up NMR based SOP for feces and urine, five specimens of male Jiulong yak (approximately three years of age, receiving no supplementation) were randomly selected for the current experiment.

Upon slaughtering, urine samples were collected by cystocentesis upon direct bladder visualization using a sterile syringe while feces were collected as the animals defecated during the slaughtering process.

3.2 Characterization of horse urine metabolome and assessment of the probiotic supplementation effects (A2 and A3)

Ten Standardbred horses (4 male and 6 female) were included in the study. The age of the horses ranged between 3 and 8 years (median 6.5 years), and the weight was between 405 and 511 kg (median 479 kg). The study was designed as a randomized, double-blinded, placebo-controlled trial with crossover and was conducted in the same racetrack where the horses were housed.

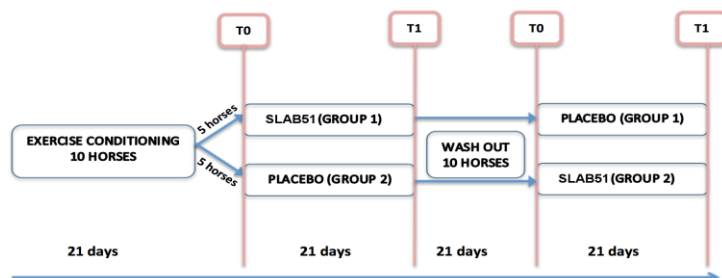


Figure 1 Schedule of the clinical trials activity. T0, before treatment. T1, after treatment.

Training sessions were always performed in the morning, immediately after consumption of the first food ration of the day. As showing in Figure 1, after 21 days of exercise conditioning, the horses were randomly divided into two groups of five subjects each: group 1 was treated with food supplemented with Slab51 probiotic mixture for 21 days; and group 2 was fed with placebo for 21 days. In detail, 17.5 g of probiotic/placebo were administered twice a day directly in the mouth of the horse using a tube dozer. After a washout period of 21 days, the two groups were reversed so that placebo was administered to group 1, and probiotics were administered to group 2 for an additional 21 days. The details of Slab51 probiotic mixture are showed in the paper authored by Laghi *et al.* (Laghi *et al.*, 2018).

Urine was collected before (pre) and after (post) each training session both at T0 and at T1 during spontaneous urination, using a stick connected with a sterile 50 mL Falcon tube containing sodium azide, directed under the urine flow at the time of urination.

3.3 Probiotic for the treatment of infantile colics assessed through a metabolome approach (A4)

All infants enrolled were randomized to receive, either oral probiotics or the placebo. Parents were requested to administer to their infants 10 drops of the probiotics, once a day, for 21 days directly in the mouth, preferably in the morning before feeding. Ten drops of the formulation delivered a dose of 5 billion colony-forming units (CFU) of probiotic-mixture (Vivomixx®, Visbiome®, DeSimone Formulation®; Danisco-DuPont, Madison, WI, USA). The placebo was characterized by an identical formulation, but without any probiotic. The details of probiotic mixture and placebo are showed in the paper authored by Baldassarre *et al.* (Baldassarre *et al.*, 2018).

Two stool samples of infants were collected, once at enrolment and then 21 days after the beginning of the administration of study products.

3.4 $^1\text{H-NMR}$

Urine and feces sample preparations are in accordance with the NMR based SOP we set up in A2, the details are showed in the result section.

$^1\text{H-NMR}$ spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy), at a frequency of 600.13 MHz, equipped with Topspin software (Ver. 3.5). The other parameters of $^1\text{H-NMR}$ are showed in the paper authored by Zhu *et al.* (Zhu *et al.*, 2018).

3.5 Statistical analysis

Statistical analysis was conducted in R computational language (Ihaka & Gentleman, 1996). For A2 and A3, taking into account that each horse was assessed multiple times, a Kruskal–Wallis test for repeated samples was applied on a molecular basis by means of

aov function of the R package “stats” (Chambers et al., 1992) on the parameters expressed as ranks (Conover & Iman, 1981). For A4, in the fecal metabolomics evaluation, molecules whose concentration varied in relation to the investigated treatments were looked for by calculating the T21–T0 differences and then they were compared by means of Mann-Whitney U test. In the context, a cut-off *p*-value of 0.05 was accepted.

To highlight the trends characterizing the samples, we calculated principal component analysis (PCA) models, or their robust counterpart (rPCA) (Hubert et al., 2005), on the molecules accepted by univariate analysis. For each model, we calculated the scoreplot, the projection of the samples in the PC space, tailored to highlight the underlying structure of the data. Besides, we calculated the Pearson correlation plot relating the concentration of each variable to the components of the model.

4. Result and Discussion

4.1 ¹H-NMR based SOP

We created an NMR analysis solution with 3-(trimethylsilyl)-propionic-2,2,3,3-d₄ acid sodium salt (TSP) 10 mM in D₂O, set at pH 7.00 ± 0.02 by means of 1 M phosphate buffer, containing also 10 µL of NaN₃ 2 mM. TSP was employed as an NMR chemical-shift reference, while NaN₃ avoided microbial proliferation.

Urine samples were prepared for ¹H-NMR by means of thawing and centrifuging them for 15 min at 18,630 g at 4 °C. An amount of supernatant equal to 350 µL was added to 350 µL of bi-distilled water and to 200 µL of NMR analysis solution. Fecal samples were prepared for ¹H-NMR analysis by vortex mixing for 5 min 80 mg of stool with 1 mL of deionized water. The obtained mixes were then centrifuged for 15 min at 18,630 g and 4 °C, and 700 µL of supernatant was added to 200 µL of NMR analysis solution. Finally, each of the obtained samples was centrifuged again at the above conditions just before analysis.

4.2 Horse urine metabolome characterization

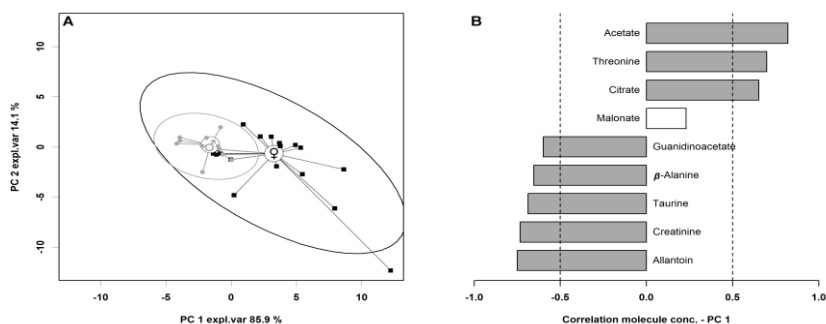


Figure 2 *rPCA* model calculated on the space constituted by the concentration of the molecules significantly different between genders. In the scoreplot A, male horses and mares are represented with black squares and gray circles respectively. The wide, empty circles represent the mean of the samples. The ellipses highlight the one standard deviation limit around the mean. In the barplot B, describing the correlation between the concentration of each molecule and its importance over PC 1, dark gray bars highlight statistically significant correlations (*p* < 0.05).

Gender - Nine of the quantified molecules were significantly affected by gender. To obtain an overview of the differences between genders, we created an rPCA model based on these molecules (Figure 2). Two components were found to describe robustly the

samples. The first component, representing 85.9% of the variance thus expressed, significantly summarized the peculiarities connected to gender ($p < 0.05$), with allantoin and creatinine high concentrations representing the main feature of male horses' urine and acetate representing the main feature of mares. Furthermore, most of molecules we found significantly altered by gender are related to discrepancies of physiology features between male horses and mares (i.e. hormones, muscle mass and exercise performance).

Age - Two molecules were found to be significantly ($p < 0.05$) affected by age, namely 3-indoxylsulfate and n-phenylacetylglutamine. Indoxyl sulfate and N-phenylacetylglutamine are mainly described as gut microorganism co-metabolite (Fujii et al., 2011; Mayneris-Perxachs et al., 2016). These pieces of information allow to speculate that the differences in horses' urine highlighted in connection to age are, at least partially, related to changes in gut microbiota profile, with particular reference to the reduction in bacteria diversity (Dougal et al., 2014).

4.3 Horse urine metabolome variation relating to probiotic supplementation

Blood lactate response to exercise can be used in field test for assessing performance and fitness, with direct consequences on the onset of fatigue in athletic horses. Comparing to a similar blood lactate concentration before all training sessions, horses at T1 treated with probiotics showed a significantly lower than the other groups observed post training (Data were provided by Dr. Fulvio Laus in University of Camerino). Since a lower blood lactate concentration has been found in treated group after exercise, probiotics seem to promote the performance of the athletic horses, which could be correlated with better performance during the race.

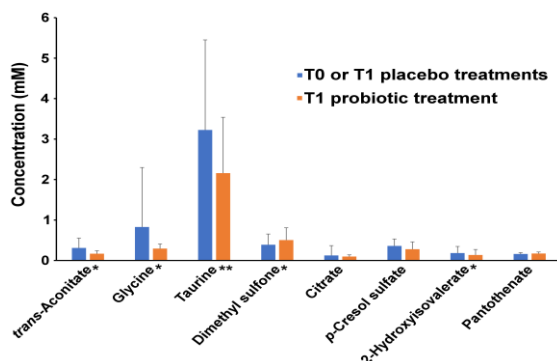


Figure 3 Concentration (mM, mean $\pm$ SD) of the molecules which concentration pre-training varied because of probiotic supplementation. For readability reasons, only comparisons characterized by a p -value lower than 0.1 are reported. The concentration of p -cresol sulfate and 2-hydroxyisovalerate was multiplied by 10 for the same reasons. * $p < 0.05$.

The molecular profile for probiotics at T1 significantly differed from the others in the concentration of five molecules (Figure 3). Among the molecules characterized by urine metabolomics, these are the only containing sulfur and the concentration of all of them has been found altered by probiotics administration. Moreover, p -cresol sulfate and taurine have been found also positively correlated to lactate concentration in blood post-exercise. These molecules seem therefore to suggest that sulfur metabolism may play a role in the highlighted energy metabolism changes.

4.4 Effectiveness and safety of a probiotic-mixture for the treatment of infantile colic through fecal metabolome approach

Fifty-nine molecules could be quantified pertaining to the chemical groups of amino acids, short chain fatty acids, organic acids and monomeric carbohydrates. The evolution of the fecal metabolome differed for 12 molecules. To have an overview of such findings, we calculated an rPCA model on the centered and scaled concentrations of these

molecules (Figure 4). The median scores of both groups at T21 appear at negative values along PC 1. Such PC, therefore, allows us to focus on the changes occurring to the metabolome of the infants upon growing. Along PC 2, contrastingly, samples from the placebo group appear at positive scores, while samples from the probiotic group appear separated from the previous ($p < 0.01$), with negative scores. PC 2, therefore, gives a holistic view of the different responses of infants, to the two treatments. Along PC 2, propylene glycol increased in subjects treated with probiotics, which appeared as the clearest biomarker of subjects supplemented with probiotics. Propylene glycol is normally found in newborn feces and, interestingly, a higher concentration has been found in the feces of breast-fed infants in comparison with formula-fed ones, which suggesting a beneficial effect for this molecule.

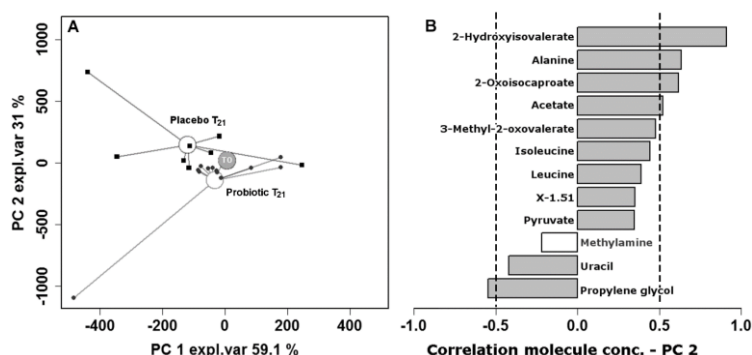


Figure 4 rPCA model built on the space, constituted the concentration of the molecules significantly different among groups. In the scoreplot (A), samples from people treated with the placebo and the probiotics are represented with squares and circles respectively. The wide, empty circles represent the median of the samples at the various time-points. Samples at T0 from the two groups are superimposed. In the barplot (B), describing the correlation between the concentration of each molecule and its importance over PC 2, gray bars highlight statistically significant correlations ($p < 0.05$).

5. Conclusions

A1 - This work gives, for the first-time, quantitative information about 54 molecules mostly representative of the metabolome observable by ^1H -NMR analysis on horse urine. Moreover, we were able to describe how gender, age and exercise affected their concentration, by means of a two steps protocol based on univariate and robust principal component analysis.

A2 - The results of this part show that probiotic supplementation could reduce postexercise blood lactate concentration in Standardbred horses in athletic activity. The tested urine molecular profile suggests that a likely mechanism underlying this positive effect is connected to a switch of energy source in muscle from carbohydrates to SCFAs.

A3 - Daily supplementation with a specific, high-concentration probiotic preparation to breastfed infants modulates infantile colic symptoms by the end of treatment (21 days). Moreover, probiotics were found to affect the fecal molecular profile.

6. References

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Programme

Wednesday, 11 September, 2019

14.00-15.00 Registration

15.00-15.30 **Opening session, Auditorium**

Marco Bindi, pro-rector for national and international scientific research, University of Florence
Marco Remaschi, Councillor for Agriculture, Regione Toscana
Simone Orlandini, Director of the Department of Agriculture, Food, Environment and Forestry, University of Florence
Marco Poiana, chair of the Italian Society of Food Sciences and Technologies (SISTAL)
Cinzia Caggia, vice-chair of the Italian Society of Agro-Food and Environmental Microbiology (SIMTREA)
Erminio Monteleone, chair of the Italian Sensory Science Society (SISS)

15.30-16.00 **Plenary lecture: "New frontiers in sensory food science for health", Auditorium**
Paula Varela, Nofima | University of Life Sciences, Norway
Chair: Erminio Monteleone, University of Florence

16.00-17.00 **Parallel session #1: Food Processing and Sustainability, Auditorium**
Chairs: Paolo Masi (University of Naples, Federico II), Maria Cristina Nicoli (University of Udine)

16.00-16.15 **Ghislain Fotie, University of Milan**
Effective Replacement in Food Packaging Laminates of the Conventional Oil Based Oxygen Barrier Polymers with Bio-Composite Laminates Containing Cellulose Nanocrystals (CNCs) Extracted from Waste and Biomasses

16.15-16.30 **Valentina Goffi, University of Tuscia**
Effects of Different Storage Technologies and Temperatures on the Quality and Nutritional Value of Yellow-Fleshed Kiwifruit

16.30-16.45 **Begum Onal, University of Salerno**
Drying of Fruits and Vegetables: Improvement of Quality and Process Modelling

16.45-17.00 **Marco Spaggiari, University of Parma**
Cereal Milling By-products Valorisation: from Unexploited Residues to Valuable Resources

16.00-16.45 **Parallel Session #2: Food Microbiology and Biotechnology, Poccetti Room**
Chairs: Cinzia Caggia (University of Catania), Costantino Fadda (University of Sassari)

16.00-16.15 **Walter Fiore, University of Milan**
Overall Assessment of a Model Probiotic Bacterium: from Gut Colonization to Clinical Efficacy

16.15-16.30 **Manolo Laiola, University of Naples Federico II**
Changes in Oral and Gut Microbiota Composition in Overweight Subjects upon Dietary Intervention Based on Mediterranean Dietary Model



16.30-16.45 **Livia Vanessa Storti, University of Basilicata**

Exploitation of Energy Metabolism in Obligate Heterofermentative Lactic Acid Bacteria

17.00-17.30 *Coffee break*

17.30-18.30 **Parallel Session #3: Food Processing and Sustainability, Auditorium**

Chairs: Mauro Moresi (University of Tuscia), Chiara Dall'Asta (University of Parma)

17.30-17.45 **Umberto Cancelli, University of Modena and Reggio Emilia**

Circular Economy Strategies for Management and Valorisation of Some By-Products of the Wine Industry

17.45-18.00 **Rodrigo Gonzalez Ortega, University of Teramo**

Development of Encapsulated Olive Oil and Olive Extracts as Innovative Natural and Health Food Ingredients

18.00-18.15 **Marina Mefleh, University of Sassari**

Are New Ancient Wheats Suitable for Breadmaking?

18.15-18.30 **Manuela Oriente, University of Molise**

Development of Barley Based Couscous with High Nutritional Value and Functional Properties

17.30-18.30 **Parallel Session #4: Food Quality and Preference, Poccetti room**

Chairs: Caterina Dinnella (University of Florence), Antonella Verzera (University of Messina)

17.30-17.45 **Giulia Baldi, University of Bologna**

Poultry Meat Quality as Affected by Growth-Related Muscular Abnormalities and Early Post-Mortem Metabolism

17.45-18.00 **Camilla Cattaneo, University of Milan**

New Perspective on Taste: Exploring Association among Oral Perception, Tongue Physiology and Oral Microbiota Composition

18.00-18.15 **Daniele Cavanna, University of Parma**

Innovative Analytical Methods Merged with Chemometric for Food Integrity Assurance

18.15-18.30 **Delvana Mutarutwa, University of Teramo**

Aroma Volatile Compounds of Coffee: Role in Authenticity, Traceability and Well-being of Consumers

16.00-18.30 **Poster session: Poster & Miniposter**

19.00 *Apericena, Chiostro di Sant'Apollonia*

Thursday, 12 September, 2019

9.00-9.30 **Plenary lecture: "Food Company dilemma: to innovate or not to Innovate - a necessary evil?", Auditorium**

Guido Calò, Sammontana

Chair: Bruno Zanoni, University of Florence

9.30-10.30 **Parallel Session #5: Food Quality and Preference, Auditorium**
Chairs: Silvana Cavella (University of Naples, Federico II), Elena Vittadini (University of Camerino)

9.30-9.45 **Alessandra De Toffoli, University of Florence**
Influence of Psychological Traits on the Acceptability of Healthy Foods

9.45-10.00 **Davide Rossi, University of Teramo**
Use of Tensiometric Techniques to Design and Develop Food and Nutraceutical Formulations

10.00-10.15 **Francesca Saitta, University of Milan**
Artificial Insulin Secretory Granules' Membranes: Thermodynamic Stability and Interaction with Food Fatty Acids

10.15-10.30 **Chenglin Zhu, University of Bologna**
1H-NMR Spectroscopy to Investigate the Connection Between Probiotics and Health

9.30-10.30 **Parallel Session #6: Food Microbiology and Biotechnology, Poccetti Room**
Chairs: Angela Capece (University of Basilicata), Severino Zara (University of Sassari)

9.30-9.45 **Vincenzo Cantatore, University of Bari Aldo Moro**
Functional Exploitation of the Metabolism of Lactic Acid Bacteria during Fermentation of Plant Matrices

9.45-10.00 **Igor Chiarelli, University of Milan**
Production of Natural Aroma Compounds by Biocatalysis in Water with Acyltransferase

10.00-10.15 **Michela Verni, University of Bari Aldo Moro**
Use of Selected Lactic Acid Bacteria for Fermentation of Non-Conventional Vegetable Matrices: Nutritional and Functional Implications

10.15-10.30 **Priya Vizzini, University of Udine**
Development of an Electrochemical Biosensor for Food Safety: Detection of Food Pathogens

10.30-11.00 *Coffee break*

11.00-12.00 **Parallel Session #7: Food Processing and Sustainability, Auditorium**
Chairs: Vincenzo Gerbi (University of Turin), Maurizio Servili (University of Perugia)

11.00-11.15 **Giulia Diamante, University of Udine**
New Insights into Pasta Cooking: The Continuous Cooking Procedure in Professional Appliances

11.15-11.30 **Federica Flamminii, University of Teramo**
Development, Characterization and Application in Food Systems of Innovative Functional Ingredients Obtained from Olive By-products Phenolic Extracts

11.30-11.45 **Melania Grassia, University of Molise**
Microencapsulation of Cocoa Polyphenols by Spray-Drying



11.45-12.00 **Giuseppe Perri, University of Bari**

The Production of an Innovative Ingredient Based on Leguminous Flour and Germinated Cereals for the Improvement of Baked Goods Obtained by Sourdough Technology

11.00-12.00 **Parallel Session #8: Food Microbiology and Biotechnology, Poccetti Room**
Chairs: Elena Sara Crotti (University of Milan), Patrizia Romano (University of Basilicata)

11.00-11.15 **Carla Cervini, University of Foggia**

Aspergillus from Grapes: Ochratoxin A Risk in Relation to Climate Change and New Strategies for Reducing Contamination

11.15-11.30 **Jatziri Mota Gutierrez, University of Turin**

Microbes in Cocoa Beans: What Microbial Communities Can Do on the Formation of Cocoa Aroma during Fermentation

11.30-11.45 **Armin Tarrah, University of Padova**

In Vitro Probiotic Properties of Newly Isolated Lactobacillus Strains from Infant Feces

11.45-12.00 **Paola Vitellio, University of Bari Aldo Moro**

Role of Functional Food Supplements in Patients with Gastrointestinal Disorders and Food Intolerances (Lactose and/or Fructose)

12.30-14.00 *Lunch*

14.00-14.30 **Plenary lecture: "Wine flavor: From Vineyard to Glass", Auditorium**

Susan Ebeler, University of California, Davis, USA

Chair: Bruno Zanoni, University of Florence

14.30-16.15 **Plenary session: "What for" Award 4th Edition - Federalimentare**

Chairs: Gianpaolo Andrich (University of Pisa), Ella Pagliarini (University of Milan)

14.30-14.45 **Marcello Alinovi, University of Parma**

Chemometric and Computational Approaches to Improve Quality and Increase Market Share of Fresh and Long-Ripened Italian Cheeses

14.45-15.00 **Marilisa Alongi, University of Udine**

Functional Food Development through Novel Sustainable Processing and Formulation

15.00-15.15 **Matteo Cibelli, University of Tuscia**

Cradle-to-Grave Environmental Profile of Dry Pasta: Assessment and Mitigation Measures

15.15-15.30 **Rosa Palagano, University of Bologna**

Advanced Solutions for Authenticity and Quality of Virgin Olive Oils

15.30-15.45 **Lapo Pierguidi, University of Florence**

Individual Differences in Cocktails Perception and Appreciation

15.45-16.00 **Sharon Puleo, University of Naples Federico II**

Texture and Trigeminal Sensations: New Approaches to Measure the Human Sensitivity

16.00-16.15 *Video presentation and voting*

- 16.15-17.15 *Coffee break*
- 17.30-19.00 **Coordinator Meeting, Poccetti Room**
- 9.30-18.30 **Poster session: Poster & Miniposter**
- 19.00 *Apericena, Chiostro di Sant'Apollonia*

Friday, 13 September, 2019

9.30-10.00 **Plenary Lecture, "PhD, what a chance!", Auditorium**
Monica Borgogno, Mérieux Nutrisciences
Chair: Erminio Monteleone, University of Florence

10.00-10.30 **Plenary session: Awarding ceremony "What for" Award 4th Edition**
Federalimentare, Auditorium
Maria Cristina Di Domizio, Federalimentare

10.30-11.00 *Coffee break*

11.00-12.30 **Plenary session with testimonials from industries: "Industrial research on innovative topics: healthy foods and sustainability", Auditorium**
Moderator: Alessandra Gemmiti, Regione Toscana Direzione "Agricoltura e Sviluppo rurale" Settore "Consulenza, formazione ed innovazione"

Speakers:

Giovanni Belli, Biscottificio Belli S.r.l.
Gianfranco Betti, Centrale del Latte della Toscana S.p.A.
Anna Cane, Deoleo
Francesco Parri, Ruffino S.r.l.
Ilio Pasqui, Toscana Certificazione Agroalimentare S.r.l.

12.30-13.00 **Closing remarks and delivery of the certificates of attendance, Auditorium**

