UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN

Area del Farmaco e Trattamenti Innovativi

CICLO XXVIII

COORDINATORE Prof. Teodori Elisabetta

ADMINISTRATION OF POLYPHENOLS-BASED BOTANICALS: INFLUENCE OF INTESTINAL MICROBIOTA'S METABOLISM, FUNCTIONALITY AND COMPOSITION IN *IN VITRO* SIMULATION TECHNOLOGY SHIME®.

Settore Scientifico Disciplinare CHIM/10

Dottorando
Dott. Giuliani Camilla

Tutore
Prof. Mulinacci Nadia

Coordinatore
Prof. Teodori Elisabetta

Anni 2012/2015

TABLE OF CONTENTS

CHAPTER 1: DIETARY SUPPLEMENT BASED ON STILBENOIDS: A FOCUS ON GUT MICROBIAL METABOLISM BY IN VITRO SIMULATOR M-SHIME® 49

Introduction

Intestinal microbiota

Composition and features of microbiota

Intestinal microbiota is defined as a distinct and highly variable composition of symbiotic microorganisms that live in the human gut. It is responsible of many functions such as to provide nutrients to the host and, at the same time, contribute to the maintenance of health status. Qualitative and quantitative alterations of gut microbiota could also lead to many diseases at gastrointestinal and systemic levels [1]

The human gastrointestinal tract is composed of approximately $10^{11\text{-}12}$ cells per ml (mainly anaerobic microorganisms), a quantity ten times higher than total number of cells of the human body [2]. However, the distribution and the concentration of bacteria change along the gastrointestinal tract due to the presence of cells belonging to the human immune system, to enzymes, to concentration of bile acids, pH and redox potential that determine different habitats and can promote the growth of different bacterial species [3].

Although the knowledge on the microbiota composition is still largely incomplete for intrinsic difficulty of determination and extreme variety of components, it's well known that the dominant bacteria in adult humans belong to a restricted number of phyla: mostly *Firmicutes* and *Bacteroidetes*, and to a lesser extent *Actinobacteria* and *Proteobacteria* and *Verrucomicrobia* [4]. The *Firmicutes* are Gram-positive, facultative or obligate anaerobic, that can produce endospores and are present in various sections of the human gut. This phylum includes many genres including *Lactobacillus*, *Enterococcus* and bacteria belonging to the class of *Clostridia*. The *Bacteroidetes* are Gram-negative, asporogenous, obligate anaerobic, and include *Prevotella* and *Bacteroides*. The *Proteobacteria* are Gram-negative, facultative anaerobic, including a number of pathogens such as *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia* and *Escherichia*. Finally the *Actinobacteria* are Gram-positive anaerobic, including *Bifidobacterium*.

In the oral cavity, there is a high concentration of bacteria, while in the stomach, due to acid pH, the concentration is low and few species are able to grow, especially *Lactobacilli*, *Streptococci* and yeasts. A similar situation can also be observed in the duodenum: the acid pH of bile prevents the growth of most bacteria and transit time, reduced by luminal contents, prevents a stable colonization. At ileum level, a gradual increase in density microbial is observed up to the high concentrations in colon tract ($10^{11\text{-}12}$ cells / ml) due to positive environmental conditions: slow transit, suitable pH, available nutrients for bacteria.

At birth, the intestine is essentially sterile and colonization by bacteria depends on the kinds of birth and feeding. During a normal birth, the baby is exposed to the vaginal and faecal flora of the mother, while in case of caesarean section the first contact is usually with breastfeeding [5,6]. Especially breastfed babies develop a microbial composition dominated by *Bifidobacterium* [7], while bottle-fed have a lower percentage [8]. All these aspects determine the final composition of the mature microbiota. Later, introducing solid food in children's diets, the microbiota grows and evolves until the composition becomes similar to adult's one [9].

Once adult, microbiota reaches a well-defined composition, with individual variability, and remains relatively stable over time [10]. Even after a change of diet or short-term antibiotic therapy, composition returns to its initial status due to community resilience [11,12].

A characterization of the mature microbiota shows that, despite the inter-individual differences represented by a group of bacteria called auxiliary component, certain bacterial species are constantly present constituting a real "core" [11,13] However, many factors can influence the composition, including psychological and genetic factors [14]. Moreover, the diet plays a very important role. Several studies confirm that changing the amount or type of carbohydrates for more than four weeks occur deep and quick changes in the composition of the intestinal microbiota [11,15]. A further confirmation comes from the comparison of the intestinal microbial community among African populations, with high vegetable carbohydrates consume, and populations of the EU or the US diets,

richer in protein. Children in Africa have a higher amount of *Bacteroidetes* (73%) than children with a number of EU *Bacteroidetes* 27% and 51% of *Firmicutes*. In addition, the species of *Bacteroidetes* dominant in the African, and absent in others, belongs to the genera *Prevotella* and *Xylanibacter*, responsible of the fermentation of xylans and other components of plant fibre [16].

Studies conducted on the microbiota provide, in most cases, a use of human faeces, in order to obtain a composition intestinal microbial closer to real one; it represents also the simplest technique and easy access from sampling. Studies carried out on samples of microbiota recovered after colon surgery are rarer.

Microbiota functionality

Metabolic role

In balanced diet, about 40 g of carbohydrates reach daily the colon without any metabolism by human enzymes. Most of them are represented by resistant starch (RS), non-starch polysaccharides (NSP) and oligosaccharides, although a small part of the mono- and disaccharides can reach the colon as well [4].

The microbiota is able to degrade some non-digestible food compounds and the endogenous mucus produced by epithelium; they represent their main source of energy in the colon. The fermentation of these carbohydrates leads to the production of important and useful molecules for the guest, the Short Chain Fatty Acids (SCFAs), mainly acetate, propionate and butyrate in the ratio 3: 1: 1 [17]. SCFAs provides multiple functions, for example they are energetic source, promote cell differentiation and regulate the immune system of the host [18]. SCFAs also promote the release of hormones as GlucagoneLikePeptide1 - GPL1 from intestinal cells, leading to an increased release of insulin, a reduction in the release of glucagon, gastric slowdown and satiety feeling, reduction of gastric and pancreatic secretion and reduction absorption of nutrients [19]. In particular, the butyrate is the main source of energy for the colonocytes, has an anti-inflammatory action, inhibits cell proliferation, and has been observed its ability *in vitro* to stimulate the differentiation of epithelial neoplastic cell lines in addition

to promoting the reversion from the neoplastic phenotype in non-neoplastic [20,21]. Propionate is transported to the liver and participates in gluconeogenesis; in the liver it also plays a role in the inhibition of the cholesterol's biosynthesis [22]. Acetate, once into the systemic circulation, mainly reaches the liver and muscle tissue where it is used for lipogenesis [23].

The amount of SCFA varies along the gastrointestinal tract, depending on the environmental conditions and the availability of nutrients. For example, in cecum and ascendant colon, fermentation it is very intense and provides a high SCFA concentration due to acidic pH values (5-6) and to the presence of bacteria with strong saccharolytic activity. On the contrary, at the level of the left colon and the distal one, it proteolytic activity is lower due to the lower amount of available substrate and a pH close to neutral. In this case, main metabolism is the anaerobic, in particular of peptides and proteins with the production of SCFAs, but with production of isobutyrate, 2-methylbutyrate and isovalerate (Branched Chain Fatty Acids, BFCA) [24].

The main source of protein that reaches the colon is derived from the diet, such as elastin and collagen, but also from epithelial cells, lysed bacterial and pancreatic enzymes. Instead of carbohydrate fermentation, the microbial metabolism of proteins and peptides leads to the formation of potentially toxic metabolites, able to contribute to the development of intestinal diseases or tumours, such as ammonia, amines, phenols, thiols and indoles [25].

Some bacterial species, especially those belonging to the genera *Bacteroides*, *Clostridium*, *Bifidobacterium* and *Lactobacillus* are involved in deconjugation of bile salts and their reabsorption in the colon [26]. Finally, the intestinal microbiota is responsible for the synthesis of vitamins (especially in the group B) such as thiamine, riboflavin, pyridoxine and niacin, and contributes to the absorption of minerals such as calcium, magnesium and iron [27].

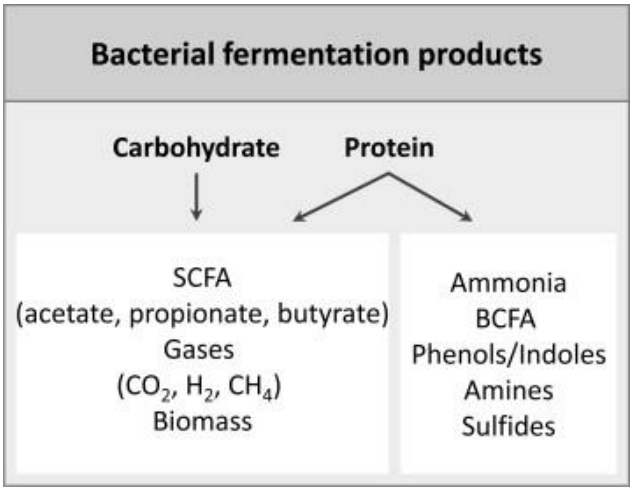


Figure 1: Microbiota metabolites from carbohydrates and protein fermentation [4]

Microbiota and immune system

Microbiota contributes to the development and regulation of the intestinal immune system as demonstrated by several studies on germ-free animals [28,29]. Furthermore, immune response due to microbiota can prevent the development of inflammatory states, which would cause an alteration in the composition with adverse consequences for human health. There is therefore a host-microbiota symbiosis necessary for intestinal homeostasis. On the other hand it was shown that dysbiosis of intestinal bacterial flora contributes to cause the onset of pathological states such as diabetes, allergies, and even obesity [30].

In the gastrointestinal tract are numerous aggregates of lymphoid cells as pharyngeal tonsils, Peyer's patches (PPs), isolated lymphoid follicles (ILFS) and mesenteric lymph nodes (MLNs) which together constitute a system called GALT (Gut Associated Lymphoid Tissue) [31]. Peyer's patches are located in the mucosa and submucosa of the small intestine and consist mainly of type B lymphocytes, able to secrete antibodies (mostly IgA) that are transported in the lumen of the intestine, where they bind to antigens and trigger other components of the immune system [32]. The isolated lymphoid follicles (ILFs) contains M cells, which improve the uptake of antigens and microbes from the lumen to the underlying

lymphoid tissues [33]. The ILFs are similar to the Peyer's patches for both function and structure, but they are smaller and are also found in the large intestine [34].

Intestinal epithelium consists of a single cell layer that prevents the invasion of pathogens, acting as a physical barrier. A layer of mucus separates the lumen of the intestine from the epithelial surface, avoiding direct contact of pathogenic microorganisms, and produces antimicrobial peptides such as C-type lectins 3β/γ (REGIIIβ and REGIIIγ). These cells can express numerous receptors that contribute to the organism's defence. The pattern recognition receptors (PRRs) (i.e. Toll-like receptor) have a crucial role in immunity, since they are able to recognize molecular structures of pathogens and start the reactions that lead to the innate immune response [31]. In addition, the intraepithelial lymphocytes (IEL_S) are part of the GALT and consist mainly of T cells CD8 positive. These cells help in preserving the integrity of the epithelial surfaces damaged, promoting the secretion of the "keratinocyte growth factor" (KGF), but on the other hand are able to produce interferon-gamma, favouring the development of inflammatory diseases [30,35,36].

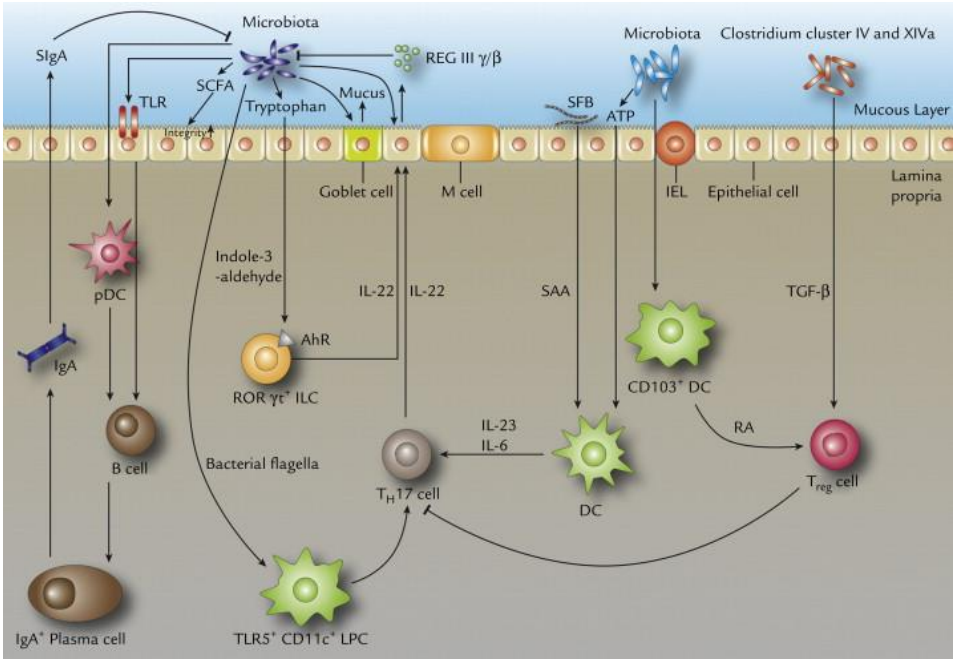


Figure 2: Interaction among intestinal microbiota and immune system [30]

Studies on germ-free animal (GF) reveal that microbiota contributes to the development of the GALT and promotes the barrier function of the intestinal epithelium. In rat GF, Peyer's patches are less active and less developed [37] and there is a lower amount of IEL_S than animals non GF [38,39]. The mucosal layer is significantly thinner as well. However the exposure of GF animals to intestinal bacteria leads to the restoration of an appropriate organization of the intestinal immune system [40] and a proper thickness of the layer of mucus, due to bacterial products such as lipopolysaccharide or peptidoglycan [41]. The lack of antimicrobial peptides and REGIIIβ - REGIIIγ leads to increased susceptibility of mice to attack by pathogens [42]. These observations indicate that the microbiota induces the development of lymphoid tissue through a detection innate system [30].

Among the products of microbial fermentation, those contributing mostly to organism's defence are the SCFA such as acetate, propionate and butyrate. *Bifidobacteria* inhibit the transport of toxins produced by *Escherichia Coli* from the intestinal lumen to the blood producing acetate [43]. Moreover, most of the SCFA bind to receptor coupled with protein G GPR43 that has a big role in inflammatory responses [44]. Mice without this receptor or GF mice that express it have severe inflammatory responses resulting in disease states such as colitis. In particular, butyrate is able to reduce the production of proinflammatory molecules by the intestinal cells, such as cytokine MCP-1, IL-6 and IFN-γ and together with the propionate decreases nitric oxide production by neutrophils and tumour necrosis factor. The production of SCFA leads to an anti-inflammatory effect, which has a positive role in the prevention of chronic inflammatory diseases such as metabolic syndrome, obesity and contributes in the slowing of aging [45].

The T-helper lymphocytes 17 are found in high concentrations in the lamina propria and have a role in defence and development of autoimmune diseases by producing proinflammatory cytokines IL-17 and IL-22. The microbiota appears to have an important role in their expression and in the GF mice the number of these cells is extremely small [46].

In addition to the active role in development and maintenance of innate and acquired immunity, microbiota contrasts the growth of pathogens by competing for substrates. A kind of tolerance by the host against him was observed, maintaining the ability to fight pathogens. The interaction between the host and the bacterial microflora is not yet fully clarified, but some bacteria (such as *Bacteroides fragilis*) are able to survive through their ability of modulating their antigenicity surface. The microbiota is capable of forming a wide variety of combinations of surface polysaccharides, escaping immuno-surveillance of the host [47].

Probiotics and prebiotics

The intake of probiotics and prebiotics can influence the composition of the intestinal microbiota, increasing the growth of bacteria with beneficial effects on human health [48], or could have positive effect on metabolic disorders as obesity [49]. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Interesting probiotic bacteria belong to genera *Lactobacillus* and *Bifidobacterium* and are widely used in fermented foods [50]. These bacteria showed the ability to modulate the signalling pathways of intestinal epithelial secretions, to influence cytokines and IgA antibodies and to increase the production of mucin by improving the functionality of epithelial barrier [51].

Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" [52]. Prebiotics avoid digestion by enzymes in the upper gastrointestinal tract and, at the colon level, affect the growth of specific microbial populations and the production of SCFA, influencing the intestinal functionality [53]. More studied prebiotics are fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and inulin, which are selectively utilized by *Bifidobacteria* and *Lactobacilli* [54].

Finally, the symbionts are combinations of probiotics and prebiotics useful to provide a synergic beneficial effect for health that cannot be obtained using the two components separated.

In vitro gastrointestinal simulation technology SHIME®

In order to understand deeply the activity and balance of gut microbiota, many different *in vitro* models were developed so far. The most common and easy way is using static models as batch culture fermentation. *Lactobacillus* and *Bifidobacteria* are extensively employed to study different aspects of microbial response to diet, and in particular to phenolic compounds [55]. Batch cultures are a suitable technique to carry out preliminary studies on stability of both bacteria and polyphenols, however more complex system are necessary for a more complete knowledge [56]. In addition to short-term, static batch culture models, other systems were designed, operating in continuous and reproducing the whole microbial community [57–59].

The SHIME® (Simulator of Human Intestinal Microbial Ecosystem) is a validated and dynamic *in vitro* model of complete gastrointestinal tract, useful for studying chemical-physical, enzymatic and microbial parameters. A careful control of environmental parameters allows to obtaining a stable and complex microbial community, very similar to the composition of different regions of the human gastrointestinal tract. The system consists of a series of vessel where it is reproduced the environment of each tract, simulating stomach, small intestine and colon.

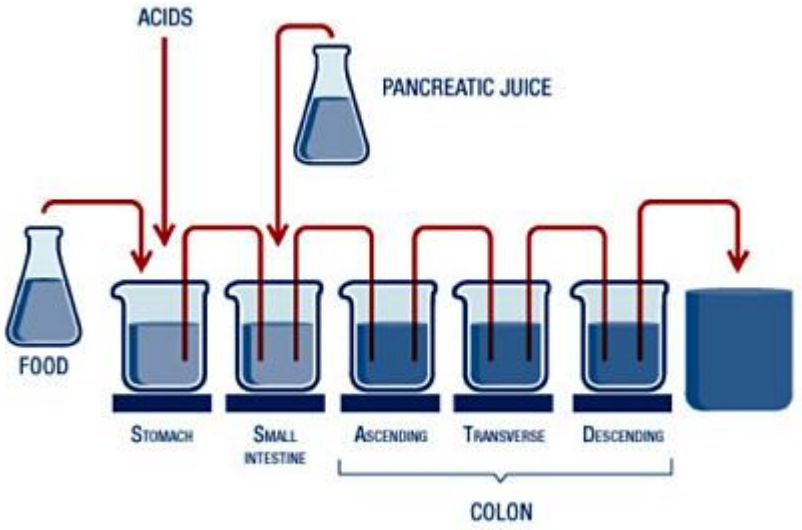


Figure 3 – Basic design of SHIME® system [www.prodigest.eu]

Depending on the type of experiment is possible to choose among three configurations representing the colon:

• 1 colon region (pH = 6.15-6.4, V = 500 mL) [60]

• 2 colon regions: proximal (pH = 5.6-5.9, V = 500 mL) and distal (pH = 6.6-6.9, V = 800 mL) [60]

• 3 colon regions: ascending (pH = 5.6-5.9, V = 500 mL), transverse (pH = 6.15-6.4, V = 800 mL) and falling (pH = 6.6-6.9; V = 600 mL) [61]

Vessels are hermetically closed to preserve simulated conditions, and each one communicates with the next vessel through tubes and pumping systems. There are also additional channels to maintain proper pH value and to access content without disturbing the balance. The whole system is treated with N_2 to eliminate the air and in particular O_2 that would affects the optimal conditions.

The SHIME® is suitable for in vitro studies on different compounds, such as foods, botanicals, prebiotics, probiotics, synbiotics or drugs, even with long-term treatments, up to 8-10 weeks.

The information obtained with this system are the production of SCFA and ammonia, the evaluation of changes in the microbial community and the definition of the metabolic fate of specific compounds, in particular from food matrices.

The study can include also any information about the activities of microbial adhesion to intestinal mucosa, using the M-SHIME® (Mucus - SHIME®), where the conventional configuration of SHIME® is improved by the addition of inert compartment covered in mucin. These carriers are replaced every 2-3 days to simulate the renewal of the layer of mucus [62].

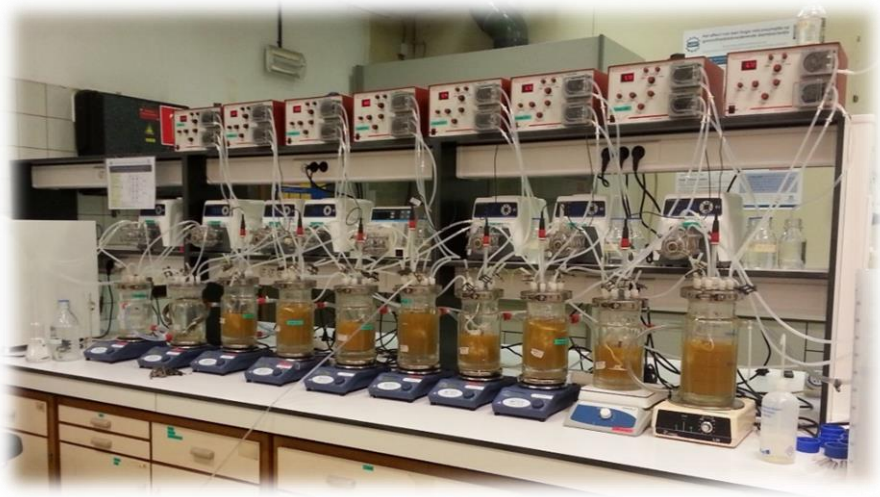


Figure 4: Gut simulator M-SHIME®

Food products

Dietary supplements and functional foods

Food supplements consumption is becoming one of most trendy topic. The research of "food supplements" in scientific literature provides around 270,000 results, 19,000 just in 2015. Much attention is focused on beneficial effects of supplements [63], in particular on polyphenols, due to their well-known anti-oxidant activity and several positive effects.

However, many issues were considered about this topic. The most important is about the control of polyphenols content in botanicals. The European Commission promoted studies to improve guidelines and knowledge [64], while a survey on the bioactive compounds in botanicals used as food supplements was recently published [65].

Once defined supplements content, it is important to understand the fate of main compounds once introduced in the organism. In order to define correct dosages for daily intake, different aspects need to be considered, such as in vivo metabolism, bioavailability, absorption and elimination, interaction with microbial community of gastrointestinal tract [55]. Beneficial or adverse effects depend on concentration of each molecule included in food supplements and its derivatives. Since big amount of polyphenols arrive at colon level without being processed, the main compounds and their metabolites can exert important effects on gut balance [66]. Microbial community is also responsible of huge degradation of phenolic compounds [67], but further knowledge of this interaction is required to completely understand the activity of food supplements containing phenolic compounds.

Food by-products

Food wastes are defined as "any food, and inedible parts of food, removed from the food supply chain to be recovered or disposed (including composted, crops ploughed in/not harvested, anaerobic digestion, bio-energy production, co-generation, incineration, disposal to sewer, landfill or discarded to sea)"[68]. The sources of wastes are several through the Food Supply Chain (FSC). At farming levels, the production of food provide large amount of sub-products, in particular part of vegetables with low commercial value. Food processing in another source of food waste, including inedible parts and matters affected by the processes. Even during the last step, the retail system, big amount of wastes are generated [69]. The consequences on environment of generating high volumes of food waste are several: it can contributes to emission of Green House Gas (GHG) and to depletion of natural sources, and has a strong social and economic impacts [70].

In 2011 FAO reported that "one-third of the edible parts of food produced for human consumption, gets lost or wasted globally, which is about 1.3 billion ton per year" [71]. Big discards are also due to an improper treatment of sub-products, in particular in agricultural sector and food processing industry. Post-harvesting

and processing are responsible of more than 40% of losses, mainly in industrial countries [72].

In last years, the interest for food waste valorisation increased much. Many by-products, recovered from different vegetable food, are widely investigated for the development of biofuel or bioenergy production processes [73]. Most wastes from plants resulted to be good candidate also for recovery of nutraceutical ingredients, suitable to be included in dietary supplements or food formulations. Cereals, roots, oil crops and fruit/vegetables by-products are all potential sources of bioactive and functional compounds [74].One of the main goal for the future is to define processes and new techniques to transform wastes in suitable extracts enriched in bioactive compounds.

One of the most investigated food process is the olive oil production. Residues of milling process are well-known to own negative properties, such as phytotoxicity, pollution of natural waters, affection of aquatic life [75]. Different strategies are already evaluated to recover wastewaters and pomace, including physical, physicochemical and biological treatments [76–82].

At the same time, also pomegranate wastes (seeds and peel) were studied to become a source of polyphenols, in particular phenolic acids, flavonoids, tannins [83,84]. Even in this case, several kind of extraction are reported to obtain maximum yield of main interesting molecules, as ultrasound-assisted extraction, highlighting a variability in the results and according with the high content of active compounds [85,86]. Recently it has been reported how during the milling process the recovery of polyphenols is in olive oil reaches a average of 2%, while the largest part of bioactive compounds is discarded with wastes [87].

These examples show the possible high valorisation of food by-products. Their safety in human use need to be studied deeply for formulation of food supplements and as nutraceutical ingredients.

Polyphenols

General features and classification

Polyphenols constitute a very heterogeneous group of molecules and usually enter in the organism through vegetable food. Fruits, vegetables, whole grains and other foods and beverages such as tea, chocolate and wine are rich [88]. Diets enrich in polyphenols are beneficial to health, and in particular help in the prevention of cancer, heart disease and neurodegenerative diseases [88]. These molecules are produced by the secondary metabolism of plants, which use them in the processes of growth and reproduction, but also as defensive agents. Polyphenols are strong antioxidants and *in vivo*, with vitamins and enzymes, contrast oxidative stress caused mainly by an excess of "reactive oxygen species" (ROS).

From the chemical point of view, polyphenols are characterized by the presence of several phenolic groups, associated in structures, more or less complex, generally of high molecular weight. To date more than 8000 phenolic structures have been identified and are classifiable from the chemical point of view in different subclasses.

- **Phenolic acids:** non-flavonoids polyphenolic compounds, which can be classified into benzoic acid derivatives and derivatives of cinnamic acid. Fruits and vegetables contain many free phenolic acids, while in crop and seeds these molecules are mainly linked to other molecules. These phenolic acids may be hydrolysed by chemical reactions acidic or alkaline, and by enzymes.

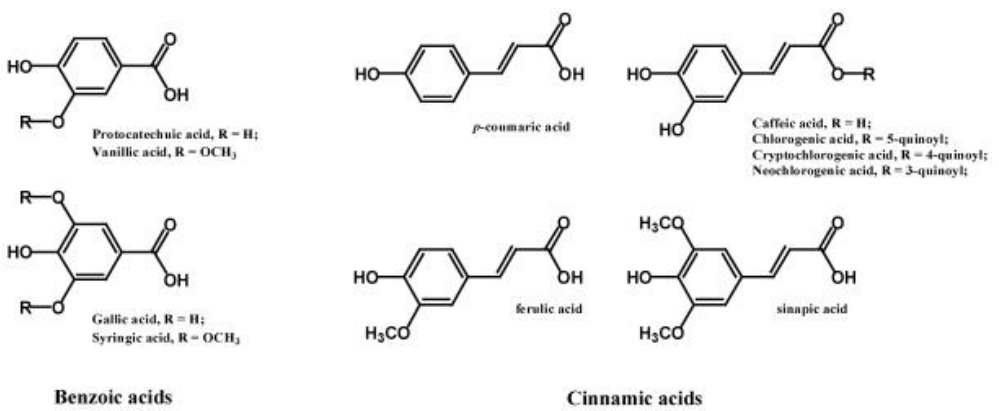


Figure 5: Main food phenolic acids: benzoic acids and cinnamic acids [89]

- **Flavonoids:** the basic structure is characterized by two phenolic rings (A and B) of 6 carbon atoms, bound to each other through 3 carbon atoms, which are arranged linear or form a third oxygenated cyclic ring (C). Flavonoids can be further divided, due to variation of the ring C and the number and arrangement of hydroxyl, into subgroups such as anthocyanins, flavan-3-ols (also called flavanols or catechins), flavones, flavanones and flavonols. Most of the flavonoids have the ring B bound to the ring C in position C2, but others, such as isoflavones and neoflavonoids, have the ring B linked to the C in position 3 and 4. These last two categories are present in a few natural sources and are smaller subgroups compared to flavonols and flavones. Even if they do not present the heterocyclic ring C, chalcones are always classified as flavonoids, and come from the degradation of anthocyanins. Proanthocyanidins are traditionally included in the class of condensed tannins and are formed by the polymerization of catechin and epicatechin (*cis* configuration), typically found for instance in honey and grapeseed. These structures are indicated as aglycones when they do not contain glycosidic groups, however most of these compounds are found in nature as glycosides.
- **Other polyphenols:** some polyphenols have functional groups containing N such as capsaicinoids, usually classified as alkaloids and responsible for the stinging sensation of hot peppers (Capsicum genus).
- **Other non-flavonoid phenols:** other non-flavonoids are considered important for human health. Some examples are resveratrol, belonging to stilbenes and mainly found in wine, grapes and some berries, ellagic acid and its derivatives, present in fruits, pomegranate and walnuts, curcumin, a strong antioxidant derived from turmeric, and rosmarinic acid, for the class of quinic caffeic acid esters.

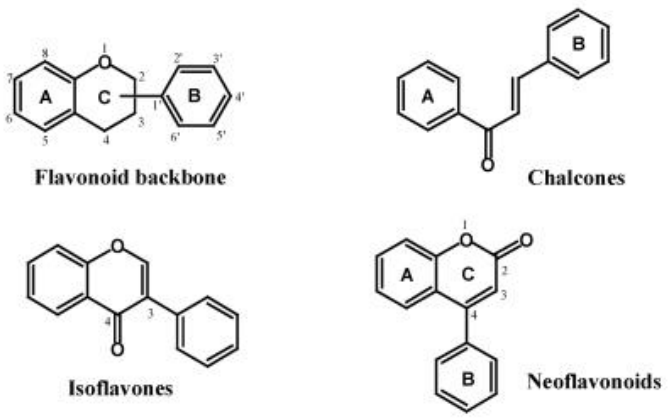


Figure 6: Structures of main flavonoids.

Polyphenols in olive "pâté"

Main polyphenols included in the olive "pâté" were already identified as tyrosol, hydroxtyrosol, oleuropein aglycone, usually developing during oil mill processing, verbascoside and caffeic acid.

Hydroxytyrosol (3,4-dihydroxyphenolethanol) and tyrosol (4-hydroxyphenylethanol) are low weight polyphenols and are classified as phenolic alcohol. The structure is similar, hydroxytyrosol just have one more hydroxyl group.

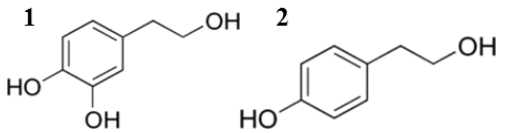


Figure 7: Hydroxytyrosol (1) and tyrosol (2).

Different studies confirmed that hydroxytyrosol already showed many positive activities on human wellness. It resulted anti-oxidant in human enterocytes, contrasting oxidative damages [90], anti-inflammatory for leukotrienes and prostaglandins inhibition [91,92] and antimicrobic [93,94]. That molecule should be used for cardioprotective and antiatherogenic properties, since it behaves as scavenger, reducing the amount of superoxide anions [95] and preventing LDL oxidation [96]. Hydroxytyrosol also showed anticancer functions, inhibiting DNA

damage peroxynitrite-dependent [97], inducing apoptosis cytochrome C-dependent [98] and also contrasting the proliferation of cancer cells [99]. Human intervention studies are in progress to assess the effects of this small molecule. Recently, a human intervention study (IMEDEA Food) was aimed to evaluate the detoxifying capacity of hydroxytyrosol and its effects on the expression of phase II enzymes involved in detoxification in the liver [100].

The verbascoside (or acteoside) is a phenylpropanoid, derived by esterification of caffeic acid, and is classified in phenolic acids.

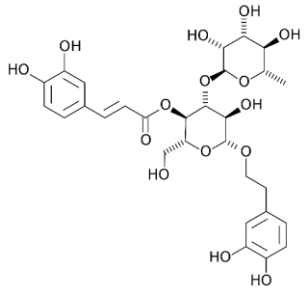


Figure 8: Verbascoside

In literature, several studies agree on anti-inflammatory, antioxidant, antiviral and antifungal activities of this molecules. Verbascoside is an effective antioxidant, due to its ability to inhibit LDL oxidation, which have an important role in atherosclerosis processes [101]. Verbascoside also showed anti-inflammatory activity in test for inhibition of macrophages functionality, involved in inflammatory processes [102]. Other studies demonstrated also the action of verbascoside on ACE inhibitor enzyme, involved in hypertensive diseases [103]. It has positive inotropic and chronotropic effects and is a vasodilator of coronary arteries [104].Verbascoside also showed to own antihyperalgesic property in treating neuropathic pain in *in vivo* studies in rats [105].

Caffeic acid belongs to phenolic acids class (or phenol carboxylic acid) and is a metabolite of verbascoside. It derived from 4-hydroxycinnamic acid and its basic structure is the catechol ring (1,2-dihydroxybenzene).

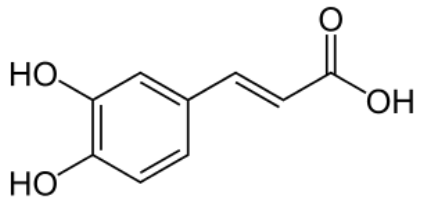


Figure 9: Caffeic acid

Caffeic acid has antioxidant, due to its ability of reducing oxidative stress [106], anti-inflammatory, inhibiting 5-lipoxygenase, antiatherogenic, contrasting the LDL oxidation [107] and chemoprotective properties, since it inhibits the oxidation of DNA in prostate cells [108].

Another group of phenolic substances present in plants belonging to the class of *Oleaceae*, is represented by secoiridoid. Oleuropein is a phenolic monoglucoside present at high concentrations in the fruit flesh; it is found in lower quantities as aglycone both in oil and mainly in by-products from virgin olive oil production. Due to its chemical structure, these derivatives are sources of hydroxytyrosol that can be easily released after hydrolysis of the ester bond, a typical process that occurs even in the extra virgin olive oils during their shelf life.

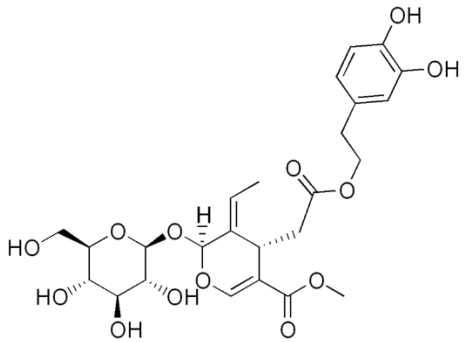


Figure 10: Oleuropein

Polyphenols in pomegranate mesocarp

The phenolic compounds present in greatest amount in the mesocarp of pomegranate (*Punica granatum* fruit), are hydrolysable tannins and, in particular, gallic acid and ellagic acid esters. Numerous combinations of monomers are

possible, which determine the formation of a high number of different structures. These are divided into gallotannins (esters of gallic acid and glucose) and ellagitannins (hexahydroxydiphenic and esters of glucose).

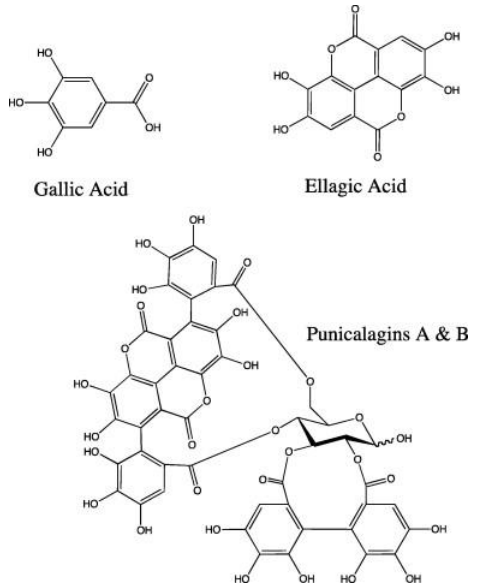


Figure 11: Main polyphenols in pomegranate mesocarp.

Ellagic acid has antioxidant, anticarcinogenic and antiatherogenic properties [109] and there are many studies on *in vivo* activity of pomegranate phenols.

Urolithins derivate from the metabolization of ellagitannins (including punicalagins) and ellagic acid by the human intestinal microbiota, after the loss of a lactone ring of ellagic acid and subsequent removal of the hydroxyl groups [110,111]. Despite punicalagins showed a strong antioxidant activity *in vitro*, they are extensively metabolized by gut microbiota into these derivatives, which show a rather insignificant antioxidant capacity [112].

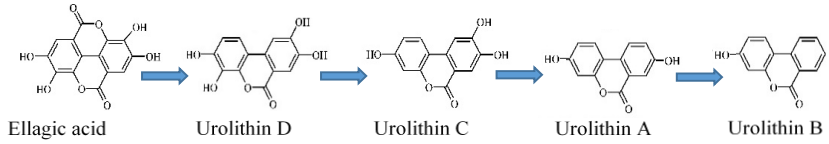


Figure 12: Chemical structure of ellagic acid, urolithins D, Uro-C, Uro-A e Uro-B. Arrows indicate metabolization sequence[113]

Urolithins were found in plasma, urine and tissues in human and in many animals (including pigs, rats, mice) after the intake of foods containing ellagitannins as pomegranate, strawberries, raspberries and nuts [113] and are recognized as *in vivo* markers of the absorption of this type of tannins.

Several *in vitro* and *in vivo* studies showed the variety of biological activities attributed to urolithins (mainly urolithin A) such as anti-cancer properties in prostate cancer [114], anti-inflammatory in colon inflammation the [115], and estrogenic / antiestrogenic activities [116].

Polyphenols in Vineatrol® 30

Vineatrol® 30 is obtained from grape vine of *Vitis vinifera*. Generally extracts of *Vitis vinifera*, leaves and fruits, contain several polyphenolic molecules, such as anthocyanins, catechins, flavonoids and stilbenes. Monomers and dimers of resveratrol belong to latter class and are more abundant in leaves and grapes contaminated with fungal infections. In fact, these molecules are recognized as factors of defence products in response to certain pests.

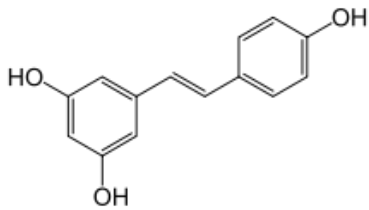


Figure 13: Trans-resveratrol

Stilbenes are photosensitive molecules and the geometry of the double bond in these molecules depends on the conditions of plant growth: little light promotes the formation of trans isomer, while the growth in a well-lit place allows the cis one. These phenols are present in particular in grapes skins, as glycosylated form, but also in leaves and petioles. Dimeric forms of resveratrol, ε-viniferin and δ-viniferin, are provided by endogenous peroxidase activity [117].

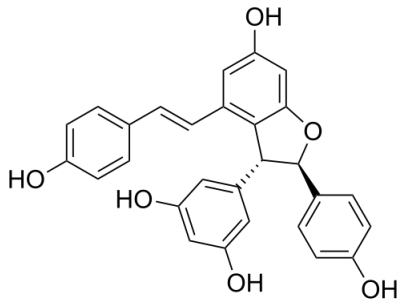


Figure 14: trans-ε-viniferin

Resveratrol is also found in a wide variety of foods and plants including the red wine, peanuts, pistachios and berries [118], even in low quantities (i.e. in wines, values are around 15 mg / L, while the other phenols reach 1 g / L). Numerous studies showed different properties of this stilbene. It has anti-inflammatory and antioxidant effects [119,120], has the ability to block human platelet aggregation and eicosanoid synthesis [121,122], has an agonistic effect on the estrogen receptors [123] and inhibitory effects on enzymatic activity of COX-2 [124]. Also it showed beneficial effects on the health and survival of mice subjected to a highly caloric diet [125]. Moreover, resveratrol may act as chemopreventive agent [126], although some data *in vivo* and *in vitro* are contradictory [127,128].

Other molecules present in Vineatrol® 30 are mostly oligomers or hydroxylated derivatives of resveratrol: ampelopsin A, hopeaphenol, trans-piceatannol, 1r-2-viniferin (vitisin A), miyabenol C, r-viniferin (vitisin B) and iso-trans-ε-viniferin. Few data are available on their biological activities.

Interaction among polyphenols and microbiota

Metabolism of polyphenols by intestinal microbiota and absorption

Once introduced in the organism through oral administration, polyphenols are recognized by the body as xenobiotics and their bioavailability is lower than the macro and micronutrients.

Low molecular weight polyphenols, such as monomeric or dimeric structures, are absorbed in the small intestine, but normally the percentage does not exceed 5-10% and the most part derived from reactions of deconjugation as deglycosylation [129]. After absorption, they undergo a phase I metabolism, such as oxidation, reduction and hydrolysis, and especially a phase II metabolism, as conjugation reactions at the level of enterocytes and hepatocytes. Hydrophilic metabolites (glucuronide, methylated and sulphates derivatives) are released into the systemic circulation and distributed in various tissues or eliminated in the urine [130].

High molecular weight polyphenols can be polymeric or oligomeric, such as condensed and hydrolysable tannins. These molecules reach the 90-95% of total dietary polyphenols intake and are not absorbed in the small intestine, but reach the colon partially altered [130]. In the large intestine their concentration can reach the millimolar range and the intestinal microbiota is responsible of their metabolism. Metabolism usually involves the cleavage of glycosidic bonds, and the breaking of the heterocyclic structure. For instance, the catabolism of proanthocyanidins (oligomers and polymers of flavan-3-ols) determines the production of lactones, aromatic and phenolic acids with different patterns of hydroxylation and length of the side chains (phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acid) [131,132].

Ellagitannins metabolism is located in intestinal lumen, through hydrolysis reactions releasing ellagic acid. In the large intestine, this compound is metabolized by the microflora and causes the production of urolithins with a decrease number of hydroxyl groups, going from urolithin D → C → A → B [133,134]. These phenolic metabolites can be partially absorbed or eliminated in the faeces. Once absorbed, they reach the liver through the portal vein, where they

are subjected to phase II metabolism (glucuronidation, methylation, sulphation or a combination of these processes). These molecules reach the systemic circulation and distribution to various organs, and are then eliminated through urine. Microbial glucuronidase and sulfatases activity can deconjugate the phase II metabolites extruded through the bile during the enterohepatic circulation, allowing the re-uptake and an increase in the bioavailability [135].

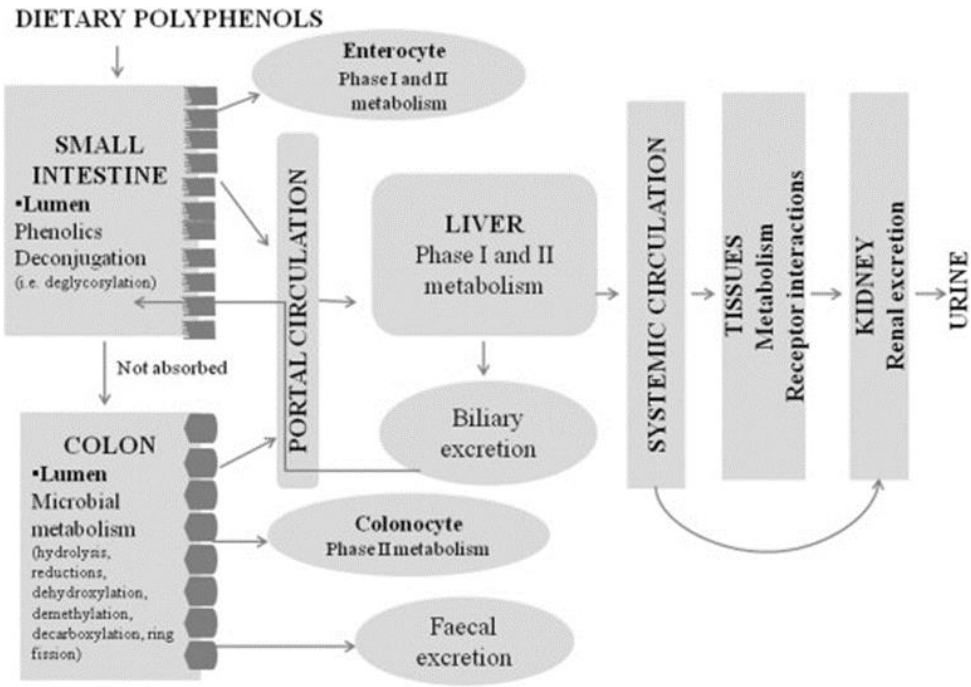


Figure 15: Metabolic fate of dietary polyphenols and derivatives [130]

Interaction mechanism of polyphenols with bacterial membrane

The influence of polyphenols on bacterial growth and metabolism depends on their structure, dose and type of microorganisms considered [56]. For example, Gram-negative bacteria are more resistant than Gram-positive, probably due to differences in the cell wall composition [136].

The mechanisms of action used by polyphenols on the bacterial flora are various. For example, polyphenols can bind the membrane of the bacterial cells, depending on the concentration, and alter its functions and inhibit the growth. Catechins act

on various bacterial species (*E. coli*, *Bordetella bronchiseptica*, *Serratia marcescens, Klebsiella pneumonias*, *choleraesis Salmonella, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis*), generating hydrogen peroxide [137] and altering microbial permeability [138]. Another proposed mechanism, observed in some studies of catechins and theaflavins, highlighted the ability of polyphenols to create hydrogen bonds between the hydroxyl groups and cell membranes [139,140].

After exposure to polyphenols presence, microbial synthesis of defensive proteins increases to protect the cell, but at the same time their metabolic activity decreases reducing the formation of biosynthetic proteins, amino acids, phospholipids and SCFA [130].

Polyphenols can also interfere in the release of small signal molecules of bacterial cells of *E. coli*, *Pseudomonas putida* and *Burkholderia cepacia*, able to activate the exponential growth of microbial population [141].

In addition, the B ring of flavonoids can intercalate or form hydrogen bonds with the bases of nucleic acids, inhibiting the synthesis of DNA and RNA [142]. Recent studies have indeed confirmed that quercetin inhibit bacterial DNA gyrase by binding to adenosine triphosphate level of the B subunit [143].

Another possible mechanism could be polyphenols ability to form complexes with metal ions, which can determine a lack of iron in the intestine and affects bacterial populations, mainly aerobes [144]. These bacteria require iron for many functions, such as for the activity of ribonucleotide reductase, useful for the synthesis of DNA, and the formation of heme groups.

Many other mechanisms of polyphenols action on specific bacterial intestinal functions are still unknown and further research is needed for a better understanding.

Modulation of microbiota by polyphenols

The inter-individual differences in the composition of the microflora bacteria can lead to differences in the bioavailability of polyphenols [145] which can be converted to bioactive compounds by microbiota, alterating intestinal microbial

balance and thus also the host's health. The administration of specific doses of polyphenols may inhibits some bacterial groups and allows others to prosper. For example, administering to rats red wine extracts rich in proanthocyanidins for a period of 16 weeks, the concentration of *Lactobacillus* and *Bifidobacterium* decreases in favor of an increased concentration of *Clostridium* and *Propionibacterium*, resulting a negative effect to health [146].

The regular intake of polyphenols from red wine in human determines the growth of *Bacteroides*, resulting in low levels of blood pressure and reduction of triglycerides and HDL cholesterol, as well as an increase of *Proteobacteria*, responsible of uric acid degradation [147].

Other studies showed that a diet rich in tannins administered to mice leads to an increase in the number of *Bacteroides*, while *Clostridium Leptum* undergoes a strong decrease [144].

Phenols from cocoa can promote the growth of *Lactobacillus* and *Bifidobacterium*, also increasing butyrate production. These changes are associated with a reduction of inflammation marker, as triacylglycerol and C-reactive protein in plasma, with a benefit to human health [148,149].

Ellagic acid is metabolized in the gut microbiota to urolithins, but there are inter-individual variability, both qualitative and quantitative, in its bioconversion. According to a study by *Tomás-Barberán et al*., three phenotypes can be identified: most common phenotype (A) produces only urolithin A conjugate, phenotype B produces iso-urolithin A and / or urolithin B in addition to the A and phenotype 0 does not produce any kind of urolithin [150].

Recently bacteria belonging to the genus *Gordonibacter* have been isolated from human feces, and *in vitro* and *in vivo* studies have shown their ability to degrade ellagic acid [151]. Additional studies demonstrated an increase in the concentration of urolithin A, but there was a decrease in concentration of iso-urolithin A and / or urolithin B, depending on the phenotype examined. Other bacteria of the intestinal microbiota are responsible for *in vivo* production of urolithins. To date, more studies are needed for their identification and characterization. This approach could lead to the development of probiotics and

prebiotics that can increase the concentration of urolithin A metabolite with cardio protective, anti-inflammatory and anti-cancer properties [152].

Metabolism of trans-resveratrol by the microbiota differs between individuals. So far, identified metabolites *in vivo* and *in vitro* are the dihydroxy-resveratrol, the 3,4'-dihydroxy-trans-stilbene and 3,4'-dihydroxy-dibenzyl (lunarin) and their quantity and presence vary individually. Two strains, *Slackia* and *Adlercreutzia* (genera belonging to the phylum *Actinobacteria*), were identified as main responsible in the production of dihydroxy-resveratrol. However, intestinal bacteria involved in reductive processes such as the elimination of the double bond or dehydroxylation are not yet identified [153].

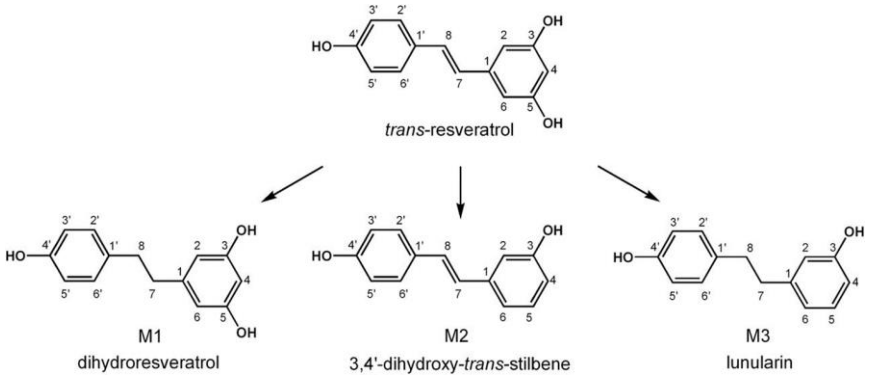


Figure 16: Possible metabolism of trans-resveratrol [153]

Further interaction between trans-resveratrol and microbiota depends on stilbene's ability to inhibit bacterial species including *Gracilibacter thermotolerans*, *Parabacteroides distasonis* and various species belonging to the class of *Clostridia* (*Clostridium aldenense*, *Clostridium hathewayi*, *Clostridium sp. MLG661*), while there is a significant increase of the *Clostridium sp. XB90*. However, the microbial composition is not strongly affected in rats [154].

To date in literature, there are a lack about the modulation of the microbiota composition by phenolic molecules derived from olive residues, such verbascoside, hydroxytyrosol and oleuropein derivatives.

References

[1] Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. Nat Rev Gastroenterol Hepatol 2012;9:577–89. doi:10.1038/nrgastro.2012.156.

[2] Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124:837–48. doi:10.1016/j.cell.2006.02.017.

[3] Savage DC. Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol 1977;31:107–33. doi:10.1146/annurev.mi.31.100177.000543.

[4] Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. Pharmacol Res 2013;69:52–60. doi:10.1016/j.phrs.2012.10.020.

[5] Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. PLoS Biol 2007;5:e177. doi:10.1371/journal.pbio.0050177.

[6] Grönlund MM, Lehtonen OP, Eerola E, Kero P. Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. J Pediatr Gastroenterol Nutr 1999;28:19–25.

[7] Favier CF, Vaughan EE, De Vos WM, Akkermans ADL. Molecular Monitoring of Succession of Bacterial Communities in Human Neonates. Appl Environ Microbiol 2002;68:219–26. doi:10.1128/AEM.68.1.219-226.2002.

[8] Roger LC, McCartney AL. Longitudinal investigation of the faecal microbiota of healthy full-term infants using fluorescence in situ hybridization and denaturing gradient gel electrophoresis. Microbiology 2010;156:3317–28. doi:10.1099/mic.0.041913-0.

[9] Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature 2012;486:222–7. doi:10.1038/nature11053.

[10] Zoetendal EG, Akkermans ADL, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable

and host-specific communities of active bacteria. Appl Environ Microbiol 1998;64:3854–9.

[11] Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J 2011;5:220–30. doi:10.1038/ismej.2010.118.

[12] Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 2008;6:e280. doi:10.1371/journal.pbio.0060280.

[13] Sekelja M, Berget I, Næs T, Rudi K. Unveiling an abundant core microbiota in the human adult colon by a phylogroup-independent searching approach. ISME J 2011;5:519–31. doi:10.1038/ismej.2010.129.

[14] Lee SM, Donaldson GP, Mikulski Z, Boyajian S, Ley K, Mazmanian SK. Bacterial colonization factors control specificity and stability of the gut microbiota. Nature 2013;501:426–9. doi:10.1038/nature12447.

[15] Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, et al. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr 2011;93:1062–72. doi:10.3945/ajcn.110.002188.

[16] De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 2010;107:14691–6. doi:10.1073/pnas.1005963107.

[17] Cummings JH. Short chain fatty acids in the human colon. Gut 1981;22:763–79.

[18] Soldavini J, Kaunitz JD. Pathobiology and potential therapeutic value of intestinal short-chain fatty acids in gut inflammation and obesity. Dig Dis Sci 2013;58:2756–66. doi:10.1007/s10620-013-2744-4.

[19] Conterno L, Fava F, Viola R, Tuohy KM. Obesity and the gut microbiota: does up-regulating colonic fermentation protect against obesity and metabolic disease? Genes Nutr 2011;6:241–60. doi:10.1007/s12263-011-0230-1.

[20] GIBSON P, MOELLER I. Contrasting effects of butyrate on the expression of phenotypic markers of differentiation in neoplastic and non-neoplastic colonic epithelial cells in vitro. J … 1992;7:165–72. doi:10.1111/j.1440-1746.1992.tb00956.x.

[21] Siavoshian S. Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. Gut 2000;46:507–14. doi:10.1136/gut.46.4.507.

[22] Evangelisti, Restani. Prodotti dietetici: Chimica, Tecnologia ed Impiego - Scheda libro | Piccin.it. n.d.

[23] Scott KP, Duncan SH, Flint HJ. Dietary fibre and the gut microbiota. Nutr Bull 2008;33:201–11. doi:10.1111/j.1467-3010.2008.00706.x.

[24] Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 1987;28:1221–7.

[25] Guarner F, Malagelada J-R. Gut flora in health and disease. Lancet 2003;361:512–9. doi:10.1016/S0140-6736(03)12489-0.

[26] Lefebvre P, Cariou B, Lien F, Kuipers F, Staels B. Role of bile acids and bile acid receptors in metabolic regulation. Physiol Rev 2009;89:147–91. doi:10.1152/physrev.00010.2008.

[27] Pierro A. Microbial Translocation in Neonates and Infants Receiving Long-term Parenteral Nutrition. Arch Surg 1996;131:176. doi:10.1001/archsurg.1996.01430140066018.

[28] Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science 2005;307:1915–20. doi:10.1126/science.1104816.

[29] Chung H, Kasper DL. Microbiota-stimulated immune mechanisms to maintain gut homeostasis. Curr Opin Immunol 2010;22:455–60. doi:10.1016/j.coi.2010.06.008.

[30] Min YW, Rhee P-L. The Role of Microbiota on the Gut Immunology. Clin Ther 2015;37:968–75. doi:10.1016/j.clinthera.2015.03.009.

[31] Kamada N, Seo S-U, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. Nat Rev Immunol 2013;13:321–35. doi:10.1038/nri3430.

[32] Newberry RD, Lorenz RG. Organizing a mucosal defense. Immunol Rev 2005;206:6–21. doi:10.1111/j.0105-2896.2005.00282.x.

[33] Owen RL, Pierce NF, Apple RT, Cray WC. M cell transport of Vibrio cholerae from the intestinal lumen into Peyer's patches: A mechanism for antigen sampling and for microbial transepithelial migration. J Infect Dis 1986;153:1108–18.

[34] Hamada H, Hiroi T, Nishiyama Y, Takahashi H, Masunaga Y, Hachimura S, et al. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. J Immunol 2002;168:57–64.

[35] Simpson SJ, Holländer GA, Mizoguchi E, Allen D, Bhan AK, Wang B, et al. Expression of pro-inflammatory cytokines by TCRαβ+ and TCRγδ+ T cells in an experimental model of colitis. Eur J Immunol 1997;27:17–25.

[36] Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A 2002;99:14338–43. doi:10.1073/pnas.212290499.

[37] Pollard M, Sharon N. Responses of the Peyer's Patches in Germ-Free Mice to Antigenic Stimulation. Infect Immun 1970;2:96–100.

[38] Bandeira A, Mota-Santos T, Itohara S, Degermann S, Hausser C, Tonegawa S, et al. Localization of γ/δ T cells to the intestinal epithelium is independent of normal microbial colonization. J Exp Med 1990;172:239–44.

[39] Bouskra D, Brézillon C, Bérard M, Werts C, Varona R, Boneca IG, et al. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 2008;456:507–10. doi:10.1038/nature07450.

[40] Hooper L V. Bacterial contributions to mammalian gut development. Trends Microbiol 2004;12:129–34. doi:10.1016/j.tim.2004.01.001.

[41] Petersson J, Schreiber O, Hansson GC, Gendler SJ, Velcich A, Lundberg JO, et al. Importance and regulation of the colonic mucus barrier in a mouse model

of colitis. Am J Physiol Gastrointest Liver Physiol 2011;300:G327–33. doi:10.1152/ajpgi.00422.2010.

[42] van Ampting MTJ, Loonen LMP, Schonewille AJ, Konings I, Vink C, Iovanna J, et al. Intestinally secreted C-type lectin Reg3b attenuates salmonellosis but not listeriosis in mice. Infect Immun 2012;80:1115–20. doi:10.1128/IAI.06165-11.

[43] Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 2011;469:543–7. doi:10.1038/nature09646.

[44] Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 2009;461:1282–6. doi:10.1038/nature08530.

[45] Kim MH, Kang SG, Park JH, Yanagisawa M, Kim CH. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. Gastroenterology 2013;145:396–406.e1–10. doi:10.1053/j.gastro.2013.04.056.

[46] Ivanov II, Frutos R de L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 2008;4:337–49. doi:10.1016/j.chom.2008.09.009.

[47] Krinos CM, Coyne MJ, Weinacht KG, Tzianabos AO, Kasper DL, Comstock LE. Extensive surface diversity of a commensal microorganism by multiple DNA inversions. Nature 2001;414:555–8. doi:10.1038/35107092.

[48] De Preter V, Hamer HM, Windey K, Verbeke K. The impact of pre- and/or probiotics on human colonic metabolism: does it affect human health? Mol Nutr Food Res 2011;55:46–57. doi:10.1002/mnfr.201000451.

[49] Delzenne NM, Neyrinck AM, Bäckhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 2011;7:639–46. doi:10.1038/nrendo.2011.126.

[50] Heller KJ. Probiotic bacteria in fermented foods: product characteristics and starter organisms. Am J Clin Nutr 2001;73:374S – 379S.

[51] Thomas CM, Versalovic J. Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbes 2010;1:148–63.

[52] Gibson GR, Probert HM, Loo J Van, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 2004;17:259–75. doi:10.1079/NRR200479.

[53] Macfarlane GT, Macfarlane S. Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. J Clin Gastroenterol 2011;45 Suppl:S120–7. doi:10.1097/MCG.0b013e31822fecfe.

[54] Watson D, O'Connell Motherway M, Schoterman MHC, van Neerven RJJ, Nauta A, van Sinderen D. Selective carbohydrate utilization by lactobacilli and bifidobacteria. J Appl Microbiol 2013;114:1132–46. doi:10.1111/jam.12105.

[55] Dueñas M, Muñoz-González I, Cueva C, Jiménez-Girón A, Sánchez-Patán F, Santos-Buelga C, et al. A Survey of Modulation of Gut Microbiota by Dietary Polyphenols. Biomed Res Int 2015;2015:850902. doi:10.1155/2015/850902.

[56] Hervert-Hernández D, Goñi I. Dietary Polyphenols and Human Gut Microbiota: a Review. Food Rev Int 2011;27:154–69. doi:10.1080/87559129.2010.535233.

[57] Aguirre M, Venema K. The use of fecal samples for studying human obesity. Eur J Epidemiol 2015;30:1067–9. doi:10.1007/s10654-015-0048-z.

[58] Minekus M, Smeets-Peeters M, Bernalier a, Marol-Bonnin S, Havenaar R, Marteau P, et al. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Appl Microbiol Biotechnol 1999;53:108–14. doi:10.1007/s002530051622.

[59] Molly K, Vande Woestyne M, Verstraete W. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. Appl Microbiol Biotechnol 1993;39:254–8. doi:10.1007/BF00228615.

[60] Van den Abbeele P, Belzer C, Goossens M, Kleerebezem M, De Vos WM, Thas O, et al. Butyrate-producing Clostridium cluster XIVa species

specifically colonize mucins in an in vitro gut model. ISME J 2013;7:949–61. doi:10.1038/ismej.2012.158.

[61] Van Den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gérard P, et al. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for bacteroidetes and Clostridium cluster IX. Appl Environ Microbiol 2010;76:5237–46. doi:10.1128/AEM.00759-10.

[62] Van den Abbeele P, Roos S, Eeckhaut V, Mackenzie D a., Derde M, Verstraete W, et al. Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. Microb Biotechnol 2012;5:106–15. doi:10.1111/j.1751-7915.2011.00308.x.

[63] Elia M, Normand C, Norman K, Laviano a. A systematic review of the cost and cost-effectiveness of standard oral nutritional supplements in the hospital setting. Clin Nutr 2015:1–11. doi:10.1016/j.clnu.2015.05.010.

[64] Pravst I. Functional Foods in Europe: A Focus on Health Claims. Sci. Heal. Soc. Asp. Food Ind., 2012, p. 165 – . doi:10.5772/31057.

[65] Plumb J, Lyons J, Nørby K, Thomas M, Nørby E, Poms R, et al. ePlantLIBRA: A composition and biological activity database for bioactive compounds in plant food supplements. Food Chem 2015;193:121–7. doi:10.1016/j.foodchem.2015.03.126.

[66] Lee HC, Jenner AM, Low CS, Lee YK. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. Res Microbiol 2006;157:876–84. doi:10.1016/j.resmic.2006.07.004.

[67] Selma M V., Espín JC, Tomás-Barberán FA. Interaction between Phenolics and Gut Microbiota: Role in Human Health. J Agric Food Chem 2009;57:6485–501. doi:10.1021/jf902107d.

[68] Östergen K, Gustavsson J, Al. E. FUSIONS Definitional Framework for Food Waste. 2014.

[69] Parfitt J, Barthel M, Macnaughton S. Food waste within food supply chains: quantification and potential for change to 2050. Philos Trans R Soc B Biol Sci 2010;365:3065–81. doi:10.1098/rstb.2010.0126.

[70] Papargyropoulou E, Lozano R, K. Steinberger J, Wright N, Ujang Z bin. The food waste hierarchy as a framework for the management of food surplus and food waste. J Clean Prod 2014;76:106–15. doi:10.1016/j.jclepro.2014.04.020.

[71] Gustavsson J, Cederberg C, Sonesson U. Global food losses and food waste. 2011.

[72] Status of FAO's Work on Post-Harvest Losses 2012.

[73] Girotto F, Alibardi L, Cossu R. Food waste generation and industrial uses: A review. Waste Manag 2015. doi:10.1016/j.wasman.2015.06.008.

[74] Galanakis CM. Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. Trends Food Sci Technol 2012;26:68–87. doi:10.1016/j.tifs.2012.03.003.

[75] Akdemir EO, Ozer A. Application of a statistical technique for olive oil mill wastewater treatment using ultrafiltration process. Sep Purif Technol 2008;62:222–7. doi:10.1016/j.seppur.2008.01.006.

[76] Rozzi A, Malpei F. Treatment and disposal of olive mill effluents. Int Biodeterior Biodegradation 1996;38:135–44. doi:10.1016/S0964-8305(96)00042-X.

[77] Mantzavinos D, Kalogerakis N. Treatment of olive mill effluents Part I. Organic matter degradation by chemical and biological processes--an overview. Environ Int 2005;31:289–95. doi:10.1016/j.envint.2004.10.005.

[78] Fountoulakis M., Dokianakis S., Kornaros M., Aggelis G., Lyberatos G. Removal of phenolics in olive mill wastewaters using the white-rot fungus Pleurotus ostreatus. Water Res 2002;36:4735–44. doi:10.1016/S0043-1354(02)00184-7.

[79] Marques IP. Anaerobic digestion treatment of olive mill wastewater for effluent re-use in irrigation. Desalination 2001;137:233–9. doi:10.1016/S0011-9164(01)00224-7.

[80] Beltran de Heredia J, Garcia J. Process Integration: Continuous Anaerobic Digestion−Ozonation Treatment of Olive Mill Wastewater. Ind Eng Chem Res 2005;44:8750–5. doi:10.1021/ie040232x.

[81] Cegarra J, Paredes C, Roig A, Bernal MP, García D. Use of olive mill wastewater compost for crop production. Int Biodeterior Biodegradation 1996;38:193–203. doi:10.1016/S0964-8305(96)00051-0.

[82] Caputo AC, Scacchia F, Pelagagge PM. Disposal of by-products in olive oil industry: waste-to-energy solutions. Appl Therm Eng 2003;23:197–214. doi:10.1016/S1359-4311(02)00173-4.

[83] Singh RP, Chidambara Murthy KN, Jayaprakasha GK. Studies on the Antioxidant Activity of Pomegranate (Punica granatum) Peel and Seed Extracts Using in Vitro Models. J Agric Food Chem 2002;50:81–6. doi:10.1021/jf010865b.

[84] Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem 2006;96:254–60. doi:10.1016/j.foodchem.2005.02.033.

[85] Goula AM, Adamopoulos KG. A Method for Preparing a Novel Solid Product from Olive Mill Wastewater: Wastewater Characterization and Product Recovery. Dry Technol 2013;31:339–49. doi:10.1080/07373937.2012.736114.

[86] Kalamara E, Goula AM, Adamopoulos KG. An integrated process for utilization of pomegranate wastes — Seeds. Innov Food Sci Emerg Technol 2015;27:144–53. doi:10.1016/j.ifset.2014.12.001.

[87] Panayotis SR, Karathano VT, Mantzavinou A. Partitioning of Olive Oil Antioxidants between Oil and Water Phases. J Agric Food Chem 2002;50:596–601.

[88] Quideau S, Deffieux D, Douat-Casassus C, Pouységu L. Plant polyphenols: chemical properties, biological activities, and synthesis. Angew Chem Int Ed Engl 2011;50:586–621. doi:10.1002/anie.201000044.

[89] Tsao R. Chemistry and biochemistry of dietary polyphenols. Nutrients 2010;2:1231–46. doi:10.3390/nu2121231.

[90] Manna C, Galletti P, Cucciolla V, Montedoro G, Zappia V. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. J Nutr Biochem 1999;10:159–65.

[91] de la Puerta R, Gutierrez VR, Hoult JRS. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. Biochem Pharmacol 1999;57:445–9. doi:10.1016/S0006-2952(98)00320-7.

[92] Petroni A, Blasevich M, Papini N, Salami M, Sala A, Galli C. Inhibition of leukocyte leukotriene b4 production by an olive oil-derived phenol identified by mass-spectrometry. Thromb Res 1997;87:315–22. doi:10.1016/S0049-3848(97)00133-3.

[93] Bisignano G, Tomaino A, Lo Cascio R, Crisafi G, Uccella N, Saija A. On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. J Pharm Pharmacol 1999;51:971–4.

[94] Capasso R, Evidente A, Schivo L, Orru G, Marcialis MA, Cristinzio G. Antibacterial polyphenols from olive oil mill waste waters. J Appl Bacteriol 1995;79:393–8.

[95] Léger CL, Kadiri-Hassani N, Descomps B. Decreased Superoxide Anion Production in Cultured Human Promonocyte Cells (THP-1) Due to Polyphenol Mixtures from Olive Oil Processing Wastewaters. J Agric Food Chem 2000;48:5061–7. doi:10.1021/jf991349c.

[96] Tsantila N, Karantonis HC, Perrea DN, Theocharis SE, Iliopoulos DG, Antonopoulou S, et al. Antithrombotic and Antiatherosclerotic Properties of Olive Oil and Olive Pomace Polar Extracts in Rabbits. Mediators Inflamm 2007;2007.

[97] Deiana M, Aruoma OI, Bianchi M de LP, Spencer JP., Kaur H, Halliwell B, et al. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. Free Radic Biol Med 1999;26:762–9. doi:10.1016/S0891-5849(98)00231-7.

[98] Ragione FD, Cucciolla V, Borriello A, Pietra VD, Pontoni G, Racioppi L, et al. Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome c-dependent apoptosis. Biochem Biophys Res Commun 2000;278:733–9. doi:10.1006/bbrc.2000.3875.

[99] Fabiani R, De Bartolomeo A, Rosignoli P, Servili M, Montedoro GF, Morozzi G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive

oil through G1 cell cycle arrest and apoptosis. Eur J Cancer Prev 2002;11:351–8. doi:10.1097/00008469-200208000-00006.

[100] Visioli F. Human Study of Hydroxytyrosol on Phase II Enzymes in Healthy Subjects. ClinicalTrials.gov 2015.

[101] Liu M-J, Li J-X, Guo H-Z, Lee K-M, Qin L, Chan K-M. The effects of verbascoside on plasma lipid peroxidation level and erythrocyte membrane fluidity during immobilization in rabbits: a time course study. Life Sci 2003;73:883–92.

[102] Díaz AM, Abad MJ, Fernández L, Silván AM, De Santos J, Bermejo P. Phenylpropanoid glycosides from Scrophularia scorodonia: In vitro anti-inflammatory activity. Life Sci 2004;74:2515–26. doi:10.1016/j.lfs.2003.10.008.

[103] Kang DG, Lee YS, Kim HJ, Lee YM, Lee HS. Angiotensin converting enzyme inhibitory phenylpropanoid glycosides from Clerodendron trichotomum. J Ethnopharmacol 2003;89:151–4. doi:10.1016/S0378-8741(03)00274-5.

[104] Pennacchio M, Alexander E, Syah YM, Ghisalberti EL. The effect of verbascoside on cyclic 3′,5′-adenosine monophosphate levels in isolated rat heart. Eur J Pharmacol 1996;305:169–71. doi:10.1016/0014-2999(96)00153-7.

[105] Isacchi B, Iacopi R, Bergonzi MC, Ghelardini C, Galeotti N, Norcini M, et al. Antihyperalgesic activity of verbascoside in two models of neuropathic pain. J Pharm Pharmacol 2011;63:594–601. doi:10.1111/j.2042-7158.2011.01264.x.

[106] Nardini M, Pisu P, Gentili V, Natella F, Di M, Piccolella F, et al. Effect of caffeic acid on tert-butyl hydroperoxide-induced oxidative stress in U937. Free Radic Biol Med 1998;25:1098–105. doi:10.1016/S0891-5849(98)00180-4.

[107] Meyer AS, Heinonen M, Frankel EN. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. Food Chem 1998;61:71–5. doi:10.1016/S0308-8146(97)00100-3.

[108] Quiles JL, Farquharson AJ, Simpson DK, Grant I, Wahle KWJ. Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. Br J Nutr 2007;88:225. doi:10.1079/BJN2002620.

[109] Seeram NP, Lee R, Heber D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (Punica granatum L.) juice. Clin Chim Acta 2004;348:63–8. doi:10.1016/j.cccn.2004.04.029.

[110] Cerdá B, Periago P, Espín JC, Tomás-Barberán FA. Identification of urolithin a as a metabolite produced by human colon microflora from ellagic acid and related compounds. J Agric Food Chem 2005;53:5571–6. doi:10.1021/jf050384i.

[111] Cerdá B, Tomás-Barberán FA, Espín JC. Metabolism of Antioxidant and Chemopreventive Ellagitannins from Strawberries, Raspberries, Walnuts, and Oak-Aged Wine in Humans: Identification of Biomarkers and Individual Variability. J Agric Food Chem 2005;53:227–35. doi:10.1021/jf049144d.

[112] Cerdá B, Espín JC, Parra S, Martínez P, Tomás-Barberán F a. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. Eur J Nutr 2004;43:205–20. doi:10.1007/s00394-004-0461-7.

[113] González-Sarrías A, Miguel V, Merino G, Lucas R, Morales JC, Tomás-Barberán F, et al. The gut microbiota ellagic acid-derived metabolite urolithin A and its sulfate conjugate are substrates for the drug efflux transporter breast cancer resistance protein (ABCG2/BCRP). J Agric Food Chem 2013;61:4352–9. doi:10.1021/jf4007505.

[114] Seeram NP, Aronson WJ, Zhang Y, Henning SM, Moro A, Lee R-P, et al. Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostate gland. J Agric Food Chem 2007;55:7732–7. doi:10.1021/jf071303g.

[115] Larrosa M, González-Sarrías A, Yáñez-Gascón MJ, Selma M V, Azorín-Ortuño M, Toti S, et al. Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. J Nutr Biochem 2010;21:717–25. doi:10.1016/j.jnutbio.2009.04.012.

[116] Larrosa M, González-Sarrías A, García-Conesa MT, Tomás-Barberán FA, Espín JC. Urolithins, ellagic acid-derived metabolites produced by human colonic microflora, exhibit estrogenic and antiestrogenic activities. J Agric Food Chem 2006;54:1611–20. doi:10.1021/jf0527403.

[117] Wilkens A, Paulsen J, Wray V, Winterhalter P. Structures of two novel trimeric stilbenes obtained by horseradish peroxidase catalyzed biotransformation of trans-resveratrol and (-)-epsilon-viniferin. J Agric Food Chem 2010;58:6754–61. doi:10.1021/jf100606p.

[118] Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventós RM, Berenguer T, Jakszyn P, Martínez C, et al. Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. Br J Nutr 2008;100:188–96. doi:10.1017/S0007114507882997.

[119] Fauconneau B, Waffo-Teguo P, Huguet F, Barrier L, Decendit A, Merillon JM. Comparative study of radical scavenger and antioxidant properties of phenolic compounds from Vitis vinifera cell cultures using in vitro tests. Life Sci 1997;61:2103–10.

[120] Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. Lancet (London, England) 1993;341:1103–4.

[121] Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. Clin Chim Acta 1995;235:207–19.

[122] Wang Z, Zou J, Huang Y, Cao K, Xu Y, Wu JM. Effect of resveratrol on platelet aggregation in vivo and in vitro. Chin Med J (Engl) 2002;115:378–80.

[123] Gehm BD, McAndrews JM, Chien P-Y, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci 1997;94:14138–43. doi:10.1073/pnas.94.25.14138.

[124] Mutoh M. Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a

resorcin-type structure. Carcinogenesis 2000;21:959–63. doi:10.1093/carcin/21.5.959.

[125] Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006;444:337–42. doi:10.1038/nature05354.

[126] Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, et al. Resveratrol: a review of preclinical studies for human cancer prevention. Toxicol Appl Pharmacol 2007;224:274–83. doi:10.1016/j.taap.2006.12.025.

[127] Wenzel E, Soldo T, Erbersdobler H, Somoza V. Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. Mol Nutr Food Res 2005;49:482–94. doi:10.1002/mnfr.200500003.

[128] Ziegler CC, Rainwater L, Whelan J, McEntee MF. Dietary Resveratrol Does Not Affect Intestinal Tumorigenesis in ApcMin/+ Mice. J Nutr 2004;134:5–10.

[129] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81:230S – 242S.

[130] Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem 2013;24:1415–22. doi:10.1016/j.jnutbio.2013.05.001.

[131] Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: Food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.

[132] Saura-Calixto F, Serrano J, Goñi I. Intake and bioaccessibility of total polyphenols in a whole diet. Food Chem 2007;101:492–501. doi:10.1016/j.foodchem.2006.02.006.

[133] Espín JC, González-Barrio R, Cerdá B, López-Bote C, Rey AI, Tomás-Barberán FA. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. J Agric Food Chem 2007;55:10476–85. doi:10.1021/jf0723864.

[134] Larrosa M, García-Conesa MT, Espín JC, Tomás-Barberán FA. Ellagitannins, ellagic acid and vascular health. Mol Aspects Med 2010;31:513–39. doi:10.1016/j.mam.2010.09.005.

[135] Selma M V, Espín JC, Tomás-Barberán FA. Interaction between phenolics and gut microbiota: role in human health. J Agric Food Chem 2009;57:6485–501. doi:10.1021/jf902107d.

[136] Puupponen-Pimiä R, Nohynek L, Hartmann-Schmidlin S, Kähkönen M, Heinonen M, Määttä-Riihinen K, et al. Berry phenolics selectively inhibit the growth of intestinal pathogens. J Appl Microbiol 2005;98:991–1000. doi:10.1111/j.1365-2672.2005.02547.x.

[137] Edwin Haslam, Terence H. Lilley, Edward Warminski, Hua Liao, Ya Cai, Russell Martin, et al. Phenolic Compounds in Food and Their Effects on Health I. vol. 506. Washington, DC: American Chemical Society; 1992. doi:10.1021/bk-1992-0506.

[138] Hattori M, Kusumoto IT, Namba T, Ishigami T, Hara Y. Effect of tea polyphenols on glucan synthesis by glucosyltransferase from Streptococcus mutans. Chem Pharm Bull 1990;38:717–20.

[139] Sirk TW, Brown EF, Friedman M, Sum AK. Molecular binding of catechins to biomembranes: relationship to biological activity. J Agric Food Chem 2009;57:6720–8. doi:10.1021/jf900951w.

[140] Sirk TW, Friedman M, Brown EF. Molecular binding of black tea theaflavins to biological membranes: relationship to bioactivities. J Agric Food Chem 2011;59:3780–7. doi:10.1021/jf2006547.

[141] Huber B, Eberl L, Feucht W, Polster J. Influence of polyphenols on bacterial biofilm formation and quorum-sensing. Z Naturforsch C 58:879–84.

[142] Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26:343–56. doi:10.1016/j.ijantimicag.2005.09.002.

[143] Plaper A, Golob M, Hafner I, Oblak M, Šolmajer T, Jerala R. Characterization of quercetin binding site on DNA gyrase. Biochem Biophys Res Commun 2003;306:530–6. doi:10.1016/S0006-291X(03)01006-4.

[144] Smith AH, Zoetendal E, Mackie RI. Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microb Ecol 2005;50:197–205. doi:10.1007/s00248-004-0180-x.

[145] Hervert-Hernández D, Pintado C, Rotger R, Goñi I. Stimulatory role of grape pomace polyphenols on *Lactobacillus acidophilus* growth. Int J Food Microbiol 2009;136:119–22. doi:10.1016/j.ijfoodmicro.2009.09.016.

[146] Dolara P, Luceri C, De Filippo C, Femia A Pietro, Giovannelli L, Caderni G, et al. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutat Res 2005;591:237–46. doi:10.1016/j.mrfmmm.2005.04.022.

[147] Queipo-Ortuño MI, Boto-Ordóñez M, Murri M, Gomez-Zumaquero JM, Clemente-Postigo M, Estruch R, et al. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. Am J Clin Nutr 2012;95:1323–34. doi:10.3945/ajcn.111.027847.

[148] Tzounis X, Rodriguez-Mateos A, Vulevic J, Gibson GR, Kwik-Uribe C, Spencer JPE. Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. Am J Clin Nutr 2011;93:62–72. doi:10.3945/ajcn.110.000075.

[149] Fogliano V, Corollaro ML, Vitaglione P, Napolitano A, Ferracane R, Travaglia F, et al. In vitro bioaccessibility and gut biotransformation of polyphenols present in the water-insoluble cocoa fraction. Mol Nutr Food Res 2011;55 Suppl 1:S44–55. doi:10.1002/mnfr.201000360.

[150] Tomás-Barberán FA, García-Villalba R, González-Sarrías A, Selma M V, Espín JC. Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. J Agric Food Chem 2014;62:6535–8. doi:10.1021/jf5024615.

[151] Selma M V, Beltrán D, García-Villalba R, Espín JC, Tomás-Barberán FA. Description of urolithin production capacity from ellagic acid of two human intestinal *Gordonibacter species.* Food Funct 2014;5:1779–84. doi:10.1039/c4fo00092g.

[152] Romo-Vaquero M, García-Villalba R, González-Sarrías A, Beltrán D, Tomás-Barberán FA, Espín JC, et al. Interindividual variability in the human metabolism of ellagic acid: Contribution of *Gordonibacter* to urolithin production. J Funct Foods 2015;17:785–91. doi:10.1016/j.jff.2015.06.040.

[153] Bode LM, Bunzel D, Huch M, Cho G-S, Ruhland D, Bunzel M, et al. In vivo and in vitro metabolism of trans-resveratrol by human gut microbiota. Am J Clin Nutr 2013;97:295–309. doi:10.3945/ajcn.112.049379.

[154] Etxeberria U, Arias N, Boqué N, Macarulla MT, Portillo MP, Martínez JA, et al. Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats. J Nutr Biochem 2015;26:651–60. doi:10.1016/j.jnutbio.2015.01.002.

Aims

The aim of the studies included in this PhD thesis, was to improve the knowledge about intestinal microbiota after oral administration of polyphenols-enriched extracts. According to literature, lack information are available to date on the interaction between phenols and intestinal microbiota. In this work, several experiments were performed using the gut simulator Mucus-SHIME® or M-SHIME®. The SHIME® is a validated model of the complete gastro-intestinal system, constituted by different vessels simulating stomach and small intestine for the first food digestion, and colon to investigate changes in the microbial community. The M-SHIME® allows investigating also the adhesion of bacteria at mucosal part using mucin compartment in colon vessels.

Three vegetable extracts were selected for administration tests on M-SHIME®.

The first extract was the dietary supplement Vineatrol® 30. It contains polyphenols derived from grape vine, in particular stilbenoids as resveratrol and its dimers (ε-viniferin) and trimers (myabenol C). The poor bioavailability of these molecules, and especially of resveratrol, represents an issue that should be overcome by using high concentrations. However, huge quantities of polyphenols can lead to antimicrobial effects, causing an alteration of the intestinal microbiota, which is closely related to human health.

The second part of the study was conducted on an olive "pâté" recovered directly during the milling process for extra virgin olive oil production. The pâté is characterized by a high content of fiber and typical polyphenols derived from olive. The second extract was obtained applying a decoction to pomegranate mesocarp. This waste represent up to 50% of fresh fruit weight and it is the main by-product from juice production. The decoction allowed recovering a good amount of ellagitannins and polysaccharides. The high polyphenols content of these by-products led to the recovery of bioactive compounds for the production of food supplements or nutraceutical ingredients.

The in vitro tests with M-SHIME® were conducted selecting two different diets: one regular feed and one high protein-low sugar diet. The aim was to collect

results about polyphenols-microbiota interaction in different environmental conditions.

The studies were carried out from different point of view, considering the mutual influence between polyphenols and microbiota. Different parameters were considered:

- polyphenols metabolites, using HPLC/DAD/MS analysis
- Short Chain Fatty Acids (SCFAs) and NH_4^+ levels determination
- microbiota composition, through PCR-DGGE and Illumina sequencing on bacterial DNA

The results were evaluated together to obtain correlations among metabolic fate of polyphenols and the effects of phenolic compounds on microbial communities, and improve then the knowledge about this complex system.

Chapter 1: Dietary supplement based on stilbenoids: a focus on gut microbial metabolism by in vitro simulator M-SHIME®

Camilla Giuliani, Marzia Innocenti, Nadia Mulinacci, Massimo Marzorati[1], Ramiro Vilchez-Vargas[1], Tom Van de Wiele[1]

Department of Neurofarba- Pharmaceutical and Nutraceutical Division, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, (FI) Italy.

[1] *LabMET, Faculty of Bioengineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium*

Introduction

Resveratrol and its derivatives (glycosides and polymers) are polyphenols classified as stilbenes. Different foods and plants contain these molecules, such as grapes, red wine, some kind of tea, berries and peanuts [1]. These molecules are well-known for several biological activities, mainly antioxidant and anti-inflammatory effects, cardio protection and cancer prevention [2–4]. Although the huge amount of studies, often *in vitro* and *in vivo* results are contradictory [5]. To date, physiological effects of resveratrol and other stilbenes are still not completely clear. One explanation involves the metabolic fate of these molecules following oral administration. It is demonstrated that only 5-10% of dietary intake of polyphenols is absorbed in the small gut while the residual part reaches the large intestine [6]. Colon microbiota is then able to metabolize and transform stilbenes through many different reactions, till complete disappearance of the original molecules in short times [4,7]. These metabolites could maintain the same positive properties of the parent compound or could be even toxic for both bacteria and human [8]. The colonic absorption of stilbenes and their metabolites is estimated between 35% and 80% and the wide range is due to inter-individual microbial diversity and consequent different metabolism [9].

At the same time, polyphenols and stilbenes influence on gut microbiota is not much studied. It's well-known that polyphenols can modulate composition and functionality of microbial community due to their antimicrobial activity [8]. Many different mechanism of action on microbial cells are described for polyphenols. Due to their structure and their ability to create H-bonds, these molecules seemed to be able to provide a huge stress to microbial cells, for instance binding and disturbing cells membrane, influencing thus its permeability [6,10,11]. However, there is not much information about the effects of these compounds on non-pathogenic bacteria and on composition and relative ratios between different microbial groups. Therefore, it is important to investigate more about mutual effects of polyphenols and microbial community in order to understand deeply the mechanism of interaction.

According to the different range of absorption, due to inter-individual variability, it is also convenient to define the highest concentration of these molecules that does not affect the microbial community, but that provide a sufficient amount of compounds to exert beneficial effects.

In this context, the use of dynamic in vitro simulators can help the research in areas of the gastrointestinal tract that are not easily accessible. Among the available simulators, the SHIME® is a validated model of the complete gastro-intestinal system, with a series of vessels simulating stomach and small intestine for the first food digestion, and the different areas of the colon to investigate changes occurring in the microbial community. The Mucus-SHIME® (M-SHIME®) allows to investigate also the adhesion of bacteria at mucosal part using mucin compartment in the colon vessels. The aim of this work was to investigate the effects of Vineatrol®, a commercial extract containing resveratrol and derivatives, on intestinal microbiota, using a gut simulator (i.e. M-SHIME®). Vineatrol® is a powder traded for application in healthy foods and dietary supplements.

A triple M-SHIME® experiment was carried out using two different concentrations of Vineatrol®. The extract was administrated directly into colon vessel, to maximize the concentration of resveratrol and the stilbenes. The effects of the extract on the microbial communities were evaluated in terms of metabolism (SCFA and NH_4^+ concentrations) and taxonomic composition (DGGE and Illumina sequencing). HPLC/DAD and HLPC/DAD/MS analyses were performed to investigate microbial metabolism on Vineatrol®.

Material and methods

Vineatrol® 30 extract

Vineatrol® (Breko GmbH, Bremen, Germany), is an extract obtained from water/ethanol extraction of resveratrol monomers and oligomers. Grapevine shoots of *Vitis vinifera* used for extraction were cultivated in the wine-growing

region Bordeaux (Fr). Main phenolic compounds (stilbenes) are declared in label around 30% of the total weight of the extract [12].

Batch experiments

Batch incubations of colonic microbial communities were performed to assess the effect of the extract on bacterial metabolic activity and composition. Faecal slurries were incubated in the presence of the extract, as described previously [13]. Negative controls without Vineatrol® were prepared with 1 mL of faecal slurry and 4 mL of feed. Two concentrations (1.5 and 2.5 g of extract/L) were selected for the experiments, in accordance with literature [9]. Each experiment was performed in duplicate.

Vineatrol® was weighed directly in sterile Hungate tubes. Then, 1 mL of faecal slurry and 4 mL of feed were added and the tubes were capped with butyl-rubber stoppers, flushed with N_2 and incubated at 37°C for 24h. The 5 mL sample was used for extraction of SCFA and HPLC/DAD/MS analysis.

M-SHIME® experiments

A triple M-SHIME® model, inoculated with faecal samples from a healthy donor, was performed. The M-SHIME® contains, in addition to traditional luminal microbial community [14], some mucin slots to host surface-attached microbes [15]. These microcosms (K1-carrier, AnoxKaldnes AB, Lund, Sweden) are submerged in mucin-agar. At the start of each experiment, 500 mL of selected feed and 80 mucin-covered microcosms were added to proximal colon (PC) unit, while the distal colon (DC) unit was filled with 800 mL and the same amount of mucin slots. Subsequently, inoculation of the colon reactors was performed with 40 mL of a 1:5 dilution of fresh stools of the healthy human donor [16]. After an initial incubation of 18 h, 140 mL nutritional medium and 60 mL pancreatic juice were supplied to each proximal colon compartment three times per day. The M-SHIME® was kept at 37 °C and anaerobic condition by flushing for 10 min with N_2 every day.

Three couples of PC and DC vessels ran simultaneously: one couple for control and the other two for different treatments with Vineatrol® [Figure 1].

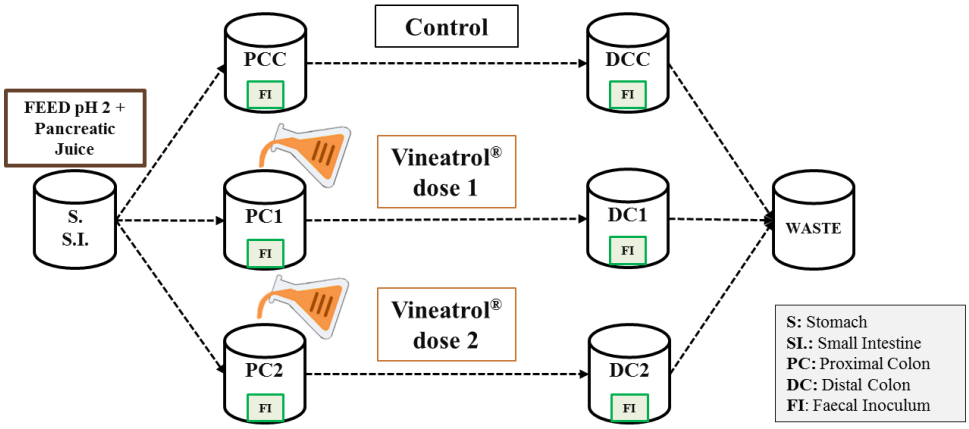


Figure 1 - M-SHIME® design

After a stabilization period of 2 weeks, daily doses of the extract were administrated in the PC vessel for 10 days [Figure 2]. At the end of the treatment, a 4-day washout period was then carried out. Two doses were selected for the experiment, 1 and 2 g/L, looking for a concentration without negative effects according to batch experiments. Three times per week, at the same time of the day, 20 mL of liquid sample were collected from each colon vessel. 1 mL was centrifuged and the pellet was stored at -20 °C for DNA extraction. Residual liquid was stored at -20 °C for metabolic analyses.

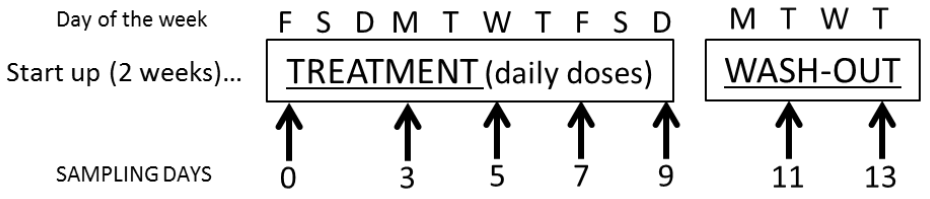


Figure 2 – M-SHIME® experiment protocol

Abbreviations

All samples were reported in plots and, throughout the text, with following abbreviations [Table 1].

PCC dX	Proximal Colon Control day X(0,3,5,7,9,11,13)
DCC dX	Distal Colon Control day X(0,3,5,7,9,11,13)
PC1 dX	Proximal Colon dose 1 day X(0,3,5,7,9,11,13)
DC1 dX	Distal Colon dose 1 day X(0,3,5,7,9,11,13)
PC2 dX	Proximal Colon dose 2 day X(0,3,5,7,9,11,13)
DC2 dX	Distal Colon dose 2 day X(0,3,5,7,9,11,13)

Table 1 – Samples abbreviations. C= Control, 1= dose 1 and 2= dose 2. dX = day of sampling

Metabolite measurement

SCFA analysis were performed according to standard method [17]. A liquid-liquid extraction with diethyl ether was applied on frozen samples, after the addiction of H_2SO_4 and internal standard. SCFA quantitative analysis were performed by capillary gas chromatography coupled with a flame ionization detector (GC-FID), to measure acetic, propionic, butyric, isobutyric, caproic, isocaproic, valeric and isovaleric acids.

NH_4^+ was analysed by steam distillation according to standard methods (4500-NH3 B; APHA, 1992). Determination of total ammoniacal nitrogen (TAN) in liquid luminal samples was performed through the quantification of NH_4^+ by the addition of MgO, distillation of NH_3 into boric acid solution and subsequent back-titration.

DNA extraction

Bacterial DNA from luminal samples was extracted as described earlier [18], using a Lysis Buffer (TrisEDTA, NaCl, PVP40, SDS, water) and glass beads for

FastPrep. DNA extract from mucin was obtained using bead-beating with same lysis buffer. Extraction was performed with phenol-chloroform and EtOH/NaOAc was used for precipitation [19]. Samples were dissolved in TrisEDTA 1X and stored at -20°C. Concentration and quality were verified by Glomax Multi Detection system (Promega, USA) and 2% agarose gel electrophoresis.

PCR-denaturing gradient gel electrophoresis (DGGE)

In order to investigate composition changes in the microbial communities a PCR-DGGE was assessed as described previously [16]. All samples for PCR were prepared starting from 1:10 dilutions. A PCR-DGGE was carried out for total bacteria, two nested PCR protocols were performed for *Lactobacilli* and *Bifidobacteria*.

External PCR for *Lactobacilli* was performed using 159F / 667R primers under the following conditions: initial denaturation 95 °C for 7 minutes; 35 cycles at 94°C for 1 minute, 56°C for 1 minute; 72°C for 2 minutes and a final extension at 72°C for 10 min [20].

External PCR for *Bifidobacteria* was performed using 164F / 662R primers under the following conditions: initial denaturation 95 °C for 7 minutes; 35 cycles at 94°C for 1 minute, 62°C for 1 minute; 72°C for 2 minutes and a final extension at 72°C for 10 min [21].

Internal PCR (*Lactobacilli* and *Bifidobacteria*) and amplification for total bacteria of 16S rRNA gene were performed with primers 338F-GC and 518R. Cycling conditions were: initial denaturation 94 °C for 5 minutes; 30 cycles at 95°C for 1 minute, 53°C for 1 minute; 72°C for 2 minutes and a final extension at 72°C for 10 min [22].

PCR products were separated on denaturant gradient of polyacrylamide gel (DGGE). DGGE (Denaturing Gradient Gel Electrophoresis) was performed [23] using the INGENY System (Ingeny International BV, The Netherlands). PCR fragments were loaded on an 8% polyacrylamide gel in 1´TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). Polyacrylamide gels were prepared with 45-60% denaturing gradients for total bacteria and *Lactobacilli*, and 50-65%

for *Bifidobacteria*. Electrophoresis was run for 16 hours at 60°C and 120 V. Staining and analysis of the gels was performed as described previously [24].

The normalization and analysis of DGGE gel patterns was done with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). During the processing, lanes were defined, background was subtracted, differences in the intensity of the lanes were compensated during normalization and band classes were detected. A matrix of similarities for the densiometric curves of the band patterns was calculated based on the Pearson product–moment correlation coefficient. A composite dataset was created by merging the information from the all the band patterns in order to obtain a combined dendrogram – using UPGMA linkage – containing the information from the gels on total bacteria, bifidobacteria and lactobacilli.

Illumina Sequencing

Samples collected from all vessels (PCC, DCC, PC1, DC1, PC2, DC2) at different times (d0, start up; d9, end of the treatment; d13 after washout) were selected for Illumina sequencing. Genomic DNA was extracted as previously described [18] with the following modifications. Samples were suspended in 1 mL Tris/HCl (100 mM pH 8.0), supplemented with 100 mM EDTA, 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) sodium dodecyl sulfate, transfered to a 2 ml Lysing Matrix E tube (Qbiogene, Alexis Biochemicals, Carlsbad, CA), subjected to mechanical lysis in a Fast Prep-24 instrument (40 s, 6.0 m s-1) and purified as described. The V1-2 region of the 16S rRNA gene was amplified as previously described [25]. In a first 20 cycles PCR reaction the 16S rRNA gene was enriched using the well-documented 27F and 338R primers [26,27] as previously specified [28]. Libraries were sequenced in a MiSeq platform (Illumina).

Pair-end Raw sequences were assembled and aligned using mothur [29] and subsequently filtered as previously described [25]. Briefly, reads were clustered allowing for two mismatches using mothur. The data-set was then filtered to consider only those phylotypes that were present in at least one sample at a relative

abundance > 0.1% or were present in all samples at a relative abundance > 0.001%. A total of 1,591,358 reads were obtained, and grouped into 1747 phylotypes. Rarefaction curves and statistics were generated using the package vegan from the R program. All phylotypes were assigned a taxonomic affiliation based on the naïve Bayesian classification (RDP classifier) [30] with a 80% of confidence. Obtained tables, ranking the identified phylotypes and their abundance, have been used to describe the relative their relative abundance - at order and phylum level - in each sample and to produce cluster dendrograms and PCA (principal component analysis) graphs.

HPLC/DAD/MS analysis of colonic metabolites

For HPLC/DAD/MS analysis two samples from PC control vessel were added with Vineatrol® at the same concentrations used for the experiment, to evaluate time zero profiles (PCCVINEA1 and PCCVINEA2). These samples were then compared with PC1, DC1, PC2 and DC2 samples collected during the experiment (d3, d5, d7, d9, d13) and with vessels content before the administration (PC1d0, DC1d0, PC2d0 and DC2d0) to identify any possible interferences due to feed.

Samples for HPLC/DAD analysis were pre-treated in order to obtain a clean solution as follows: 2 mL of surnatant from liquid luminal material of defrost suspension were added with 0.5 mL of MeOH/Acetonitrile/Acetone (1/1/1) and mixed with Vortex, then kept at room temperature for 25 min. The suspension was then centrifuged at 4 °C, 5000 rpm, 10 min. Supernatant was recovered and used for analysis.

Qualitative analysis were performed using a HP 1100 liquid chromatography coupled with HP 6200 series MS/TOF (Agilent Technologies, USA). A 150mm×2 mm i.d., 4μm, RP-18, Sinergi Fusion column (Phenomenex, USA) was used. Eluents selected were (A) H_2O pH 3.2 with formic acid and (B) CH_3CN. The used multi-step linear solvent gradient was: 0-2 min 20-20% B; 2–25 min, 20–50% B; 25–27 min 50–100% B; 27-33 min 100-100%; 33-35 min 100-20%, equilibration time 10 min; flow rate 0.2 mL/min. The UV-Vis spectra were recorded in the range 200–500 nm and the chromatograms were acquired at 240 nm, 254 nm, 280

nm, 307 nm, 320 nm. MS spectra were acquired using Dual-ESI source in negative polarity with 100 V fragmentor, 3800 V capillary voltage, 350°C of gas temperature.

Quantitative analysis were carried out using a HP 1200 liquid chromatography equipped with a DAD detector (Agilent Technologies, USA), using the same column and method applied for qualitative analysis. Stilbenes content was determined by HPLC/DAD. A MilliQ water solution of trans-Resveratrol (Sigma Aldrich) 0.0214 mg/mL was used to create a calibration curve at 307 nm in the range of linearity 0.04-0.16 ug with R^2= 0.999.

Results

Batch experiments

SCFA levels, in controls and samples treated with Vineatrol® after 24h, were investigated by GC-FID. Two replicates, A and B, were performed for each concentration (1.5 g/L and 2.5 g/L). The same SCFA levels were observed in samples treated with Vineatrol®, at both concentrations, as compared to control [Figure 3]. No significant differences were observed after the treatment with selected dosages of Vineatrol® and the microbial activity was not affected by the extract at these concentrations after 24h.

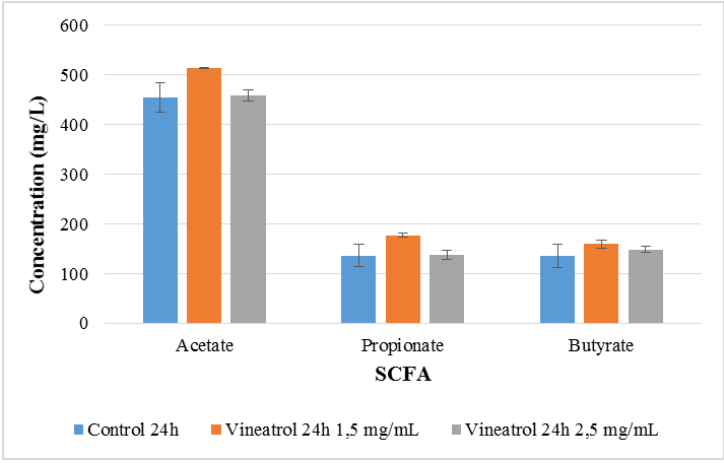


Figure 3 – Duplicates average of main SCFA concentrations in samples from 24h batch experiments

In order to investigate the Vineatrol® metabolism, control samples added with fresh Vineatrol® and samples treated (both concentrations and both replicates) were analysed by HPLC/DAD/MS. Figure 4 shows the profiles from control and replicates A and B of samples treated with the highest concentration (2.5 g/L). The concentrations of the main components suffered a strong decrease and viniferin's signal almost disappeared. The same evolution was detected also for lower concentration.

According to these results, the doses for the first SHIME® experiment were then assessed at 2 g/L and 1 g/L.

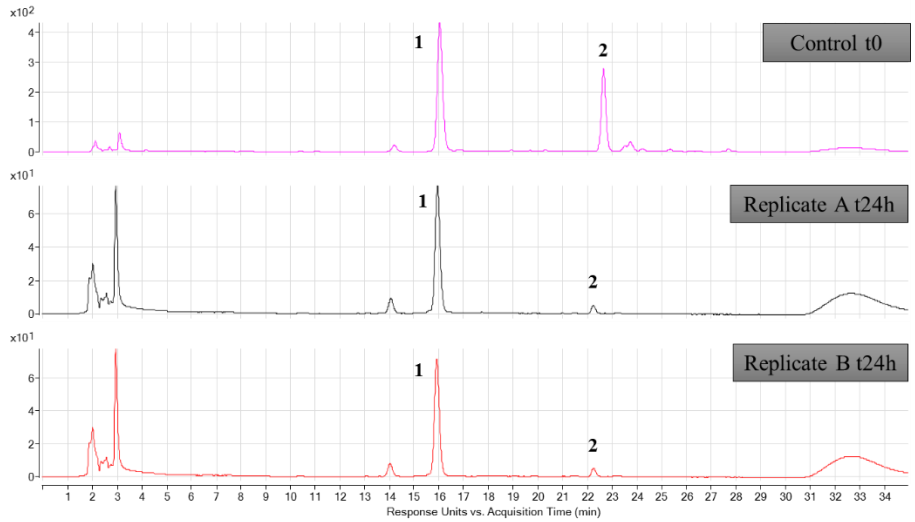


Figure 4 – HPLC/DAD profiles at 307 nm of samples from 24h batch experiments. Control and replicates (A/B) at higher concentration (2.5 g/L) are reported. Main compounds were identified as trans-resveratrol (1) and ε-viniferin (2).

Vineatrol® effects on microbial metabolism in M-SHIME® experiment: SCFA and ammonium

The effects of Vineatrol® administration in the M-SHIME® experiment were first evaluated in terms of microbial functionality, in particular SCFA and NH_4^+ production.

SCFA levels significantly decreased after treatment with both selected doses [Figure 5]. PC and DC metabolism showed similar trends and administration of the extracts provided the same effects, even with different doses. Subsequently, during the washout period, SCFA levels showed again an increasing trend. The same trend was detected for NH_4^+ concentration during and after treatment [Figure 6].

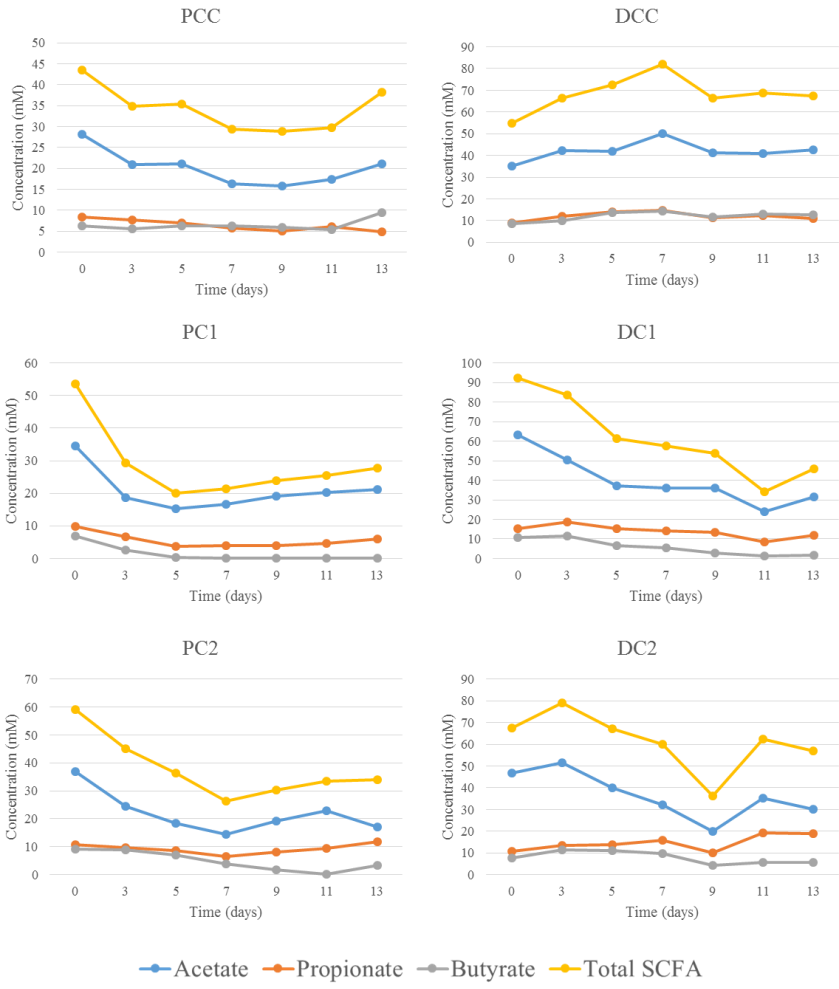


Figure 5 – SCFA concentrations progression during the experiment.

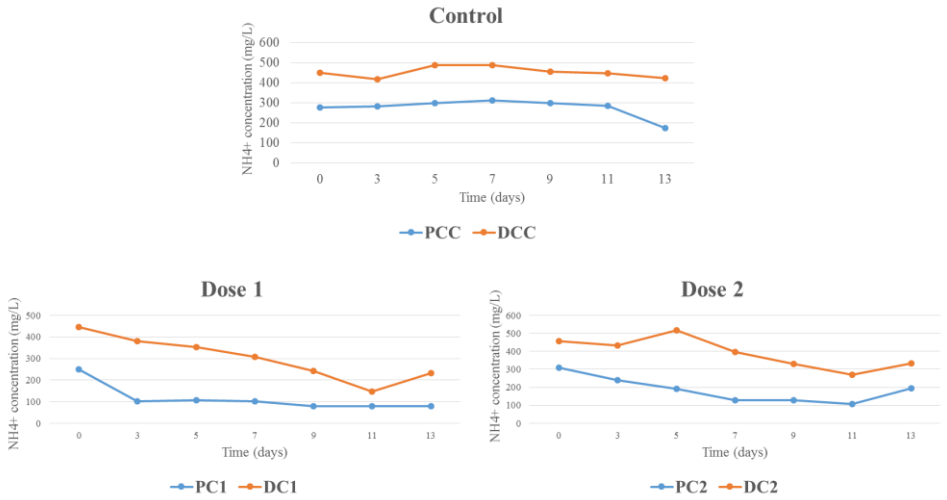


Figure 6 - NH_4^+ level evolution at different time sampling

Vineatrol® effects on microbial community composition in M-SHIME® experiment

PCR-DGGE measurement

PCR-DGGE for total bacteria, *Bifidobacteria* and *Lactobacilli* were performed on samples collected immediately before starting the treatment (Day 0), halfway and end of treatment (Day 5-9) and after washout (Day 13). DGGE images were processed through Bionumerics software to identify patterns and define the clusterization through composite data set.

Separation among control and treated samples was well defined [Figure 7]. PC1 treated samples (higher concentration) resulted different from control and starting point. In particular, correlation values of PC1 d5/d9/d13 compared with PC1 d0 and PCC were low, down to 40%, meaning that the microbial communities changed after administration of Vineatrol®. Pattern from DC1 vessels did not show the same evolution and showed 80% correlation, indicating smaller variations among d0 and samples collected during and after the treatment. PC samples treated with the lower concentration demonstrated a weak change compared to the starting point. However, correlation values are around 70%

among d0 and following times, meaning that the administration of the extract poorly affected the composition of the microbial communities. The evolution of DC2 at time zero and during/after the treatment did not show significant variations, since the samples clustered together with high correlation (> 80%).

In summary, the higher concentration had a stronger effect on proximal vessel. For the lower dosage, the effect on PC was consistent with the higher one, but the variation seemed less strong. Effects on DC1 and DC2 were not much evident during the treatment, probably due to lower concentrations reaching these vessels. The effects are more marked in PC since the extract administration was provided directly in that vessel and more time was required to reach an effective concentration in DC vessel. It is clear that Vineatrol® had an effect on the microbial composition at both tested concentrations (2 and 1 g/L).

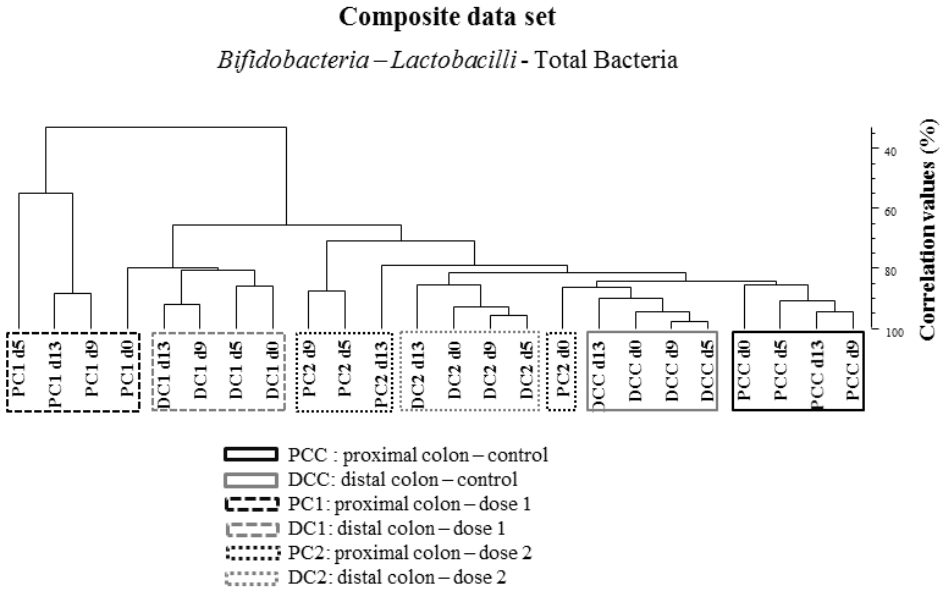


Figure 7 – Composite data set of DGGE patterns

Illumina sequencing

As shown in Figure 8 and 9, microbial communities from control and treated vessels were compared at the phylum and order level.

Despite the intrinsic variability, the microbial communities treated with higher dosage showed a strong change during the treatment. In PC1 d9 and PC1 d13 collected from lumen, it is possible to observe that the phylum *Proteobacteria* was enriched and the phylum *Bacteroidetes* decreased during the treatment. In particular, a significant enrichment of *Enterobacteriales* was observed, while the abundance of the order *Bacteroidales* decreased. This trend in PC1 vessel was more intense in mucin than in lumen samples. The interruption of treatment did not provide a fast return to original conditions, since the microbial community from lumen after the washout (PC1 d13) was similar to the microbial communities enriched during the treatment. In mucus samples, after the washout the composition returned close to starting point condition. Comparable behaviour was observed also in samples DC1 d9 and DC1 d13. The same taxonomic groups dominated the microbial communities after the treatment. The order *Synergistales* (phylum *Synergistetes*), which was abundant in the inoculum, was outcompeted during the treatment. Also the abundance of the order *Erysipelotrichales* decreased during the treatment. In samples treated with lower concentration, a similar evolution was shown. PC2 d9 and PC2 d13, from both lumen and mucus, presented the enrichment of the order *Enterobacteriales* and correspondent decrease of the order *Bacteroidales*, but in lesser extent than PC1 samples. In this case, the 4 days wash out seemed to be enough to revert the effects of the treatment. In DC2 samples, no significant changes were detected. As seen for DC1, some less represented order, *Synergistales* and *Erysipelotrichales*, was also outcompeted.

Cluster dendrogram of lumen samples, shown in Figure 10, confirmed the significant changes in PC1 and PC2 after treatment. Samples from proximal vessel from time d9 and d13 clustered together. DC1 d9 and d13 appeared separated from other DC, indicating the presence of a different microbial community. The samples collected from DC2 and the samples collected from the control grouped in the same cluster showing a weak effect of Vineatrol® on the composition of the microbial communities. Considering the samples collected from mucus, PC1 and PC2 grouped together, with a higher correlation with control than lumen,

suggesting a lower effect. Effects on DC1 and DC2 did not result remarkable, in particular DC1 d9/d13 and DC2 d9 demonstrated weak differences if compared to control, but not relevant.

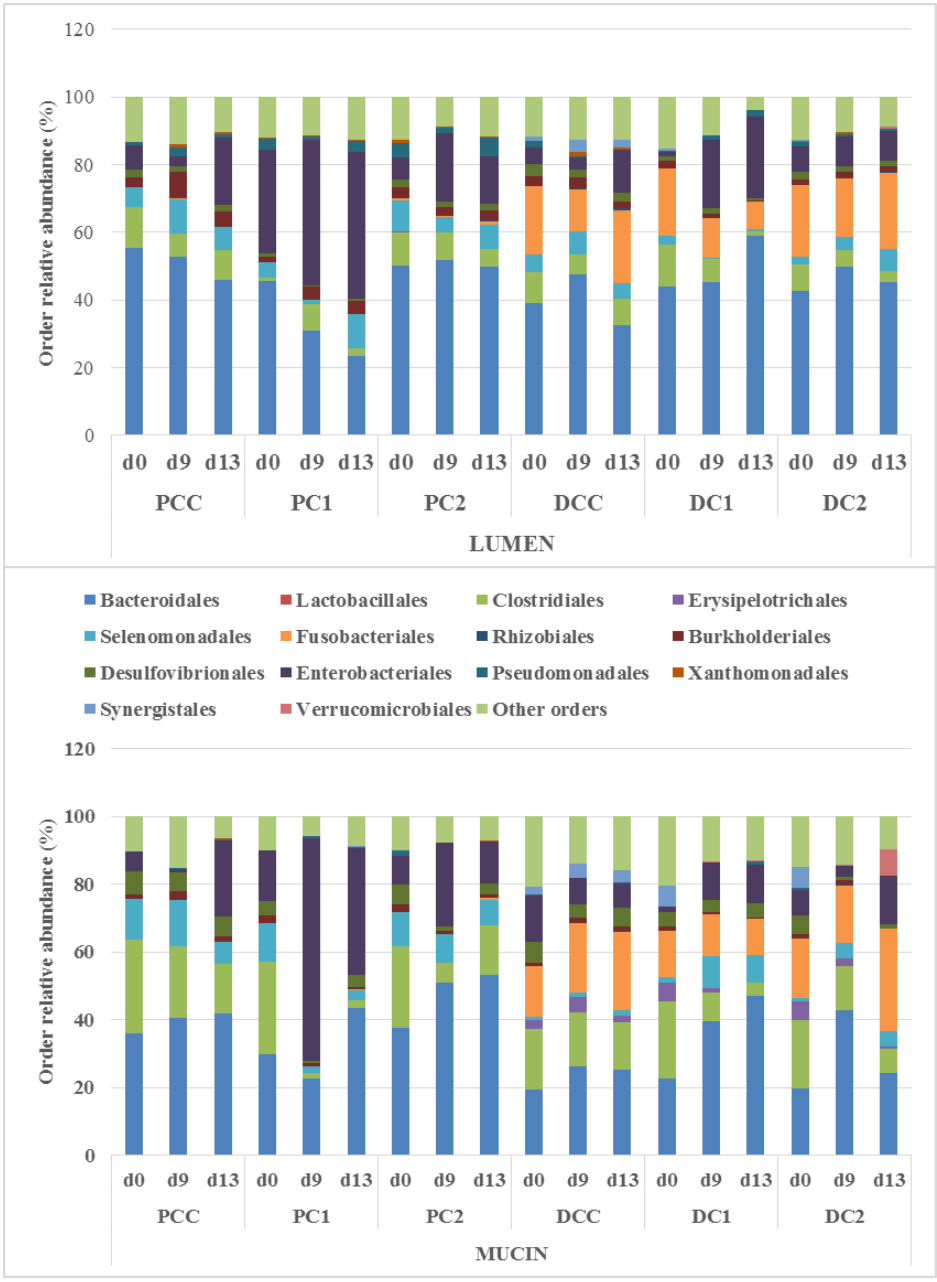


Figure 8 – Comparison of microbial distribution in samples from lumen and mucin, in terms of orders.

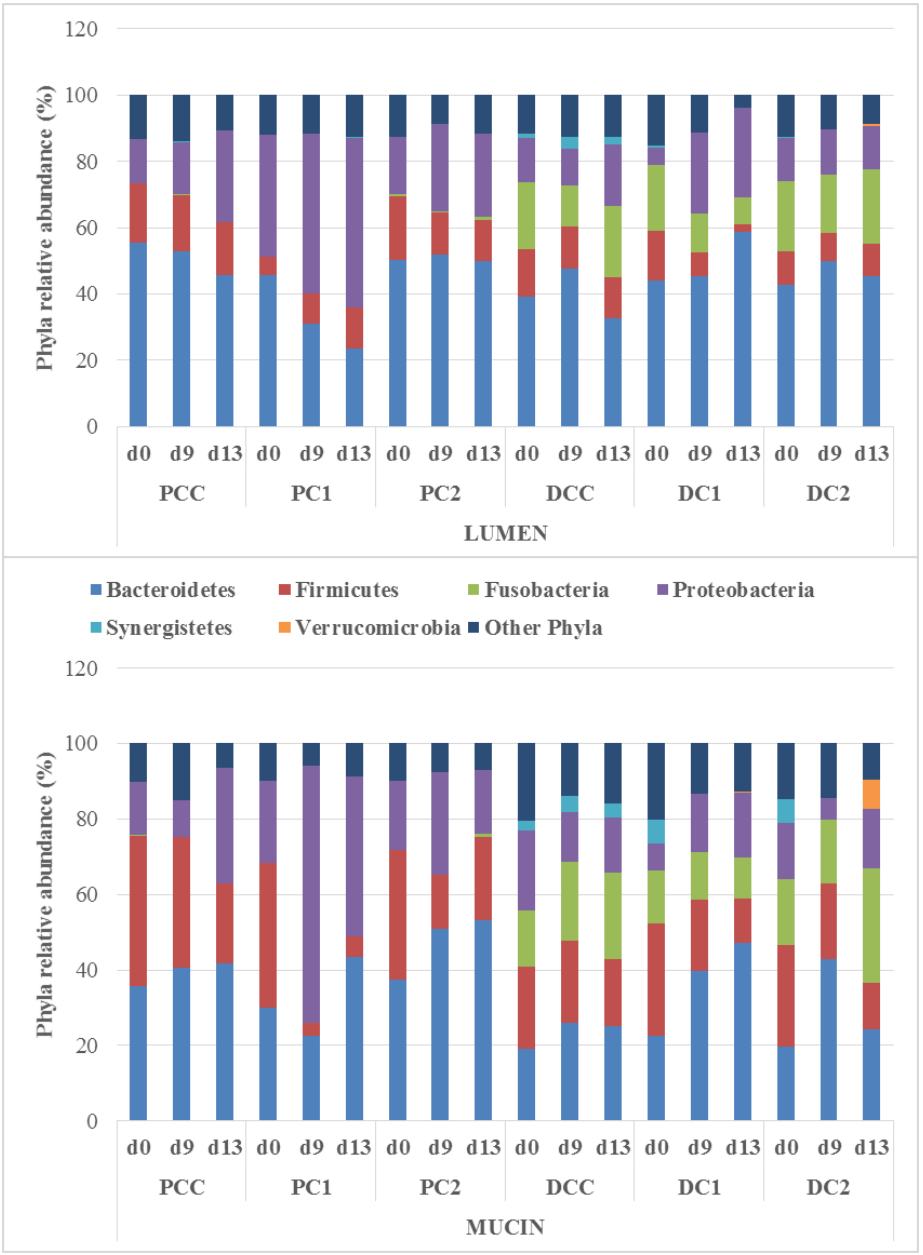


Figure 9 - Comparison of microbial distribution in samples from lumen and mucin, in terms of phyla.

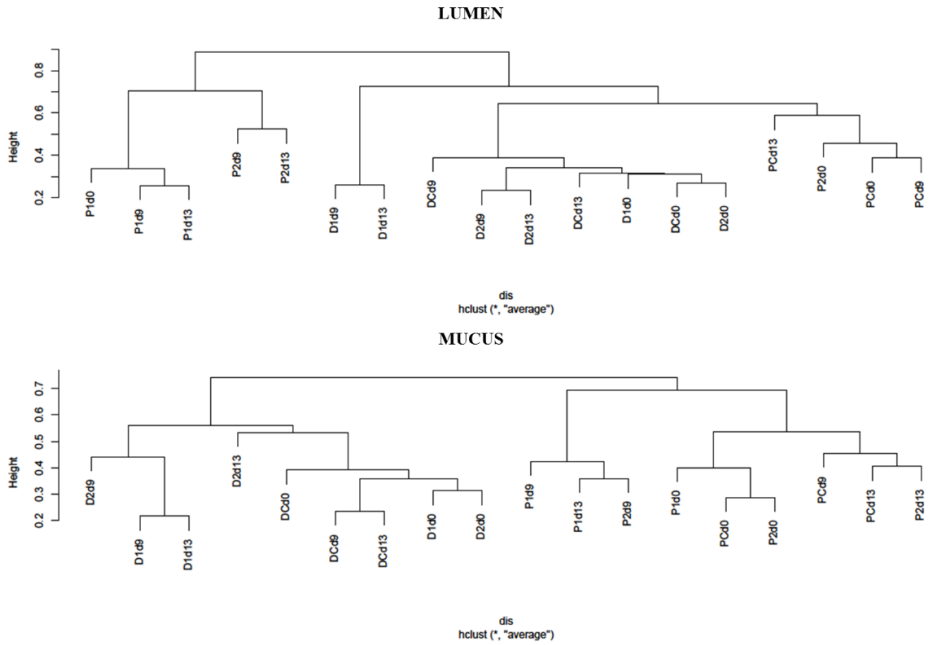
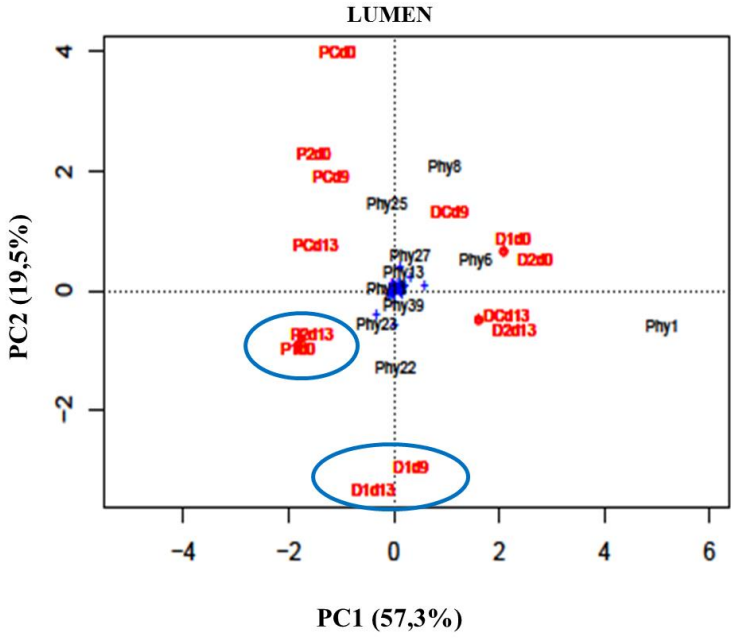


Figure 10 – Cluster dendrograms of lumen and mucus samples.

PCA for lumen and mucus confirmed the cluster analysis results [Figure 11]. In lumen plot, PC and DC from control and starting points were separated in two well-defined groups. PC treated samples showed a significant clusterization, since PC1 d9/d13 and PC2 d9 overlapped in the same area. As observed in the corresponding dendrogram, DC1 d9/d13 were isolated from other DC samples showing remarkable differences. PCA for mucus samples was consistent with cluster analysis results as well. The group including PC1d9/d13 and PC2 d9, formed a different cluster compared to PC control and inoculum (d0) indicating that these communities resulted the more affected by the treatment. DC samples did not show patterns and grouped together.

Calculation of the Shannon and richness indexes did not show significant differences among the samples (data not shown).



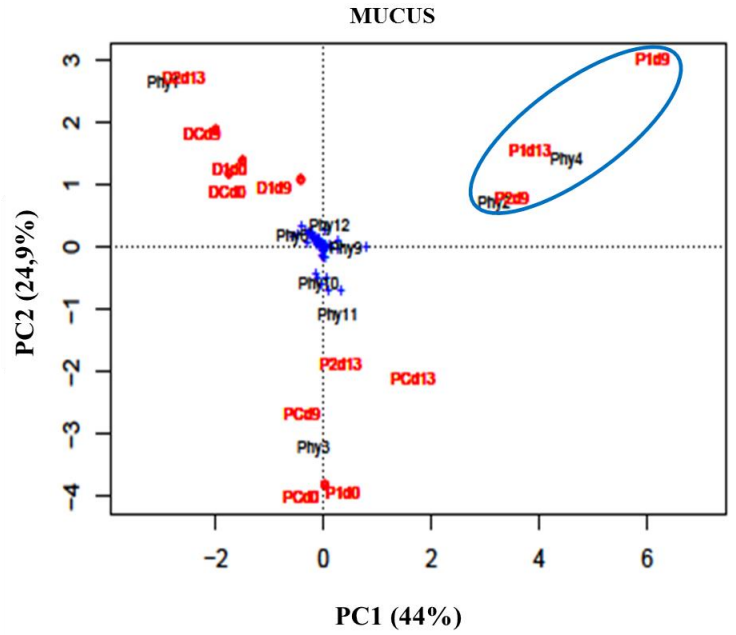


Figure 11 – PCA plots for LUMEN and MUCUS samples

Stilbenes metabolic fate in M-SHIME® experiment

The metabolic fate of resveratrol and other stilbenes was investigated by HPLC/DAD and HPLC/DAD/MS analysis. Samples collected from PC and DC at different time, a blank and a control from PC were treated in order to purify the solution for the analysis. Soluble proteins were precipitated and the clean supernatant was recovered after centrifugation for HPLC injections.

A HPLC/DAD/MS analysis was performed to evaluate differences in all samples profiles. According to Figure 12, no changes in chromatographic profiles at 307 nm of different sampling time were observed. Chromatograms were identical in control and treated samples and compounds identity was confirmed by ESI-TOF analysis. As shown in the chromatograms, two main signals were recorded; they were identified as trans-resveratrol (rt=13 min; 227.068 m/z) and trans-ε-viniferin (rt=19 min; 453.13 m/z), according to their UV and MS spectra. Other minor compounds were also identified as Myabenol (rt= 20.5; 679.188 m/z) and Resveratrol tetramer (tr=21.5 min; 905.244 m/z) [Figure 13]. The same results were obtained for both the tested concentration.

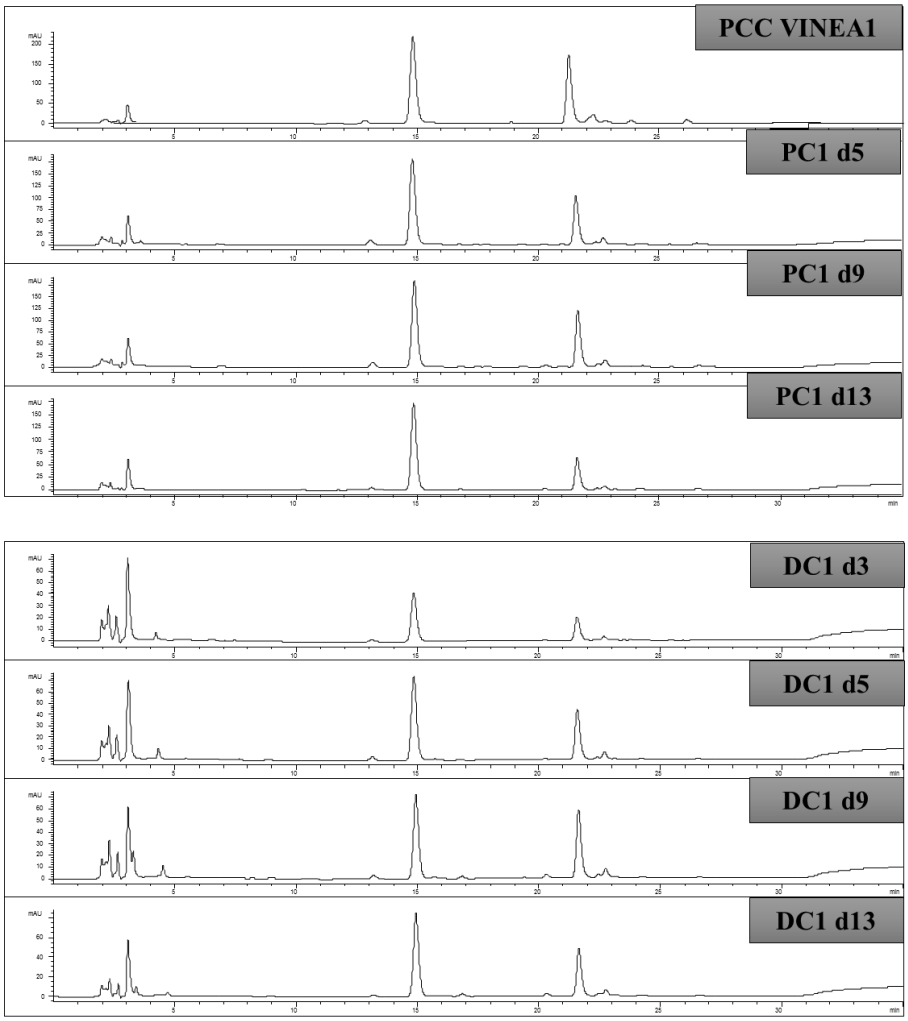


Figure 12 – HPLC/DAD profiles at 307 nm of samples treated with higher concentration of Vineatrol®, from PC and DC vessels.

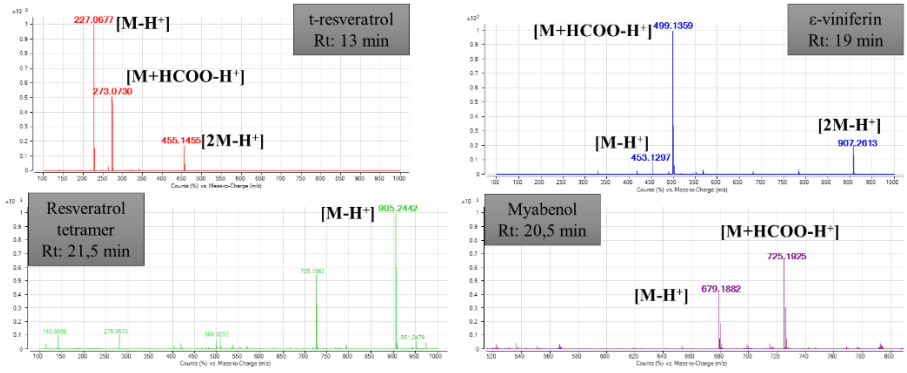


Figure 13 – MS spectra of main stilbenoids in Vineatrol®.

PCCVINEA1, PCCVINEA2, and all collected samples from PC1, DC1, PC2 and DC2 vessels were then analysed to quantify the concentration of main compounds, using the same elution method and the same column. Pure t-resveratrol solution was used to create a calibration curve and to express data. ε-viniferin and minor derivatives were quantified as resveratrol, applying a specific correction factor [31]. Concentrations of samples collected at different times are reported inFigure 14. The trends of all compounds were similar and consistent. In the PC samples, concentrations increased quickly for both doses and reach a plateau, then decreased after 4 days washout. Samples from DC showed the same trend, with a slower increase, since the extract was added in the PC and just a part of this was transferred in DC vessel during each cycle. These consistent results confirmed that the extract composition did not change significantly and no relevant metabolism was detected.

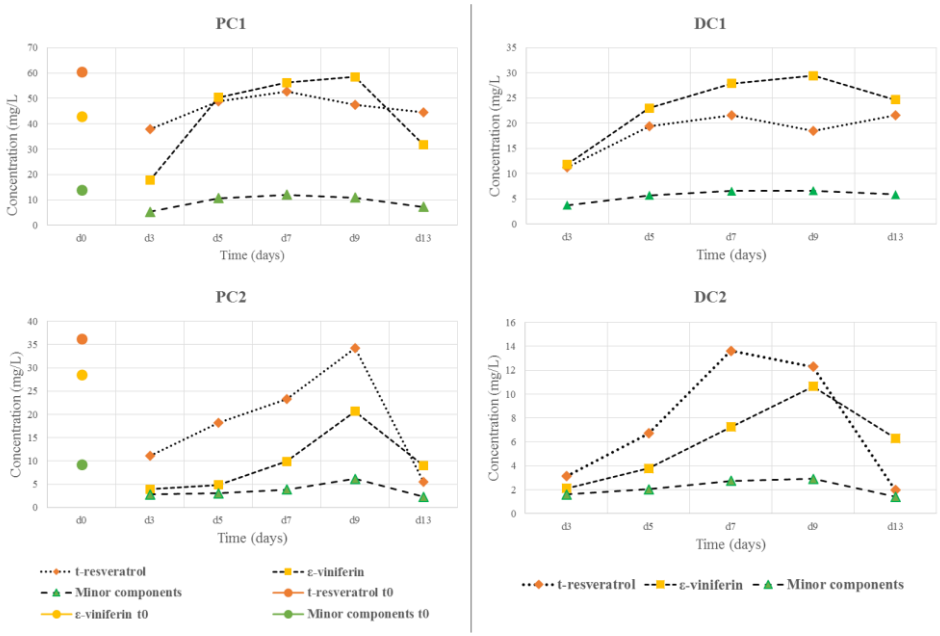


Figure 14 – Main stilbenes concentration during experiment, in PC and DC vessels administered with Vineatrol®

Discussion

It is reported that t-resveratrol and its derivatives suffer strong metabolism after a single oral administration, and their bioavailability is reduced, also due to intestinal microbial functionality. To go beyond that issue, a daily intake of Vineatrol® was administered at colon level. In this work, many aspects were evaluated to investigate the mutual effects of phenols contained in dietary supplement and gut microbial community. T-resveratrol and derivatives usually suffer several transformation, and their bioavailability is very low, as almost all phenolic compounds [4,32,33]. At the same time, t-resveratrol owns well-known antimicrobial activity [34]. The aim of this work was to highlight the administration effects of a stilbene-enriched extract, Vineatrol®, on intestinal microbial community using a gut simulator.

The doses of Vineatrol®, for the treatment in *in vitro* simulator M-SHIME®, were selected through batch experiments with single administration of different concentrations. The evaluation of microbial functionality and stilbenes metabolic fate were consistent with literature, where the metabolization of t-resveratrol and other stilbenes after a single administration was clearly observed in *in vivo* test [9]. SCFA levels were stable in control and treated samples, while the concentration of main stilbenes decreased (indicating that t-resveratrol and its derivatives suffered a metabolization by microbiota). Two dosages were then assessed for test with M-SHIME®.

The microbial communities were studied in terms of composition and functionality. SCFA and NH_4^+ are the products from sugar fermentation and proteolysis. Their levels are considered a clue of wellness of microbial population. In PC and DC control, their concentrations showed a regular trend all over the period. On the other hand, SCFA and NH_4^+ levels resulted strongly reduced by the administration of Vineatrol®: PC1, DC1, PC2 and DC2 trends showed a decrease during the treatment, with a weak return to starting value after the washout. In particular, the higher dose led to a more intense effect in both PC and DC samples. The overall functionality resulted affected.

The second step of the study was to evaluate changes in microbial composition. The preliminary study through DGGE on lumen samples highlighted significant differences among control and treated communities. These modifications were then deeply investigated using an Illumina sequencing on DNA extracted from lumen and mucus samples. The results obtained from sequencing were consistent with an alteration of the microbial functionality and pointed out that communities treated with higher dosage showed a strong change in overall composition, in both lumen and mucus materials. Variation after treatment with Vineatrol® was more evident for PC1 than for DC1 community. In PC2 and DC2, challenged with lower concentration of extract, the evolution was similar, but in less extent. Most important information is about the enrichment of *Enterobacteriales* and the decrease of *Bacteroidales* order. The *Enterobacteriaceae* are a large family of Gram-negative bacteria that includes, along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella* and *Shigella*. *Bacteroidetes* are involved in relevant functions and healthy metabolism of gut microbiota.

The results on microbial communities during the treatment were consistent with information obtained from SCFA and NH_4^+ levels. In terms of communities, a 4-day washout was not sufficient for a return to starting situation, in particular in PC1 (with higher dosage). However, SCFA and NH_4^+ levels showed a weak increased after the treatment. Microbial functionality, in terms of SCFA and NH_4^+, seemed to recover quickly, while for the establishment of the communities' composition more time could be required.

The evaluation of stilbenes metabolic fate was the last part of the work. Despite positive results from preliminary batch experiments that confirmed the metabolization of t-resveratrol and derivatives after a single administration, a continuous treatment with repeated intake for several days, led to interruption of normal metabolism. Main compounds were not transformed and their concentration increased during the experiment, indicating that the polyphenols metabolism was strongly affected.

Conclusions

Results obtained on stilbenes metabolic fate and microbial functionality and composition pointed out a strong negative affection, mainly induced by a continuous administration of this extracts. Results suggest that a similar approach did not seem a proper solution to increase the bioavailability of these compounds *in vivo*, since they modify significantly microbiota equilibrium, with potential negative consequences on human health. Further studies with lower concentrations need to be developed and, at the same time, alternative methods of administration should be taken into account to provide the maximum beneficial effects without affecting the intestinal microbiota.

References

[1] Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventós RM, Berenguer T, Jakszyn P, Martínez C, et al. Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. Br J Nutr 2008;100:188–96. doi:10.1017/S0007114507882997.

[2] Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR, et al. Resveratrol: a review of preclinical studies for human cancer prevention. Toxicol Appl Pharmacol 2007;224:274–83. doi:10.1016/j.taap.2006.12.025.

[3] Petrovski G, Gurusamy N, Das DK. Resveratrol in cardiovascular health and disease. Ann N Y Acad Sci 2011;1215:22–33. doi:10.1111/j.1749-6632.2010.05843.x.

[4] Walle T, Hsieh F, Delegge MH, Oatis JE, Walle UK. High absortion but very low bioavaibility of oral resveratrol in humans. Drug Metab Isposition 2004;32:1377–82. doi:10.1124/dmd.104.000885.sponding.

[5] Ziegler CC, Rainwater L, Whelan J, McEntee MF. Dietary Resveratrol Does Not Affect Intestinal Tumorigenesis in ApcMin/+ Mice. J Nutr 2004;134:5–10.

[6] Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem 2013;24:1415–22. doi:10.1016/j.jnutbio.2013.05.001.

[7] Rechner AR, Smith M a., Kuhnle G, Gibson GR, Debnam ES, Srai SKS, et al. Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products. Free Radic Biol Med 2004;36:212–25. doi:10.1016/j.freeradbiomed.2003.09.022.

[8] Duda-Chodak A, Tarko T, Satora P, Sroka P. Interaction of dietary compounds, especially polyphenols, with the intestinal microbiota: a review. Eur J Nutr 2015;54:325–41. doi:10.1007/s00394-015-0852-y.

[9] Bode LM, Bunzel D, Huch M, Cho G, Ruhland D, Bunzel M, et al. In vivo and in vitro metabolism of trans -resveratrol by human gut. Am J Clin Nutr 2013;97:295–309. doi:10.3945/ajcn.112.049379.INTRODUCTION.

[10] Kemperman R a., Bolca S, Roger LC, Vaughan EE. Novel approaches for analysing gut microbes and dietary polyphenols: Challenges and opportunities. Microbiology 2010;156:3224–31. doi:10.1099/mic.0.042127-0.

[11] Hattori M, Kusumoto IT, Namba T, Ishigami T, Hara Y. Effect of tea polyphenols on glucan synthesis by glucosyltransferase from Streptococcus mutans. Chem Pharm Bull 1990;38:717–20.

[12] Izard J-C. GRAPE VINE RESVERATROL Health and Beauty Benefits 2010.

[13] De Weirdt R, Coenen E, Vlaeminck B, Fievez V, Van den Abbeele P, Van de Wiele T. A simulated mucus layer protects Lactobacillus reuteri from the inhibitory effects of linoleic acid: Beneficial Microbes: Vol 4, No 4. Benef Microbes 2013:299–312.

[14] Van Den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gérard P, et al. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for bacteroidetes and Clostridium cluster IX. Appl Environ Microbiol 2010;76:5237–46. doi:10.1128/AEM.00759-10.

[15] Van den Abbeele P, Roos S, Eeckhaut V, Mackenzie D a., Derde M, Verstraete W, et al. Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. Microb Biotechnol 2012;5:106–15. doi:10.1111/j.1751-7915.2011.00308.x.

[16] Possemiers S, Verth K, Uyttendaele S, Verstraete W. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 2004;49:495–507. doi:10.1016/j.femsec.2004.05.002.

[17] Greenberg AE, Clesceri LS, Eaton AD. Standard methods for the examination of water and wastewater 18th edition. 1992.

[18] Vilchez-Vargas R, Geffers R, Suárez-Diez M, Conte I, Waliczek A, Kaser VS, et al. Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray

system. Environ Microbiol 2013;15:1016–39. doi:10.1111/j.1462-2920.2012.02752.x.

[19] Boon N, Top EM, Verstraete W, Siciliano SD. Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. Appl Environ Microbiol 2003;69:1511–20.

[20] Heilig HGHJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, de Vos WM. Molecular Diversity of Lactobacillus spp. and Other Lactic Acid Bacteria in the Human Intestine as Determined by Specific Amplification of 16S Ribosomal DNA. Appl Environ Microbiol 2002;68:114–23. doi:10.1128/AEM.68.1.114-123.2002.

[21] Satokari RM, Vaughan EE, Akkermans ADL, Saarela M, de Vos WM. Bifidobacterial Diversity in Human Feces Detected by Genus-Specific PCR and Denaturing Gradient Gel Electrophoresis. Appl Environ Microbiol 2001;67:504–13. doi:10.1128/AEM.67.2.504-513.2001.

[22] Ovreås L, Forney L, Daae FL, Torsvik V. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 1997;63:3367–73.

[23] Muyzer G, De Waal EC, Uitterlinden a. G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993;59:695–700.

[24] Boon N, Goris J, Vos P De, Verstraete W, Top EM. Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Comamonas testosteroni Strain , I2 gfp. Appl Environ Microbiol 2000;66:2906–13. doi:10.1128/AEM.66.7.2906-2913.2000.Updated.

[25] Camarinha-Silva A, Jáuregui R, Chaves-Moreno D, Oxley APA, Schaumburg F, Becker K, et al. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environ Microbiol 2014;16:2939–52. doi:10.1111/1462-2920.12362.

[26] Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid Tech. Bact. Syst., Chichester, United Kingdom: Wiley & Sons; 1991, p. 115–75.

[27] Etchebehere C, Tiedje J. Presence of two different active nirS nitrite reductase genes in a denitrifying Thauera sp. from a high-nitrate-removal-rate reactor. Appl Environ Microbiol 2005;71:5642–5. doi:10.1128/AEM.71.9.5642-5645.2005.

[28] Chaves-Moreno D, Plumeier I, Kahl S, Krismer B, Peschel A, Oxley APA, et al. The microbial community structure of the cotton rat nose. Environ Microbiol Rep 2015. doi:10.1111/1758-2229.12334.

[29] Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 2009;75:7537–41. doi:10.1128/AEM.01541-09.

[30] Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7. doi:10.1128/AEM.00062-07.

[31] Santamaria AR, Innocenti M, Mulinacci N, Melani F, Valletta A, Sciandra I, et al. Enhancement of Viniferin Production in Vitis vinifera L. cv. Alphonse Lavallée Cell Suspensions by Low-Energy Ultrasound Alone and in Combination with Methyl Jasmonate. J Agric Food Chem 2012;60:11135–42. doi:10.1021/jf301936u.

[32] Pandareesh MD, Mythri RB, Bharath MMS. Bioavailability of dietary polyphenols: Factors contributing to their clinical application in CNS diseases. Neurochem Int 2015;89:198–208. doi:10.1016/j.neuint.2015.07.003.

[33] Amri a., Chaumeil JC, Sfar S, Charrueau C. Administration of resveratrol: What formulation solutions to bioavailability limitations? J Control Release 2012;158:182–93. doi:10.1016/j.jconrel.2011.09.083.

[34] Paulo L, Oleastro M, Gallardo E, Quiroz JA, Domingues F. Antimicrobial properties of resveratrol: a review. Formatex Res Cent 2011:1225–35.

Chapter 2: Administration of polyphenols included in food by-products in high protein diets.

Camilla Giuliani, Marzia Innocenti, Mohammad Kathib, Massimo Marzorati[1], Tom Van de Wiele[1], Nadia Mulinacci

Department of Neurofarba- Pharmaceutical and Nutraceutical Division, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, (FI) Italy.
[1] LabMET, Faculty of Bioengineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

Introduction

In last years, sugar free diets with high protein content became a trendy approach for weight-loss all over the world [1]. These type of diet provides some positive effects, such as a fast decrease of fat mass and enhancement of insulin sensitivity [2]. On the other hand, it is important to remind that a severe decrease of carbohydrates affects the wellness of colonic region. A strong decrease of total short-chain fatty acid (SCFA) concentrations is reported as consequence of low carbohydrates diet [3,4]. Since SCFA demonstrated beneficial effects, concentration decreasing provides a deterioration of wellness. In addition, high protein intake could lead to many potential dangerous situations. Since more proteins arrive at colon region, their transformation provides increased amount of fermentation products, like nitrogenous metabolites [5,6]. Many studies demonstrated that these molecules are involved in increasing colon cancer risk and inflammatory disease [7–9].

The effects of polyphenols dietary intake is also widely investigated. Several studies about beneficial effects of these molecules in in vitro and in vivo models was published in last the years [10–13]. Many properties are attributed to phenolic compounds, such as anti-oxidant [10,14,15], anti-inflammatory [16–19], cardiovascular protection [20–23] and chemopreventive activities [24–28], but in vivo mechanisms are not yet clear. According to these results, polyphenols intake could then contrast adverse consequences reported for high protein diets.

Moreover, dietary fiber and non-digestible carbohydrates are fermented by microbial community, so they can contrast the SCFA decrease and provide positive effects [29–31] and also improve the release of phenolic compound from vegetable materials [32]. In 2011, a study reported that 50% of dietary polyphenols cross the small intestine linked to fiber [33]. One other work showed how the combination of fiber and phenols could improve functionality of different foods [34].

One example of vegetable matters including polyphenols are food wastes. Food by-products derive mostly from food processing and farming, where huge amounts of discards are produced [35]. These wastes are used often for biofuel or

bioenergy production processes [36]. However, many discards from plants are also considered as sources of fiber and bioactive polyphenols. There are many example of wasted plants material studied as candidates for the recovery of bioactive compounds, such as cereals, roots, oil crops and fruit/vegetables. [37]. One of the most interesting food process is the olive oil production and many approaches were studied in order to recover both polyphenols and fiber from milling by-products (wastewaters and pomace) [38–44]. Several works are reported also about pomegranate discards, in particular seeds and peel [45], as source of phenolic compounds and polysaccharides [46,47]. Different approaches were investigated to obtain the higher yields, with various results confirming high content of active compounds [45,48].

It is important to know that the body recognizes polyphenols as xenobiotics and their bioavailability is lower than common macro and micronutrients, which suffer different metabolism [11]. Moreover, the bioavailability of phenolic compounds depends also on inter-individual differences in the composition of the microbial community, which can be converted to bioactive compounds by microbiota, modifying intestinal microbial balance and thus also the host's health [49].

The aim of this work was to investigate about potential benefits of two food by-products, containing both fiber and bioactive polyphenols, when administered in addition to diets with high protein and low carbohydrates content.

The selected by-products were an olive "pâté" (OP), derived from extra virgin olive oil production and made up of wet pulp, and a decoction of pomegranate mesocarp (PM), which represent the most abundant waste from pomegranate juice production. A Triple M-SHIME® experiment was performed to investigate the effects of oral administration considering polyphenols metabolic fate (HPLC/DAD/MS analysis) and SCFA levels (GC-FID).

Material and methods

Food by-products

Two extracts were used in the experiment with simulator. Pomegranate (Wonderful cv) from Tuscan farming was selected. Mesocarp was separated from the other parts, then washed with water. Once dried, a 1h decoction was applied. Suspension was freeze-dried, grounded and used as powder (PM). Polysaccharides were precipitated by the addition of two volumes of EtOH to original decoction and keeping the solution at 0°C for 3h. Solid residue were weight and the total amount was 12%, referring to the dried extract [50].

The second sample derived from a water suspension of residual material from olive oil production, named olive "pâté" (OP) that includes wet pulp and husk. The liquid suspension was freeze-dried. Powder was defatted adding hexane and stirring for 1 h. The solvent was then recovered and powder was dried. The producer declared in label a total fiber content of 24%, according to standard method for determination of fiber in vegetable materials.

HPLC/DAD/MS analysis

Quantitative analysis were performed on PM and OP, preparing samples as described below.

PM sample was prepared solving the extract 10 mg/mL in H_2O. Two consecutives extraction with EtOH/H_2O 8:2 v/v were applied to OP. The extracts were gathered together and dried under vacuum. Final powder was solved in EtOH/H_2O 8:2 v/v. For PM characterization, stock solution of α+β punicalagins, 1 mg/mL, was prepared in DMSO, then diluted with H_2O before the HPLC analysis; solution of ellagic acid 0.78 mg/mL was prepared in MeOH. The calibration curve of α+β punicalagins (380 nm) was in a linearity range between 0.5-8 μg with a R^2 = 0,9982; the calibration curve of ellagic acid (370nm) was in the linearity range of 0,031–1,25 μg with R^2 = 0,9995. PM samples were analysed using a 150mm×2 mm i.d., 4μm, RP-18, Sinergi Fusion column (Phenomenex, USA). Eluents selected were (A) formic acid/H_2O and (B) CH_3CN. The multi-step linear solvent

gradient used was: 0-4 min 5-25% B; 4–8 min, 25–25% B; 8–14 min 25–35% B; 14-16 min 35-90%; 16-18 min 90-5%, equilibration time 10 min; flow rate 0.4 mL/min. The UV-Vis spectra were recorded in the range 200–500 nm and the chromatograms were acquired at 240 nm, 280 nm, 330 nm, 370 nm, 380 nm. MS spectra were acquired using Dual-ESI source in negative polarity with 100 fragmentor, 4000 V Capillary Voltage, 350°C of Gas Temperature.

For OP hydroxytyrosol and tyrosol were quantified using a calibration curve of tyrosol standard with $R^2 = 0.999$ and recording the areas at 280 nm. Oleuropein and its derivatives were quantified applying a four-point calibration curve obtained with standard oleuropein with $R^2 = 0.999$ and recording areas at 280 nm. Verbascoside and phenylpropanoids derivatives were quantified using caffeic acid calibration curve obtained with $R^2 = 0.999$ and recording areas at 330 nm. The amount of verbascoside in particular, was measured using a correction factor of weight equal to 3.47. A 150mm×3 mm i.d., 2.7 μm, RP-18, Poroshell column (Agilent Technologies, USA) was used. Same eluents were selected. The multi-step linear solvent gradient used was: 0-40 min 5-40% B; 40–45 min, 40–40% B; 45–50 min 40–100% B; 50-53 min 100-100%; 53-55 min 100-5%, equilibration time 10 min; flow rate 0.4 mL/min. The UV-Vis spectra were recorded in the range 200–600 nm and the chromatograms were acquired at 240 nm, 280 nm, 330 nm, 350 nm, 540 nm. MS spectra were acquired using Dual-ESI source in negative polarity with 80 V fragmentor, 3800 V Capillary Voltage, 350°C of Gas Temperature.

M-SHIME® experiments

One Triple M-SHIME® model was performed inoculating with faecal samples from a healthy donor. The M-SHIME® contained, in addition to traditional luminal microbial community [51], a special feed without sugars including: yeast extract 3.0 g/L, peptone 1.0 g/L, and pig gastric mucin 4.0 g/L. At the start of each experiment, 500 mL of selected feed was added to Proximal Colon (PC) unit, while the Distal Colon (DC) unit was filled with 800 mL. Following inoculation was performed with 40 mL of a 1:5 dilution of fresh stools of a healthy human

volunteer [52]. After an initial incubation of 18h for pH stabilisation, 140 mL nutritional medium and 60 mL pancreatic juice were supplied to each proximal colon compartment three times per day. The M-SHIME® was kept at 37 °C and anaerobic condition by flushing for 10 min with N_2 every day.

After a stabilisation period of 2 weeks, daily doses of extracts were administrated directly to PC vessel, for 10 days. The experiment was performed administering 4 g/L of OP and 2 g/L of PM [Figure 1].

Three times per week, at the same time of the day, 20 mL of liquid sample were collected from each colon vessel. Luminal liquid was stored at -20°C for metabolic analyses [Figure 2].

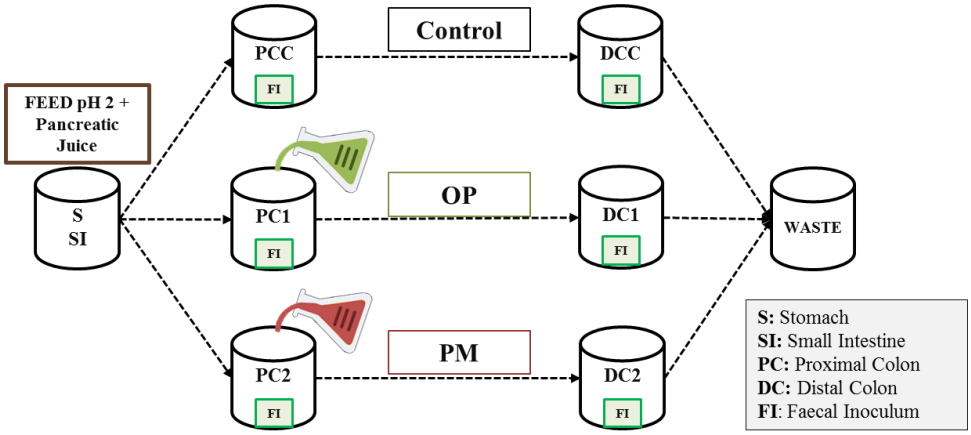


Figure 1 - SHIME® design

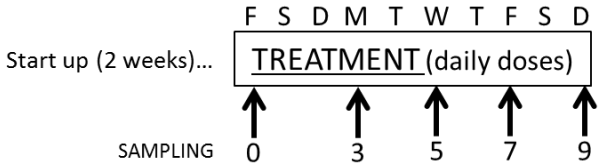


Figure 2 – SHIME® experiment

Short Chain Fatty Acids analysis

For SCFA analysis, a liquid-liquid extraction with diethyl ether was applied on frozen samples, after the addiction of H_2SO_4 and internal standard. SCFA quantitative analysis was performed using capillary gas chromatography coupled with a flame ionization detector (GC-FID), to measure acetic, propionic, butyric, isobutyric, caproic, isocaproic, valeric and isovaleric acids.

Results

Composition of PM and OP

The two materials were analyzed with optimized methods and quantitative content was assessed using pure standards.

In PM, ellagitannins content was evaluated in terms of α + β punicalagins, as most abundant compounds, and ellagic acid and its glycosides, as minor components [Figure 3 and 4]. A overall amount of 120.21 mg/g of dried sample, in terms of total tannins, was obtained. As expected, the α + β punicalagins resulted the main compounds reaching 70.7 mg/g in mesocarp decoction [Table 1]. Ellagic acid was also well represented in the extracts, with a concentration of 3.67 mg/g.

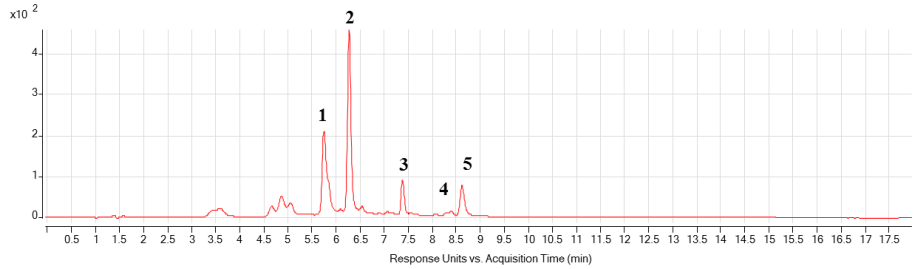


Figure 3 - Chromatograms at 370 nm of PM.

1) α-punicalagin, 2) β-punicalagin, 3) ellagic acid hexoside, 4) ellagic acid pentoside, 5) ellagic acid.

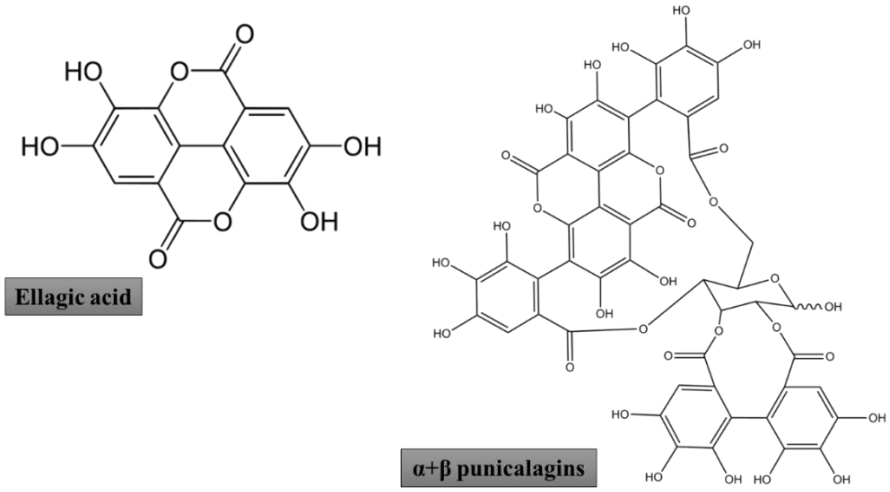


Figure 4 – Structure of main phenolic compounds in PM.

Products	Compounds	Concentration (mg/g)
PM	α+β punicalagins	70.7
	Ellagic acid and derivatives	10.70
	Total tannins	120.2

Table 1 - Tannins content of PM in terms of mg/ g of dried material

Considering the different classes of phenolic compounds included in *Olea Europea*, polyphenols content of OP was calculated using three different external standards: tyrosol (form small phenols), oleuropein (for secoiridois class) and caffeic acid (for verbascoside and derivatives) [Figure 5 and 6 – Table 2]The most abundant class correspond to oleuropein derivatives, with a total concentration of 77.77 mg/g. OP resulted rich in term of hydroxytyrosol, while tyrosol concentration was lower. Verbascoside was also significantly present in the extract. The overall amount of polyphenols was quite high, reaching a total content of 97.55 mg/g.

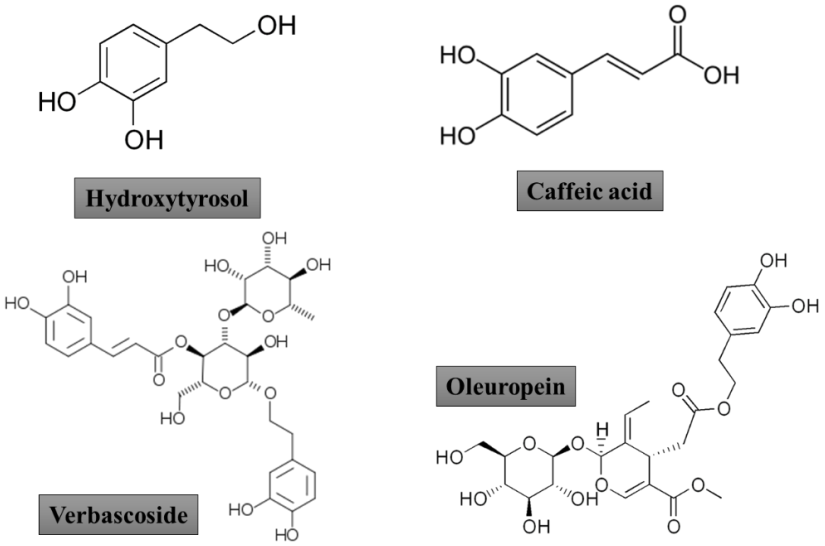


Figure 5 - Main polyphenols identified in OP

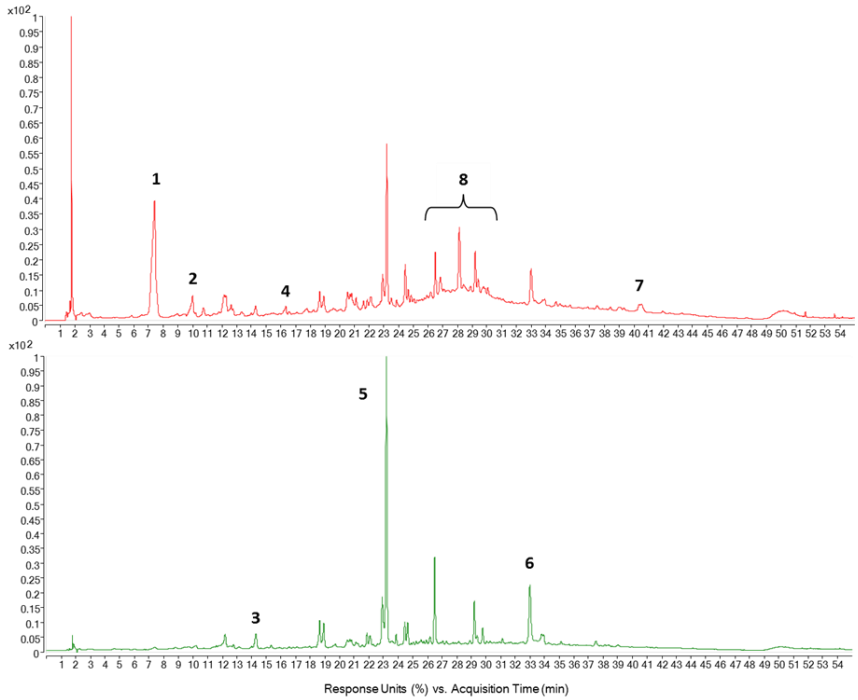


Figure 6 - HPLC profiles at 280 (red) e 330 (green) nm from OP sample. Hydroxytyrosol (1), tyrosol (2), caffeic acid (3), oleuropein-aglycone mono-aldehyde (4), verbascoside (5), luteolin aglycone (6), oleuropein aglycone (7), deacetoxy oleuropein aglycone (8)

Products	Compounds	Concentration (mg/g)
OP	Verbascoside	4.52
	Hydroxytyrosol	15.26
	Oleuropein derivatives	77.77
	Luteolin	0.42
	Total polyphenols	97.55

Table 2 – Polyphenols content of OP in terms of mg/ g of dried material

Phenolic profiles and content of these two samples, shown in Tab 1 and 2 and Figure 3 and 6, are characterize by the presence of several compounds. Part of them will be metabolized and transformed in smaller molecules.

Polyphenols effects on microbial metabolism : SCFA

SCFAs level was calculated in samples from all vessels at many times (day 0, 2, 4, 7, 9) through GC-FID. Acetate, butyrate, propionate and total SCFA concentrations are reported as most significant [Figure 7].

Samples from control vessels, both PC and DC, showed a regular trend during all experiment period. As expected, DC levels were higher than PC's, and the system resulted stable over time.

OP administrated samples presented a different trend compared with control. In both PC and DC, an increase of SCFAs level was recorded during the treatment period. Concentrations rose about 50%, meaning that fiber, included in OP, were used as carbon source by bacterial community.

Treatment with PM led to similar results. Starting level was lower than other samples, but SCFAs showed the same evolution, increasing during all period. Even in this case levels got higher in PC and DC along the experiment, probably due to polysaccharides, included in PM, which are transformed by microbial population.

Moreover, the increase provided by OP resulted higher than PM. This result is consistent with the overall content of the two samples. OP includes 24% (w/w) of total fiber, while for PM the amount of polysaccharides was assessed to 12% (w/w).

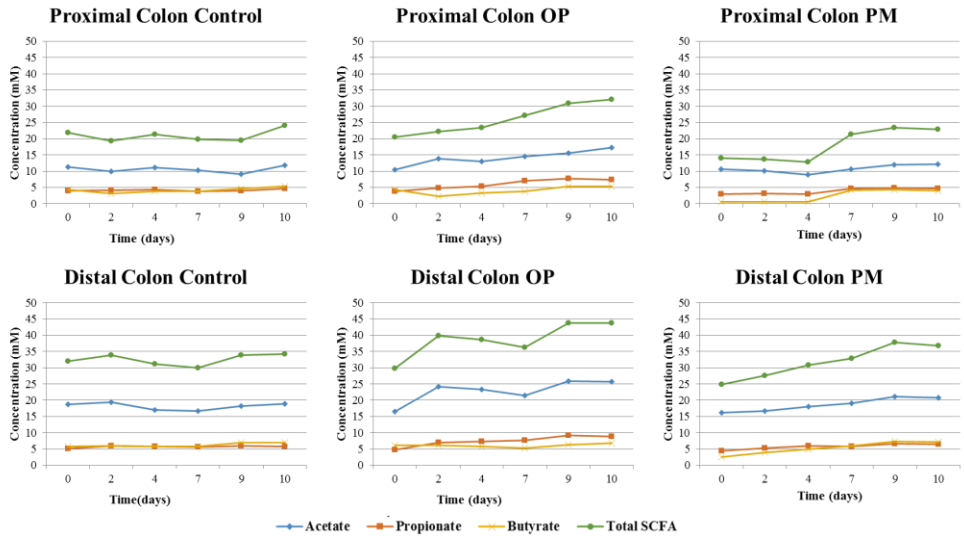


Figure 7 – SCFA levels during the M-SHIME® experiment.

Discussion

The two by-products selected for this study were analysed in order to define mainly the polyphenols content. PM showed a high amount of total ellagitannins, in particular punicalagins and ellagic acid derivatives. The decoction applied as extraction method is then effective to recover main compounds, including polyphenols and polysaccharides. This method provides a soluble extract ready for the oral administration.

The profile of OP showed a significant content of phenolic molecules. The dominant compound was hydroxytyrosol, but also other molecules, like oleuropein derivatives, verbascoside and luteolin, are well represented in the extract.

It can be assessed the two selected by-products resulted as a good source of polyphenols.

The evaluation of microbial functionality showed low concentrations in control samples, but a significant improvement in SCFA levels was provided by the administration for 10 days of these extracts. PM and OP contains molecules that are used as C-source by intestinal bacteria, probably fiber and polysaccharide included in original raw materials. The same evolution was observed in PC and DC vessels administered with the two by-products.

This preliminary study showed potential benefits of the use of food by-products in addition to a high protein/no sugars diet. The content of polyphenols found in OP and PM, together with dietary fiber, was very interesting. In addition, the increase of SCFA, obtained with the administration of OP and PM, could contrast the negative effects of extreme diets, improving microorganism and gut wellness. Further investigation will be carried out on metabolic fate of polyphenols and effects on microbiota composition, in order to better understand the interaction between phenolic compound and bacteria.

References

[1] Vogels N, Westerterp-Plantenga MS. Categorical strategies based on subject characteristics of dietary restraint and physical activity, for weight maintenance. Int J Obes 2005;29:849–57. doi:10.1038/sj.ijo.0802984.

[2] Johnstone AM, Horgan GW, Murison SD, Bremner DM, Lobley GE. Effects of a high-protein ketogenic diet on hunger, appetite, and weight loss in obese men feeding ad libitum. Am J Clin Nutr 2008;87:44–55. doi:“It felt like a mild high”...

[3] Brinkworth GD, Noakes M, Clifton PM, Bird AR. Comparative effects of very low-carbohydrate, high-fat and highcarbohydrate, low-fat weight-loss diets on bowel habit and faecal short-chain fatty acids and bacterial populations. Br J Nutr 2009;101:1493–502.

[4] Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE. Reduced Dietary Intake of Carbohydrates by Obese Subjects Results in Decreased Concentrations of Butyrate and Butyrate-Producing Bacteria in Feces. Appl Environ Microbiol 2007;73:1073–8. doi:10.1128/AEM.02340-06.

[5] Magee E a, Richardson CJ, Hughes R, Cummings JH. Contribution of dietary protein to sulfide production in the large intestine: an in vitro and a controlled feeding study in humans. Am J Clin Nutr 2000;72:1488–94.

[6] Gill CIR, Rowland IR. Diet and cancer: assessing the risk. Br J Nutr 2002;88 Suppl 1:S73–87. doi:10.1079/BJN2002632.

[7] Norat T, Riboli E. Meat consumption and colorectal cancer: a review of epidemiologic evidence. Nutr Rev 2001;59:37–47.

[8] Hughes R, Cross a J, Pollock JR, Bingham S. Dose-dependent effect of dietary meat on endogenous colonic N-nitrosation. Carcinogenesis 2001;22:199–202. doi:10.1093/carcin/22.1.199.

[9] Rumney CJ, Rowland IR, O’Neill IK. Conversion of IQ to 7-OHIQ by gut microflora. Nutr Cancer 1993;19:67–76. doi:10.1080/01635589309514237.

[10] Quideau S, Deffieux D, Douat-Casassus C, Pouységu L. Plant polyphenols: chemical properties, biological activities, and synthesis. Angew Chem Int Ed Engl 2011;50:586–621. doi:10.1002/anie.201000044.

[11] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81:230S – 242S.

[12] Edwin Haslam, Terence H. Lilley, Edward Warminski, Hua Liao, Ya Cai, Russell Martin, et al. Phenolic compounds in food and their effects on health I. vol. 506. Washington, DC: American Chemical Society; 1992. doi:10.1021/bk-1992-0506.

[13] Agrawal M. Natural polyphenols based new therapeutic avenues for advanced biomedical applications. Drug Metab Rev 2015;2532:1–11. doi:10.3109/03602532.2015.1102933.

[14] Meyer AS, Heinonen M, Frankel EN. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. Food Chem 1998;61:71–5. doi:10.1016/S0308-8146(97)00100-3.

[15] Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventós RM, Berenguer T, Jakszyn P, Martínez C, et al. Concentrations of resveratrol and derivatives in foods and estimation of dietary intake in a Spanish population: European Prospective Investigation into Cancer and Nutrition (EPIC)-Spain cohort. Br J Nutr 2008;100:188–96. doi:10.1017/S0007114507882997.

[16] Martínez JA, Etxeberría U, Galar A, Milagro FI. Role of dietary polyphenols and inflammatory processes on disease progression mediated by the gut microbiota. Rejuv Res 2013;16:435–7. doi:10.1089/rej.2013.1481.

[17] Dolara P, Luceri C, De Filippo C, Femia A Pietro, Giovannelli L, Caderni G, et al. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutat Res 2005;591:237–46. doi:10.1016/j.mrfmmm.2005.04.022.

[18] Larrosa M, González-Sarrías A, Yáñez-Gascón MJ, Selma M V, Azorín-Ortuño M, Toti S, et al. Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. J Nutr Biochem 2010;21:717–25. doi:10.1016/j.jnutbio.2009.04.012.

[19] Simpson SJ, Holländer GA, Mizoguchi E, Allen D, Bhan AK, Wang B, et al. Expression of pro-inflammatory cytokines by TCRαβ+ and TCRγδ+ T cells in an experimental model of colitis. Eur J Immunol 1997;27:17–25.

[20] de Lorgeril M, Salen P, Guiraud A, Boucher F, de Leiris J. Resveratrol and non-ethanolic components of wine in experimental cardiology. Nutr Metab Cardiovasc Dis 2003;13:100–3.

[21] Petrovski G, Gurusamy N, Das DK. Resveratrol in cardiovascular health and disease. Ann N Y Acad Sci 2011;1215:22–33. doi:10.1111/j.1749-6632.2010.05843.x.

[22] Larrosa M, García-Conesa MT, Espín JC, Tomás-Barberán FA. Ellagitannins, ellagic acid and vascular health. Mol Aspects Med 2010;31:513–39. doi:10.1016/j.mam.2010.09.005.

[23] Gehm BD, McAndrews JM, Chien P-Y, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci 1997;94:14138–43. doi:10.1073/pnas.94.25.14138.

[24] Fabiani R, De Bartolomeo A, Rosignoli P, Servili M, Montedoro GF, Morozzi G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. Eur J Cancer Prev 2002;11:351–8. doi:10.1097/00008469-200208000-00006.

[25] Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 2009;461:1282–6. doi:10.1038/nature08530.

[26] Cardinali A, Pati S, Minervini F, D'Antuono I, Linsalata V, Lattanzio V. Verbascoside, isoverbascoside, and their derivatives recovered from olive mill wastewater as possible food antioxidants. J Agric Food Chem 2012;60:1822–9. doi:10.1021/jf204001p.

[27] Cerdá B, Tomás-Barberán FA, Espín JC. Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. J Agric Food Chem 2005;53:227–35. doi:10.1021/jf049144d.

[28] Wenzel E, Soldo T, Erbersdobler H, Somoza V. Bioactivity and metabolism of trans -resveratrol orally administered to Wistar rats. Mol Nutr Food Res 2005;49:482–94. doi:10.1002/mnfr.200500003.

[29] Flint HJ, Duncan SH, Scott KP, Louis P. Interactions and competition within the microbial community of the human colon: links between diet and health. Environ Microbiol 2007;9:1101–11. doi:10.1111/j.1462-2920.2007.01281.x.

[30] Tazoe H, Otomo Y, Kaji I, Tanaka R, Karaki S-I, Kuwahara A. Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. J Physiol Pharmacol 2008;59 Suppl 2:251–62.

[31] Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: The role of butyrate on colonic function. Aliment Pharmacol Ther 2008;27:104–19. doi:10.1111/j.1365-2036.2007.03562.x.

[32] Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, et al. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr 2011;93:1062–72. doi:10.3945/ajcn.110.002188.

[33] Saura-Calixto F. Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. J Agric Food Chem 2011;59:43–9. doi:10.1021/jf1036596.

[34] Quirós-Sauceda AE, Palafox-Carlos H, Sáyago-Ayerdi SG, Ayala-Zavala JF, Bello-Perez LA, Alvarez-Parrilla E, et al. Dietary fiber and phenolic compounds as functional ingredients: interaction and possible effect after ingestion. Food Funct 2014;5:1063–72. doi:10.1039/c4fo00073k.

[35] Status of FAO's Work on Post-Harvest Losses 2012.

[36] Girotto F, Alibardi L, Cossu R. Food waste generation and industrial uses: A review. Waste Manag 2015. doi:10.1016/j.wasman.2015.06.008.

[37] Galanakis CM. Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. Trends Food Sci Technol 2012;26:68–87. doi:10.1016/j.tifs.2012.03.003.

[38] Rozzi A, Malpei F. Treatment and disposal of olive mill effluents. Int Biodeterior Biodegradation 1996;38:135–44. doi:10.1016/S0964-8305(96)00042-X.

[39] Mantzavinos D, Kalogerakis N. Treatment of olive mill effluents Part I. Organic matter degradation by chemical and biological processes--an overview. Environ Int 2005;31:289–95. doi:10.1016/j.envint.2004.10.005.

[40] Fountoulakis M., Dokianakis S., Kornaros M., Aggelis G., Lyberatos G. Removal of phenolics in olive mill wastewaters using the white-rot fungus Pleurotus ostreatus. Water Res 2002;36:4735–44. doi:10.1016/S0043-1354(02)00184-7.

[41] Marques IP. Anaerobic digestion treatment of olive mill wastewater for effluent re-use in irrigation. Desalination 2001;137:233–9. doi:10.1016/S0011-9164(01)00224-7.

[42] Beltran de Heredia J, Garcia J. Process integration: continuous anaerobic digestion−ozonation treatment of olive mill wastewater. Ind Eng Chem Res 2005;44:8750–5. doi:10.1021/ie040232x.

[43] Cegarra J, Paredes C, Roig A, Bernal MP, García D. Use of olive mill wastewater compost for crop production. Int Biodeterior Biodegradation 1996;38:193–203. doi:10.1016/S0964-8305(96)00051-0.

[44] Caputo AC, Scacchia F, Pelagagge PM. Disposal of by-products in olive oil industry: waste-to-energy solutions. Appl Therm Eng 2003;23:197–214. doi:10.1016/S1359-4311(02)00173-4.

[45] Kalamara E, Goula AM, Adamopoulos KG. An integrated process for utilization of pomegranate wastes — Seeds. Innov Food Sci Emerg Technol 2015;27:144–53. doi:10.1016/j.ifset.2014.12.001.

[46] Singh RP, Chidambara Murthy KN, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (punica granatum) peel and seed extracts using in vitro models. J Agric Food Chem 2002;50:81–6. doi:10.1021/jf010865b.

[47] Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem 2006;96:254–60. doi:10.1016/j.foodchem.2005.02.033.

[48] Goula AM, Adamopoulos KG. A method for preparing a novel solid product from olive mill wastewater: wastewater characterization and product recovery. Dry Technol 2013;31:339–49. doi:10.1080/07373937.2012.736114.

[49] Hervert-Hernández D, Pintado C, Rotger R, Goñi I. Stimulatory role of grape pomace polyphenols on *Lactobacillus acidophilus* growth. Int J Food Microbiol 2009;136:119–22. doi:10.1016/j.ijfoodmicro.2009.09.016.

[50] Kathib M, Innocenti M, Giuliani C, Al-Tamimi A, Rossi F, Adessi A, et al. Characterization of polysaccharides from the wonderful and laffan pomegranate fruit cultivars. Carbohydrates Res n.d.:Submitted.

[51] Van Den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gérard P, et al. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for bacteroidetes and Clostridium cluster IX. Appl Environ Microbiol 2010;76:5237–46. doi:10.1128/AEM.00759-10.

[52] Possemiers S, Verth K, Uyttendaele S, Verstraete W. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 2004;49:495–507. doi:10.1016/j.femsec.2004.05.002.

Chapter 3: By-products from olive and pomegranate as polyphenols source: simulation of oral administration in *in vitro* intestinal model M-SHIME®

Camilla Giuliani, Marzia Innocenti, Mohammad Kathib, Massimo Marzorati[1], Tom Van de Wiele[1], Nadia Mulinacci

Department of Neurofarba- Pharmaceutical and Nutraceutical Division, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, (FI) Italy.

[1] *LabMET, Faculty of Bioengineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium*

Introduction

In 2011 FAO reported that “one-third of the edible parts of food produced for human consumption, gets lost or wasted globally, which is about 1.3 billion ton per year” [1]. Big discards are also due to an improper treatment of sub-products, in particular in agricultural sector and food processing industry. Post-harvesting and processing are responsible of more than 40% of losses, mainly in industrial countries [2]. In last years, the interest toward food waste valorisation and reuse increased much. Some by-products, recovered from different vegetable food, have been widely investigated mainly for the development of biofuel or bioenergy production [3]. Most wastes from plants resulted to be good candidate also for recovery of nutraceutical ingredients, suitable to be included in dietary supplements or food formulations. Cereals, roots, oil crops and fruit/vegetables by-products are all potential sources of bioactive and functional compounds [4].The goal is to define new sustainable processes and techniques to recover and transform wastes in suitable extracts enriched in bioactive compounds.

One of the most investigated food process is the olive oil production. Residues of milling process are well-know to own negative properties, such as phytotoxicity, pollution of natural waters, affection of aquatic life, due to lipid and phenolic content [5]. Different strategies have been proposed and evaluated to manage wastewaters and pomace and reduce negative effects, including physical, physicochemical and biological treatments [6–12]. However, it was estimated that only 2-4% of total phenolic compounds included in olives could be recovered in virgin oil during the milling process, while the largest part is thrown away with wastes [13]. Different methods to recover these molecules have been studied and many processes (industrial or laboratory scale) have been developed [14–17].

At the same time, also pomegranate wastes from juice production (seeds and peel) were studied as a source of polyphenols, in particular phenolic acids, flavonoids, tannins [18,19]. The peel and mesocarp from pomegranate, discarded during juice production, could be up to 50% weight of the whole fresh fruit [20–22]. Even in this case, different extractions were evaluated in order to obtain maximum yield

of main interesting molecules. Despite the variability in the results, all studies agree with the high content of active compounds in these by-products [23,24].

According to the huge interest on these matrices, we selected two by-products: an olive "pâté" (OP), derived from extra virgin olive oil production and made up of wet pulp, and a decoction of pomegranate mesocarp (PM), from a widespread variety, the Wonderful cultivar. These samples were selected due to previous analytical screening.

It is crucial to remind that, once introduced in the organism through oral administration, the body recognizes polyphenols as xenobiotics and their bioavailability is lower than that of macro and micronutrients. Low molecular weight polyphenols, such as monomeric or dimeric structures, are absorbed in the small intestine, but normally the percentage does not exceed 5-10% and the most part derives from deconjugation and deglycosylation reactions [25]. High molecular weight polyphenols represent 90-95% of total dietary polyphenols intake and are not absorbed in the small intestine, but reach the colon partially altered [26].

The metabolic fate of main polyphenols from olive oil is still not clear and few works are reported in literature on colonic transformation of main molecules [27–29]. It is demonstrated that microorganisms usually degrade main molecules, for instance hydroxytyrosol and oleuropein aglycone, but there are not data on the effect of the total human microbiota metabolism.

Regarding pomegranate, ellagitannins metabolism is located in the intestinal lumen, through hydrolysis reactions releasing ellagic acid. In the large intestine, this compound is metabolized by bacteria and is transformed in urolithins with a decrease number of hydroxyl groups, going from urolithin D → C → A → B [30,31].

Moreover, polyphenols can influence bacterial growth and metabolism depending on their structure, dose and type of microorganisms considered [32]. After exposure to polyphenols presence, microbial synthesis of defensive protein increase to protect the cell, but at the same time their metabolic activity decrease reducing the formation of biosynthetic proteins, amino acids, phospholipids and

Short Chain Fatty Acids (SCFA) [26]. In the end, the inter-individual differences in the composition of the microflora bacteria can lead to differences in the bioavailability of polyphenols [33] which can be converted to bioactive compounds by microbiota, modifying intestinal microbial balance and thus also the host's health.

This work focused on the effects of two by-products (OP and PM) on intestinal microbiota, using a gut simulator (Mucus-SHIME or M-SHIME®). A duplicate of a Triple M-SHIME® experiment was performed to investigate the effects of oral administration for several days. These effects were evaluated studying the polyphenols metabolic fate (HPLC/DAD/MS analysis), the microbial functionality (SCFAs and NH_4^+ levels) and the microbiota composition (PCR-DGGE on bacterial DNA).

The objective was to carry out a study from different point of view, considering the mutual influence between polyphenols and microbiota.

Material and methods

Food by-products

Two extracts were used in the study. Pomegranate (Wonderful cv) from Tuscan farming was selected; fruits were harvested in Grosseto (Tuscany). Mesocarp was manually separated from the other parts, then a 1h decoption was applied. Suspension was freeze-dried, grounded and used as powder (PM).

The second derived from a water suspension of residual material from olive oil production, named olive "pâté" (OP), that includes wet pulp and husk. The liquid suspension was freeze-dried. Powder was defatted adding hexane and stirring for 1 h. The solvent was then recovered and powder was dried.

M-SHIME® experiments

Two Triple M-SHIME® models, inoculated with faecal samples of the same healthy donor, were performed in different times. The M-SHIME® contains, in addition to traditional luminal microbial community [34], some mucin

microcosms to host surface-attached microbes [35]. These microcosms (K1-carrier, AnoxKaldnes AB, Lund, Sweden) are submerged in mucin-agar. The feed selected included (g/L): arabinogalactan (1), pectin (2), xylan (1), D-(+)- glucose (0.4), starch (4), yeast extract (3.0), peptone (1.0), and pig gastric mucin (4.0). At the start of each experiment, 500 mL of selected feed and 80 mucin-covered microcosms were added to proximal colon (PC) unit, while the distal colon (DC) unit was filled with 800 mL and the same amount of mucin. Subsequently, the inoculation was performed with 40 mL of a 1:5 dilution of fresh stools of a healthy human volunteer [36]. Three couples of colon vessels ran simultaneously, one for control, the other two for OP and PM treatment [Figure 1]. After an initial incubation of 18h for pH stabilisation, 140 mL nutritional medium and 60 mL pancreatic juice were supplied to each proximal colon compartment three times per day. The M-SHIME® was kept at 37 °C and anaerobic condition by flushing for 10 min with N_2 every day.

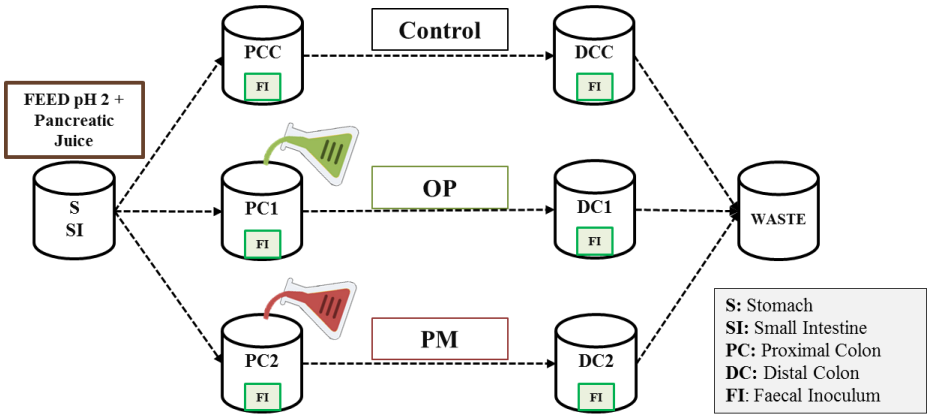


Figure 1 – M-SHIME® design

After a stabilisation period of 2 weeks, daily doses of the extract were administrated in the PC vessel for 10 days. At the end of the treatment, a 4 days washout was carried out. Experiments were performed with different extract dosages, 4 g/L of OP and 2 g/L of PM. Three times per week, at the same time of the day, 20 mL of liquid sample were collected from each colon vessel [Figure 2]. 1 mL was centrifuged and the pellet was stored at -20°C for DNA extraction. Residual liquid was stored at -20°C for metabolic analyses.

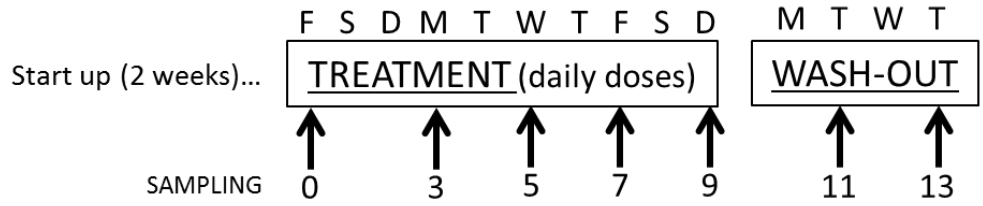


Figure 2 – M-SHIME® experiment

HPLC/DAD/MS analysis

Samples from proximal control vessels were added with fresh extracts at the same concentrations used for experiments, to evaluate starting point profiles (PCC_OLIVE and PCC_POME) for HPLC/DAD/MS analysis. These samples were then compared with proximal and distal samples recovered during treatment with extracts (PC1d9, DC1d9, PC2d9 and DC2d9). Samples of vessels content collected before the administration (PC1d0, DC1d0, PC2d0 and DC2d0) were also analysed to identify any possible interferences due to feed components.

Samples were pre-treated in order to obtain a clear solution: 2 mL of luminal liquid from each sample were added with 0.5 mL of MeOH/Acetonitrile/Acetone in ratio 1/1/1, then mixed with Vortex and kept at room temperature for 25 min as previously described [37]. Samples from vessels administrated with PM received the same treatment, but original supernatant was also acidified with HCOOH. The suspension was then centrifuged at 4°C, 5000 rpm, 10 min. 1 mL of supernatant was recovered and dried using N_2 gas. The dried residue was dissolved in 200 µL of EtOH/H_3O^+ 70/30. Final ultracentrifugation (10 min, 14000 rpm) was applied to all samples before HPLC/DAD/MS analysis.

Qualitative analysis were performed using a HP 1100 liquid chromatography (Agilent Technologies, USA) coupled with HP 6200 series MS/TOF.

For OP treated samples a 150mm×3 mm i.d., 2.7 µm, RP-18, Poroshell column (Agilent Technologies, USA) was used. Eluents selected were (A) H_2O at pH 3.2 by formic acid and (B) CH_3CN. The multi-step linear solvent gradient used was: 0-40 min 5-40% B; 40–45 min, 40% B; 45–50 min 40–100% B; 50-53 min 100%;

53-55 min 100-5%, equilibration time 10 min; flow rate 0.4 mL/min. The UV-Vis spectra were recorded in the range 200–600 nm and the chromatograms were acquired at 240 nm, 280 nm, 330 nm, 350 nm. MS spectra were acquired using Dual-ESI source in negative polarity with 80 V fragmentor, 3800 V Capillary Voltage, 350°C of Gas Temperature.

PM treated samples were analysed using a 150mm×2 mm i.d., 4μm, RP-18, Sinergi Fusion column (Phenomenex, USA). Same eluents used for OP were. The multi-step linear solvent gradient used was: 0-4 min 5-25% B; 4–8 min, 25–25% B; 8–14 min 25–35% B; 14-16 min 35-90%; 16-18 min 90-5%, equilibration time 10 min; flow rate 0.4 mL/min. The UV-Vis spectra were recorded in the range 200–500 nm and the chromatograms were acquired at 240 nm, 280 nm, 330 nm, 370 nm, 380 nm. MS spectra were acquired using Dual-ESI source in negative polarity with 100 fragmentor, 4000 V Capillary Voltage, 350°C of Gas Temperature.

Metabolite measurement

Solvents and standards with analytical purity were purchased from SIGMA Aldrich. For SCFA analysis, a liquid-liquid extraction with diethyl ether was applied on frozen samples, after the addiction of H_2SO_4 and internal standard. SCFA quantitative analysis were performed by capillary gas chromatography coupled with a flame ionization detector (GC-FID), to measure acetic, propionic, butyric, isobutyric, caproic, isocaproic, valeric and isovaleric acids, as previously described [38].

NH_4^+ level was analysed by steam distillation according to Standard methods (4500-NH_3 B; APHA, 1992). Determination of total ammoniacal nitrogen (TAN) in liquid luminal samples was performed through the quantification of NH_4^+ by the addition of MgO, distillation of NH_3 into boric acid solution and subsequent back-titration.

DNA extraction

Bacterial DNA from luminal samples was extracted as described earlier [39], using a Lysis Buffer (TrisEDTA, NaCl, PVP40, SDS, water) and glass beads for FastPrep. Extraction was performed with phenol-chloroform and EtOH/NaOAc was used for precipitation [40]. Samples were dissolved in TrisEDTA 1X and stored at -20°C. Concentration and quality were verified by Glomax Multi Detection system (Promega, USA) and 2% agarose gel electrophoresis.

PCR-denaturing gradient gel electrophoresis (DGGE)

In order to investigate potential composition changes in the microbial community a PCR-DGGE was assessed as seen previously [36]. PCR amplification for total bacteria of 16S rRNA gene was performed with primers 338F-GC and 518R and cycling conditions were: : initial denaturation 94 °C for 5 minutes; 30 cycles at 95°C for 1 minute, 53°C for 1 minute; 72°C for 2 minutes and a final extension at 72°C for 10 min [41].

PCR products were separated on denaturant gradient of polyacrylamide gel. DGGE (Denaturing Gradient Gel Electrophoresis) was performed [42] using the INGENY System (Ingeny International BV, The Netherlands). PCR fragments were loaded onto 8% polyacrylamide gels in 1´TAE buffer (Tris 1 acetate, EDTA pH 7.4). Polyacrylamide gels were made with 45-60% denaturing gradients. Electrophoresis was run for 16 hours at 60°C and 120 V. Staining and analysis of the gels were performed as described previously [43].

The normalization and analysis of DGGE gel patterns was done with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). In the process the different lanes were defined, background was subtracted, differences in the intensity of the lanes were compensated during normalization and band classes were detected.

Results

OP and PM composition

Quantitative analysis were preliminary performed on PM and OP to evaluate the total content of polyphenols and fiber. Results are reported in Table 1.

Polyphenols content of OP was evaluated in term of tyrosol, oleuropein and caffeic acid derivatives. Oleuropein derivatives resulted the most abundant, with a total concentration of 77.77 mg/g. Other polyphenols were characterized in OP, in particular hydroxytyrosol, verbascoside and luteolin. Total fiber content was defined according to standard methods for fiber determination and declared in label from the producer.

The ellagitannins content was calculated in terms of α + β punicalagins, ellagic acid and its glycosides. α + β punicalagins resulted the main compounds (70.7 mg/g), but ellagic acid was also well represented in the extract, with a concentration of 3.67 mg/g. Polysaccharides content was obtained adding two volumes of EtOH to original decoction and keeping the solution at 0°C for 3h, inducing polysaccharides precipitation [44].

Products	Compounds	Concentration (mg/g)	Fiber/Polysaccharides content
OP	Verbascoside	4.52	Total fiber 24%
	Hydroxytyrosol	15.26	
	Oleuropein derivatives	77.77	
	Luteolin	0.42	
	Total polyphenols	97.55	
PM	α+β punicalagins	70.7	Polysaccharides 12%
	Ellagic acid and derivatives	10.70	
	Total ellagitannins	120.2	

Table 1 Content of polyphenols and fiber in OP and PM

Polyphenols metabolic fate

Metabolic fate of main phenolic compounds was investigated by HPLC/DAD/MS analysis for both OP and PM. Samples collected from PC and DC at different time, a blank and a control with a fresh addition of extract from both experiments were treated in order to purify the solution for the analysis. Soluble proteins were precipitated and the clean supernatant was recovered and used for HPLC/DAD/MS analysis, aimed to point out any differences among samples profiles.

According to chemical structure of the phenolic compounds in OP, two wavelength were selected to evaluate the metabolism of main components: 280 nm (for tyrosol, hydroxytyrosol and derivatives) and 330 nm (for verbascoside and derivatives). According to Figure 3, the presence of hydroxytyrosol was well-defined in control samples and identity was confirmed by MS spectra with [M-H^+] =153.16 m/z. After the treatment, the relative intensity of hydroxytyrosol strongly decreased and a new signal appeared in both PC and DC samples. m/z value of the new signal was 137.16, corresponding to tyrosol. The identity was also confirmed comparing UV spectra and rt with a pure standard [Figure 4].

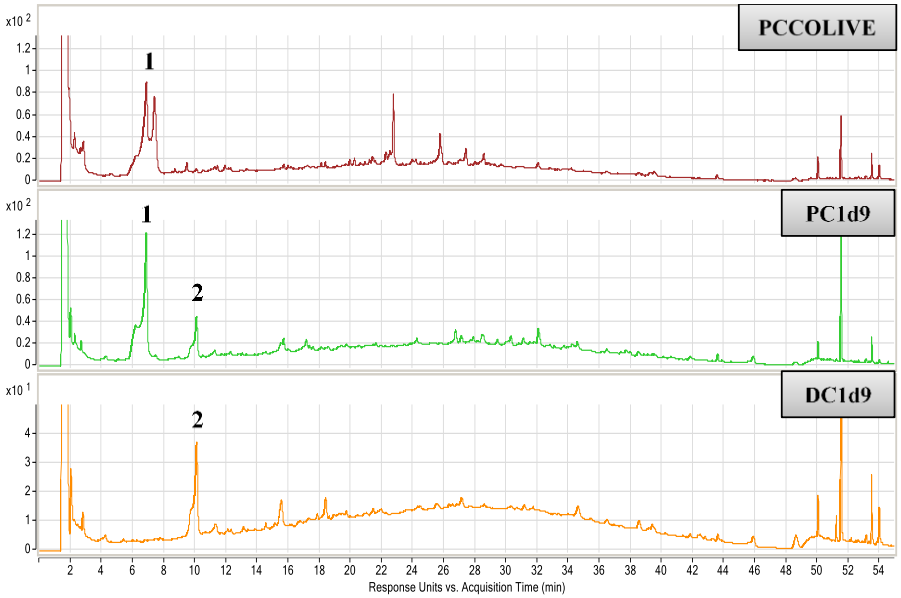


Figure 3 – Chromatograms at 280 nm of control added with fresh extract (PCCOLIVE), and samples after treatment (PC1d9 and DC1d9). Two main compounds were detected: hydroxytyrosol (1) and tyrosol (2).

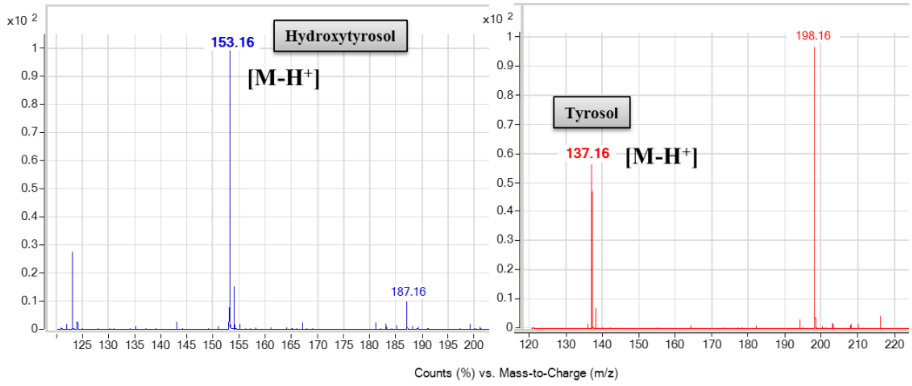


Figure 4 – MS spectra of peaks detected in profiles at 280 nm showed in Fig.3

Verbascoside, a typical phenyl propanoid of OP, was also metabolized. Despite the intensity of related signal in the control chromatogram, it disappeared in treated samples profiles and DAD did not record new peaks [Figure 5]. To investigate the metabolism of verbascoside, the structure of the molecule was studied to guess possible derivatives, taking into account the fragmentation shown

in Figure 6. The exact mass of derivatives was calculated. Applying Extract Ion processing, two main compound were identified: caffeic acid (179.15 m/z) and tyrosol (137.16 m/z) were found in treated samples [Figure 7].

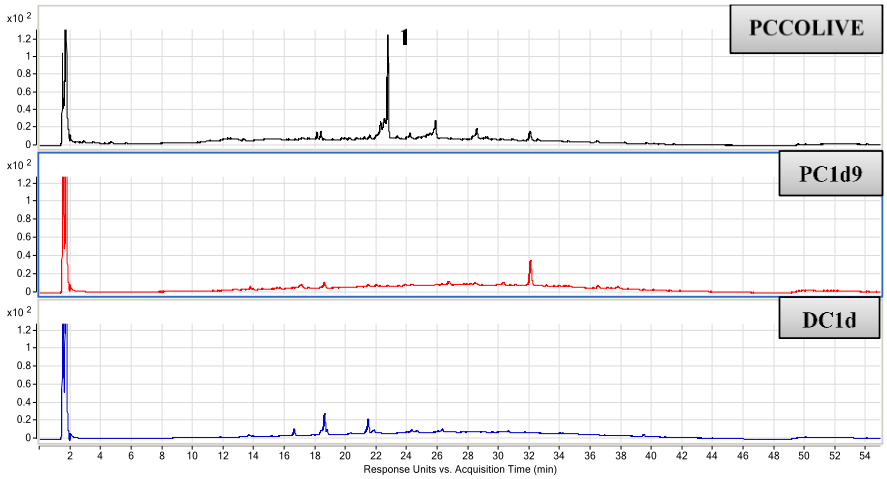


Figure 5- Chromatograms at 330 nm of the control added with fresh OP (PCCOLIVE), and samples after treatment (PC1d9 and DC1d9). Verbascoside (1) was identified in control sample, but not in other profiles.

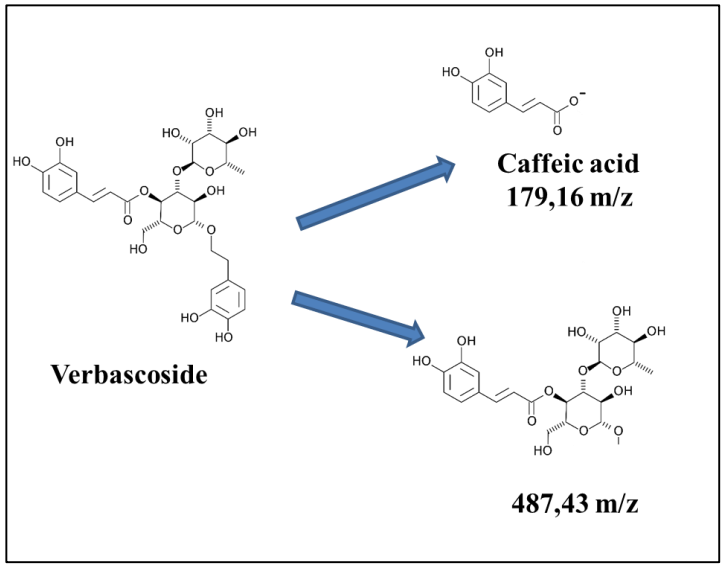


Figure 6 – Possible fragmentations guessed for verbascoside

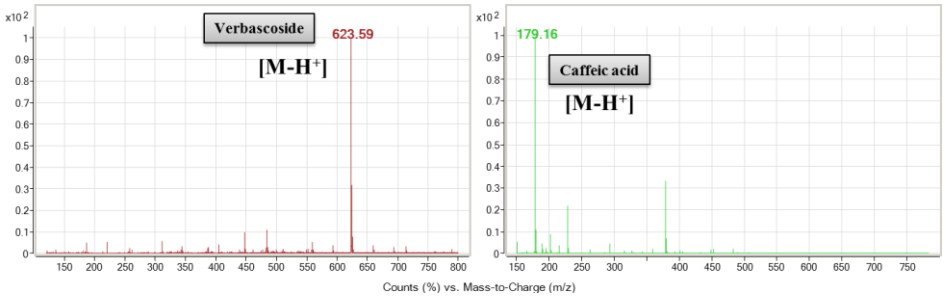


Figure 7 - MS spectra of peaks identified as verbascoside and caffeic acid.

The same approach was applied to the PM samples, focusing on main compounds included in the extract, α and β punicalagins and ellagic acid. The wavelengths selected to investigate these molecules were 380 nm and 370nm, respectively, according to their maximum absorption wavelengths.

On the other hand, punicalagins were not detected, probably due to their ability of arranging insoluble complexes with soluble protein, highly included in liquid luminal suspension. After the treatment, PC chromatogram appeared similar to the control one. The same signals were recorded; in particular, ellagic acid was still well defined and there was no clue of the presence of punilagins in the solution [Figure 8]. The presence of ellagic acid in control was confirmed by UV and MS spectra ([M-H^+ =301 m/z) [Figure 9].

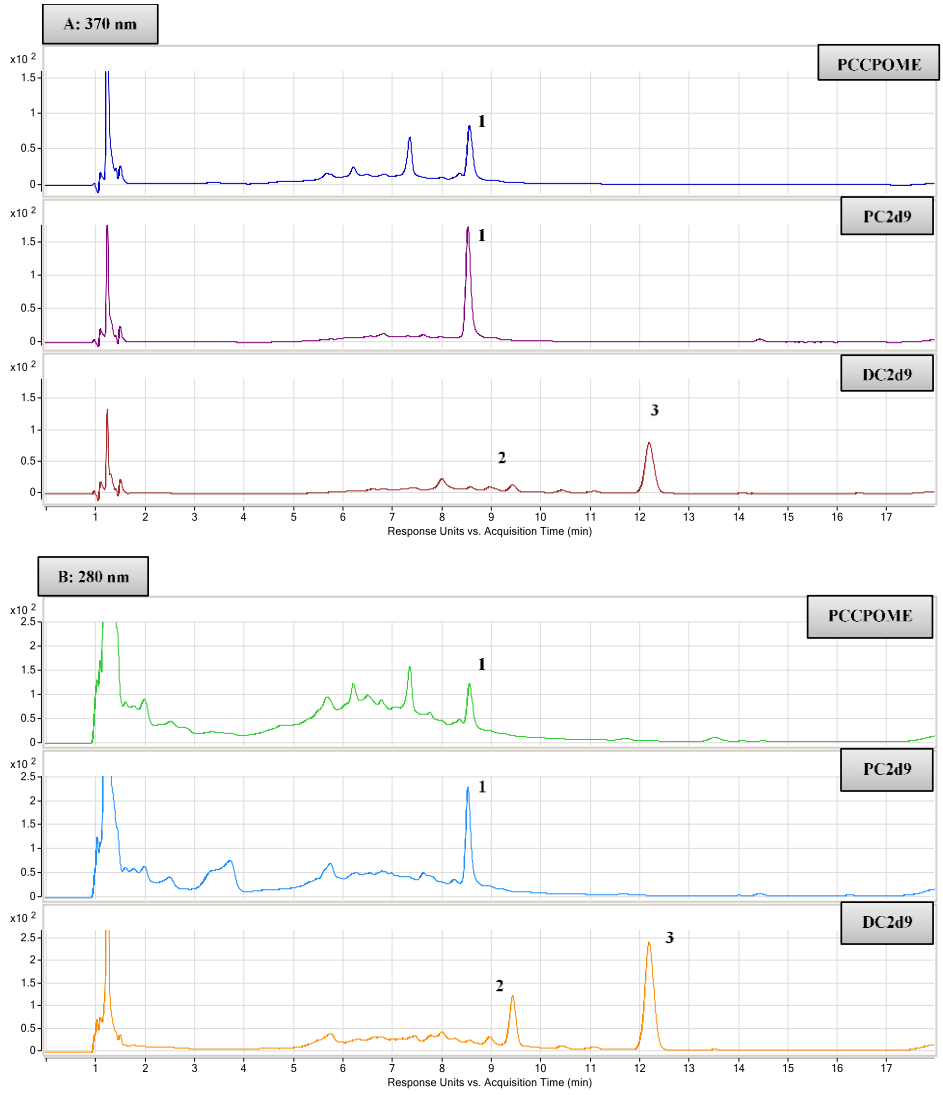


Figure 8 - Chromatograms at 370 (A) and 280 (B) nm of control added with fresh PM (PCCPOME), and samples after treatment (PC2d9 and DC2d9). Ellagic acid (1) was identified in PCCPOME and PC2d9, but not in DC2d9 profile. Urolithin A (2) and Urolithin C (3) were detected in DC2d9.

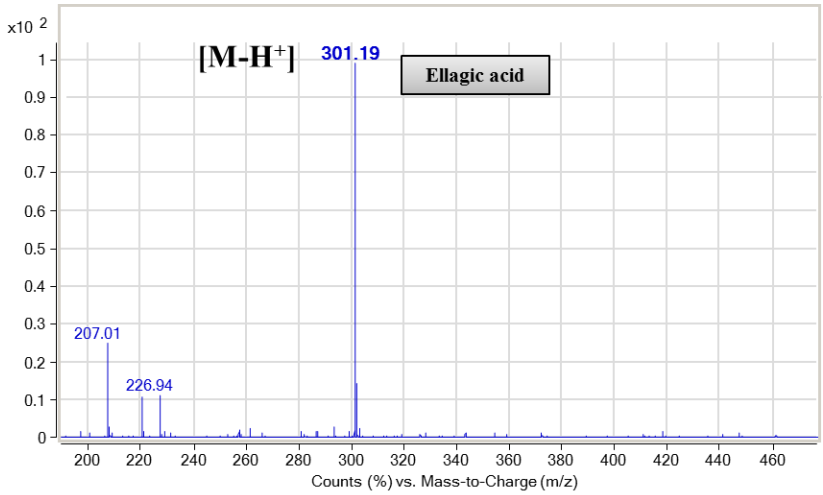


Figure 9 – MS spectra of peak identified as ellagic acid.

Treated samples from DC showed a different profile. The signal of ellagic acid disappeared, while two new signals were recorded at 280 nm [Figure 8], with corresponding m/z values of 227.15 and 243.03 [Figure 10]. The molecules were identified as Urolithin A and Urolithin C [Figure 11]. These compounds are well-known metabolites of ellagic acid, and it is very interesting how this metabolism worked only in the distal colon vessels.

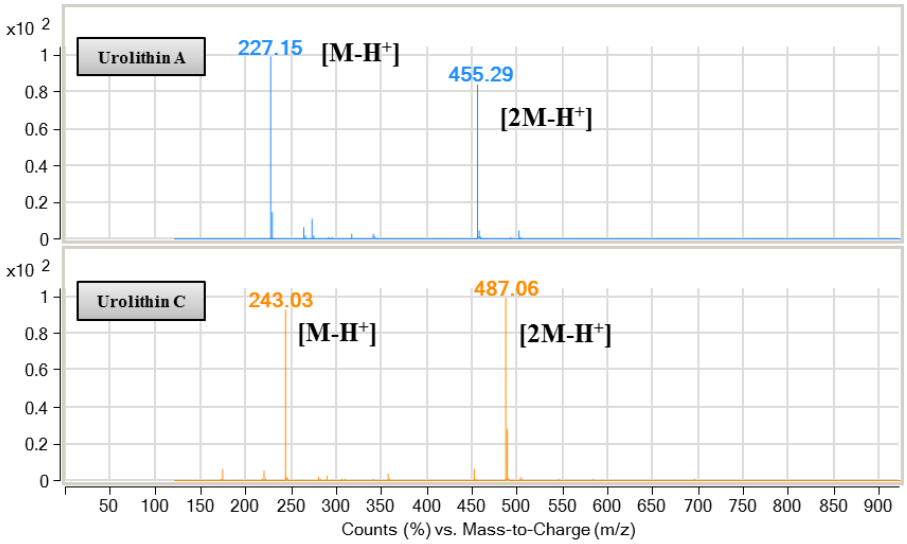


Figure 10 - MS spectra of Urolithin A (227 m/z) and Urolithin C (243 m/z)

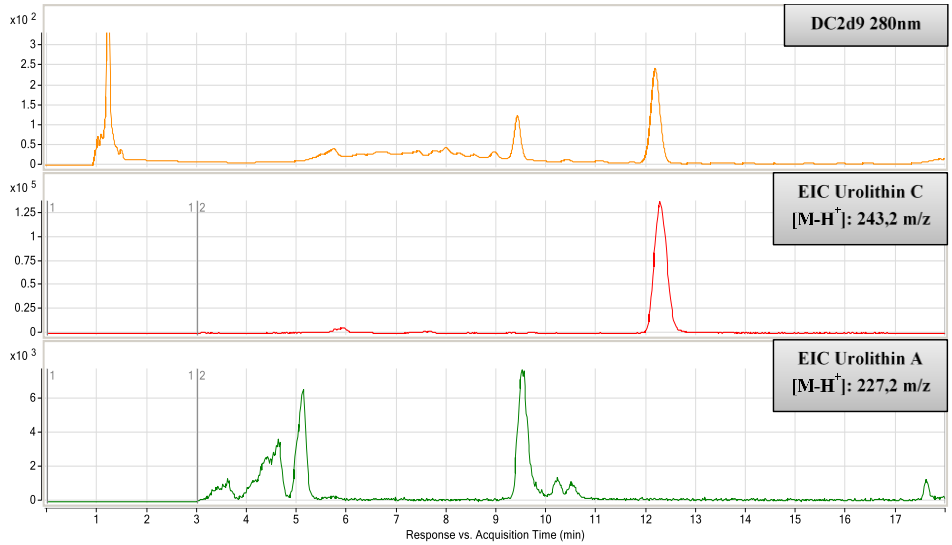


Figure 11 – Chromatogram at 280 nm and Extrac Ion profiles at [M-H$^+$]: 243.03 m/z for Urolithin C and at [M-H$^+$]: 227.15 m/z for Urolithin A from sample DC2d9.

Polyphenols effects on microbial metabolism : SCFA and NH_4^+

Metabolic activity of bacteria was investigated through quantitative analysis of SCFA and NH_4^+ concentration. Results from the two experiments were processed as average and resulting charts are shown in Figure 12 and 13.

SCFA average levels showed a quite regular trend in control vessels, except for the intrinsic variability of the system. Concentrations of DC samples were higher than PC ones, but the evolution was consistent. Similar behaviour was showed for NH_4^+ concentration in both DC and PC vessels. The system resulted quite stable with a weak variability.

Samples from vessels treated with OP showed a trend comparable with control, in both proximal and distal vessels. No significant changes were recorded, even when the treatment was interrupted and after a 4 days washout. NH_4^+ concentrations were also linear during and after the treatment.

In PC vessels treated with PM, a weak increase in SCFA levels was observed, with a decrease after administration interruption and a new increase - close to starting values - after washout. In the DC vessel the trend was less stable, with a

weak but not significant decrease of concentrations. NH_4^+ concentrations were on the other hand more regular during the treatment and the washout.

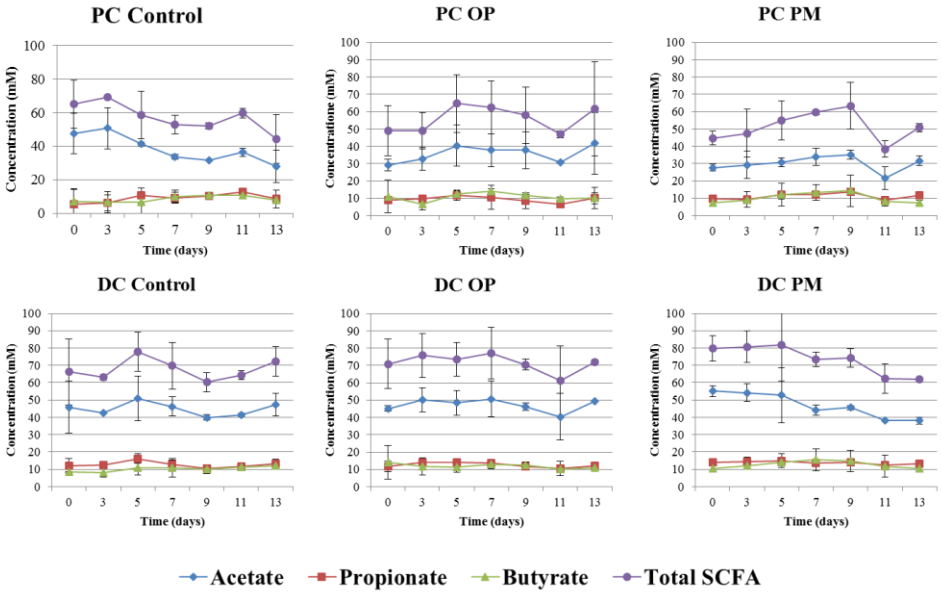


Figure 12 – SCFA levels during and after treatment in PC and DC from control, PO and PM treated vessels.

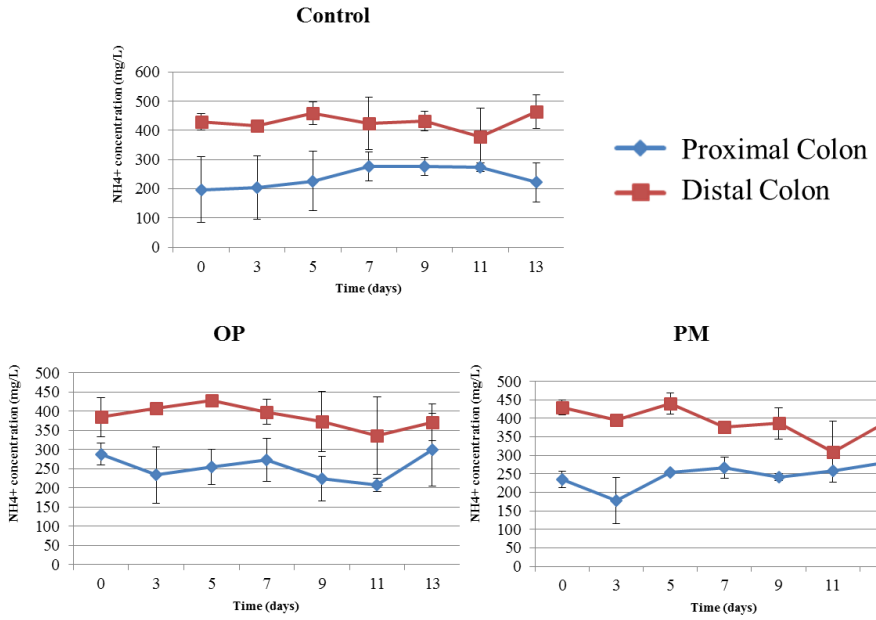


Figure 13 – NH_4^+ levels during and after treatment in PC and DC from control, PO and PM treated vessels.

Polyphenols effects on microbial composition: PCR-DGGE measurement

Different time point samples from the two experiments were selected to investigate the effects on the microbial community. PCR-DGGE for total bacteria was performed on samples collected before starting the treatment (Day 0), during and end of treatment (Day 5-9) and after washout (Day 13).

DGGE pictures were processed with Bionumerics software in order to identify patterns and define a clusterization, as shown in Figure 14.

Samples from control and vessels treated with OP formed a quite clear clusterization. Despite this, clusters showed high correlation values, meaning that no big differences were highlighted among samples.

Referring to PM, clusterization was not well defined. Even in this case high correlation values were calculated and no significant changes in microbial community were stressed out.

These results pointed out to a very selective and limited effect to low abundant microorganisms of the extracts on the microbiota composition, due probably to low concentration of the main phenolic components in these samples.

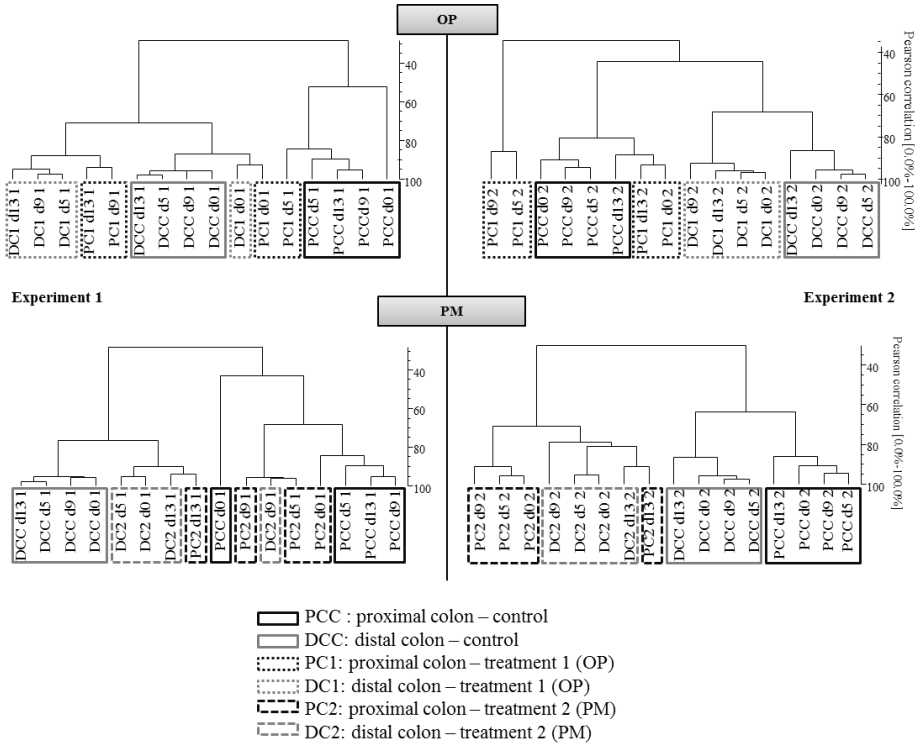


Figure 14 – Clusterization of DGGE patterns from starting point (d0), during and end of treatment (d5, d9), after washout (d13). For each experiment, treated samples were compare with control.

Discussion

The experiments performed to investigate mutual effects of microbial community and polyphenols took into account different parameters, to obtain more complete information.

The metabolic fate of polyphenols was the main aspect investigated in this study: secoiridoids and phenyl propanoids presence was evaluated for OP, while the ellagitannins were studied for PM samples. It is important to understand how these molecules should affect the metabolism they usually undergo depending on microbiota, since these transformations are a clear clue of microbial wellness. At the same time, it is needed to evaluate which metabolites are produced. It is

established that, at colon level, there is the maximum absorption of phenolic compounds and their derivatives [25,26], but not all metabolites maintain the same positive effects of the original molecules. OP content was first evaluated, identifying two main molecules, hydroxytyrosol and verbascoside. These compounds own positive effects, like anti-oxidant, anti-inflammatory, antimicrobic, anti-atherosclerotic, anti-hypertensive effects [45–52]. Therefore, a complete metabolism was detected in PC and DC, demonstrating the normal activity of microbiota. Moreover, the molecules identified as derivatives were tyrosol and caffeic acid, that also showed many beneficial properties, such as antioxidant, anti-inflammatory, anti-atherogenic and chemoprotective [53–55]. Referring to PM, the first result was the absence of punicalagins, the main components of pomegranate juice and mesocarp. In literature the capability of these compounds to complex soluble protein and produce precipitate is already reported [56]. According to this property, it is possible then to assume that they are involved in this reaction, due to high protein content in the feed selected for the experiments. Ellagic acid turned out to be the main component in control sample, and its metabolites were not detected in PC, but were identified in DC, where new signals appeared and urolithinsA and C were identified. Even in this case, the obtained result is consistent with previous *in vivo* studies that showed the metabolic path of ellagic acid to urolithins in the distal tract of intestinal system [30,31]. These resulted confirmed also the ability of SHIME® system to well simulate the human metabolism of specific intestinal regions.

Microbial functionality was then investigated. Since polyphenols could affect various bacterial functions, levels of SCFA, derived from sugars fermentation, and NH_4^+, proteolysis index, are usually quantified to evaluate the activity of microbial community. In duplicated experiments, both materials did not show any significant effects on normal levels. Compared to PC and DC from control, samples administrated with OP and PM showed a regular trend of SCFA and NH_4^+, with concentrations comparable with those of control. Few variations were recorded, but they were too weak to be considered significant. For this reason, it can be assessed that the selected products do not affect microbial functionality.

In addition to previous results, also a PCR-DGGE for total bacteria and relative data processing through Bionumerics were performed to obtain qualitative information on microbial composition. High correlation values calculated for comparison among control and treated samples, demonstrated that the overall composition did not change significantly after the treatment, maintaining similar pattern and distribution. These results confirmed that the low concentrations of phenolic compounds in selected by-products are not able to affect and modulate the microbial community, which maintains its composition during continuous daily administration.

Conclusions

In this study we demonstrated that the use of by-product derived from olive and pomegranate, containing medium amount of polyphenols, did not provide any significant change in colon microbial community and functionality. The products did not provide positive effects in addition to a normal diet, but at the same time no negative impact was detected. According to these results, OP and PM resulted as good candidates to be included in food supplements or to be used as excipients in nutraceutical formulations. They can be considered a vehicle for a further intake of polyphenols and beneficial metabolites, associated with fiber and polysaccharides which could provide also prebiotics effects.

Further investigation are necessary to understand the real bioavailability of main compounds, in order to exert positive effects at systemic level.

References

[1] Gustavsson J, Cederberg C, Sonesson U. Global food losses and food waste. 2011.

[2] Status of FAO's Work on Post-Harvest Losses 2012.

[3] Girotto F, Alibardi L, Cossu R. Food waste generation and industrial uses: A review. Waste Manag 2015. doi:10.1016/j.wasman.2015.06.008.

[4] Galanakis CM. Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. Trends Food Sci Technol 2012;26:68–87. doi:10.1016/j.tifs.2012.03.003.

[5] Akdemir EO, Ozer A. Application of a statistical technique for olive oil mill wastewater treatment using ultrafiltration process. Sep Purif Technol 2008;62:222–7. doi:10.1016/j.seppur.2008.01.006.

[6] Rozzi A, Malpei F. Treatment and disposal of olive mill effluents. Int Biodeterior Biodegradation 1996;38:135–44. doi:10.1016/S0964-8305(96)00042-X.

[7] Mantzavinos D, Kalogerakis N. Treatment of olive mill effluents Part I. Organic matter degradation by chemical and biological processes--an overview. Environ Int 2005;31:289–95. doi:10.1016/j.envint.2004.10.005.

[8] Fountoulakis M., Dokianakis S., Kornaros M., Aggelis G., Lyberatos G. Removal of phenolics in olive mill wastewaters using the white-rot fungus Pleurotus ostreatus. Water Res 2002;36:4735–44. doi:10.1016/S0043-1354(02)00184-7.

[9] Marques IP. Anaerobic digestion treatment of olive mill wastewater for effluent re-use in irrigation. Desalination 2001;137:233–9. doi:10.1016/S0011-9164(01)00224-7.

[10] Beltran de Heredia J, Garcia J. Process integration: continuous anaerobic digestion−ozonation treatment of olive mill wastewater. Ind Eng Chem Res 2005;44:8750–5. doi:10.1021/ie040232x.

[11] Cegarra J, Paredes C, Roig A, Bernal MP, García D. Use of olive mill wastewater compost for crop production. Int Biodeterior Biodegradation

1996;38:193–203. doi:10.1016/S0964-8305(96)00051-0.

[12] Caputo AC, Scacchia F, Pelagagge PM. Disposal of by-products in olive oil industry: waste-to-energy solutions. Appl Therm Eng 2003;23:197–214. doi:10.1016/S1359-4311(02)00173-4.

[13] Panayotis SR, Karathano VT, Mantzavinou A. Partitioning of Olive Oil Antioxidants between Oil and Water Phases. J Agric Food Chem 2002;50:596–601.

[14] Agalias A, Magiatis P, Skaltsounis A-L, Mikros E, Tsarbopoulos A, Gikas E, et al. A new process for the management of olive oil mill waste water and recovery of natural antioxidants. J Agric Food Chem 2007;55:2671–6. doi:10.1021/jf063091d.

[15] Sabatini N. Recent patents in olive oil industry: New technologies for the recovery of phenols compounds from olive oil, olive oil industrial by-products and waste waters. Recent Pat Food Nutr Agric 2010;2:154–9.

[16] El-Abbassi A, Khayet M, Hafidi A. Micellar enhanced ultrafiltration process for the treatment of olive mill wastewater. Water Res 2011;45:4522–30. doi:10.1016/j.watres.2011.05.044.

[17] Frankel E, Bakhouche A, Lozano-Sánchez J, Segura-Carretero A, Fernández-Gutiérrez A. Literature review on production process to obtain extra virgin olive oil enriched in bioactive compounds. Potential use of byproducts as alternative sources of polyphenols. J Agric Food Chem 2013;61:5179–88. doi:10.1021/jf400806z.

[18] Singh RP, Chidambara Murthy KN, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. J Agric Food Chem 2002;50:81–6. doi:10.1021/jf010865b.

[19] Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem 2006;96:254–60. doi:10.1016/j.foodchem.2005.02.033.

[20] Al-Said F a., Opara LU, Al-Yahyai R a. Physico-chemical and textural

quality attributes of pomegranate cultivars (*Punica granatum L.*) grown in the Sultanate of Oman. J Food Eng 2009;90:129–34. doi:10.1016/j.jfoodeng.2008.06.012.

[21] Levin GM. Pomegranate Roads: a soviet botanist's exile from eden. Pomegranate roads; 2006.

[22] Kathib M. Bioactive compounds into edible syrian plants: pomegranate and capper. 2015.

[23] Goula AM, Adamopoulos KG. A method for preparing a novel solid product from olive mill wastewater: wastewater characterization and product recovery. Dry Technol 2013;31:339–49. doi:10.1080/07373937.2012.736114.

[24] Kalamara E, Goula AM, Adamopoulos KG. An integrated process for utilization of pomegranate wastes — Seeds. Innov Food Sci Emerg Technol 2015;27:144–53. doi:10.1016/j.ifset.2014.12.001.

[25] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81:230S – 242S.

[26] Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem 2013;24:1415–22. doi:10.1016/j.jnutbio.2013.05.001.

[27] Mosele JI, Martín-Peláez S, Macià A, Farràs M, Valls R-M, Catalán Ú, et al. Faecal microbial metabolism of olive oil phenolic compounds: in vitro and in vivo approaches. Mol Nutr Food Res 2014;58:1809–19. doi:10.1002/mnfr.201400124.

[28] Corona G, Tzounis X, DessÌ MA, Deiana M, Debnam ES, Visioli F, et al. The fate of olive oil polyphenols in the gastrointestinal tract: Implications of gastric and colonic microflora-dependent biotransformation. Free Radic Res 2009.

[29] Lin P, Qian W, Wang X, Cao L, Li S, Qian T. The biotransformation of oleuropein in rats. Biomed Chromatogr 2013;27:1162–7.

doi:10.1002/bmc.2922.

[30] Espín JC, González-Barrio R, Cerdá B, López-Bote C, Rey AI, Tomás-Barberán FA. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. J Agric Food Chem 2007;55:10476–85. doi:10.1021/jf0723864.

[31] Larrosa M, García-Conesa MT, Espín JC, Tomás-Barberán FA. Ellagitannins, ellagic acid and vascular health. Mol Aspects Med 2010;31:513–39. doi:10.1016/j.mam.2010.09.005.

[32] Hervert-Hernández D, Goñi I. Dietary Polyphenols and Human Gut Microbiota: a Review. Food Rev Int 2011;27:154–69. doi:10.1080/87559129.2010.535233.

[33] Hervert-Hernández D, Pintado C, Rotger R, Goñi I. Stimulatory role of grape pomace polyphenols on Lactobacillus acidophilus growth. Int J Food Microbiol 2009;136:119–22. doi:10.1016/j.ijfoodmicro.2009.09.016.

[34] Van Den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gérard P, et al. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for bacteroidetes and Clostridium cluster IX. Appl Environ Microbiol 2010;76:5237–46. doi:10.1128/AEM.00759-10.

[35] Van den Abbeele P, Roos S, Eeckhaut V, Mackenzie D a., Derde M, Verstraete W, et al. Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. Microb Biotechnol 2012;5:106–15. doi:10.1111/j.1751-7915.2011.00308.x.

[36] Possemiers S, Verth K, Uyttendaele S, Verstraete W. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 2004;49:495–507. doi:10.1016/j.femsec.2004.05.002.

[37] Crowe KM. Optimizing protein precipitation efficiency for assessing the contribution of low molecular weight compounds to serum antioxidant capacity. Clin Biochem 2014;47:116–8. doi:10.1016/j.clinbiochem.2014.06.021.

[38] Greenberg AE, Clesceri LS, Eaton AD. Standard methods for the examination of water and wastewater 18th edition. 1992.

[39] Vilchez-Vargas R, Geffers R, Suárez-Diez M, Conte I, Waliczek A, Kaser VS, et al. Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system. Environ Microbiol 2013;15:1016–39. doi:10.1111/j.1462-2920.2012.02752.x.

[40] Boon N, Top EM, Verstraete W, Siciliano SD. Bioaugmentation as a tool to protect the structure and function of an activated-sludge microbial community against a 3-chloroaniline shock load. Appl Environ Microbiol 2003;69:1511–20.

[41] Ovreås L, Forney L, Daae FL, Torsvik V. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 1997;63:3367–73.

[42] Muyzer G, De Waal EC, Uitterlinden a. G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993;59:695–700.

[43] Boon N, Goris J, Vos P De, Verstraete W, Top EM. Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading Comamonas testosteroni Strain , I2 gfp. Appl Environ Microbiol 2000;66:2906–13. doi:10.1128/AEM.66.7.2906-2913.2000.Updated.

[44] Kathib M, Innocenti M, Giuliani C, Al-Tamimi A, Rossi F, Adessi A, et al. Characterization of polysaccharides from the Wonderful and Laffan pomegranate fruit cultivars. Carbohydrates Res n.d.:Submitted.

[45] Manna C, Galletti P, Cucciolla V, Montedoro G, Zappia V. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. J Nutr Biochem 1999;10:159–65.

[46] de la Puerta R, Gutierrez VR, Hoult JRS. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. Biochem Pharmacol 1999;57:445–9. doi:10.1016/S0006-2952(98)00320-7.

[47] Petroni A, Blasevich M, Papini N, Salami M, Sala A, Galli C. Inhibition of leukocyte leukotriene b4 production by an olive oil-derived phenol identified by Mass-Spectrometry. Thromb Res 1997;87:315–22. doi:10.1016/S0049-3848(97)00133-3.

[48] Bisignano G, Tomaino A, Lo Cascio R, Crisafi G, Uccella N, Saija A. On the *in-vitro* antimicrobial activity of oleuropein and hydroxytyrosol. J Pharm Pharmacol 1999;51:971–4.

[49] Capasso R, Evidente A, Schivo L, Orru G, Marcialis MA, Cristinzio G. Antibacterial polyphenols from olive oil mill waste waters. J Appl Bacteriol 1995;79:393–8.

[50] Liu M-J, Li J-X, Guo H-Z, Lee K-M, Qin L, Chan K-M. The effects of verbascoside on plasma lipid peroxidation level and erythrocyte membrane fluidity during immobilization in rabbits: a time course study. Life Sci 2003;73:883–92.

[51] Díaz AM, Abad MJ, Fernández L, Silván AM, De Santos J, Bermejo P. Phenylpropanoid glycosides from *Scrophularia scorodonia*: In vitro anti-inflammatory activity. Life Sci 2004;74:2515–26. doi:10.1016/j.lfs.2003.10.008.

[52] Kang DG, Lee YS, Kim HJ, Lee YM, Lee HS. Angiotensin converting enzyme inhibitory phenylpropanoid glycosides from *Clerodendron trichotomum*. J Ethnopharmacol 2003;89:151–4. doi:10.1016/S0378-8741(03)00274-5.

[53] Nardini M, Pisu P, Gentili V, Natella F, Di M, Piccolella F, et al. Effect of caffeic acid on tert-butyl hydroperoxide-induced oxidative stress in U937. Free Radic Biol Med 1998;25:1098–105. doi:10.1016/S0891-5849(98)00180-4.

[54] Meyer AS, Heinonen M, Frankel EN. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL

oxidation. Food Chem 1998;61:71–5. doi:10.1016/S0308-8146(97)00100-3.

[55] Quiles JL, Farquharson AJ, Simpson DK, Grant I, Wahle KWJ. Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. Br J Nutr 2007;88:225. doi:10.1079/BJN2002620.

[56] Quideau S, Deffieux D, Douat-Casassus C, Pouységu L. Plant polyphenols: chemical properties, biological activities, and synthesis. Angew Chem Int Ed Engl 2011;50:586–621. doi:10.1002/anie.201000044.

Final remarks

Food supplements with high polyphenols content are widely used. The properties of phenolic compounds were defined through many studies in *in vitro* and *in vivo* models. However, after oral administration these molecules can suffer many metabolism, in particular due to intestinal microbial communities (microbiota). Microbiota activity could be also influenced by phenolic compounds, which can interact with bacterial cells using many mechanisms.
In this work, we evaluated the mutual influence between polyphenols and intestinal microbiota, considering different aspects: polyphenols metabolic fate by HPLC/DAD and HPLC/MS-TOF, microbial functionality measuring Short Chain Fatty Acids (SCFA) and NH_4^+ levels, and microbiota composition by PCR-DGGE / Illumina sequencing. All studies were performed reproducing a continuous oral administration in the *in vitro* gastrointestinal simulator M-SHIME®.

Three main studies were conducted, using different botanicals, with various classes of phenolic compounds, and applying different experimental conditions.

The first study was performed with Vineatrol®, a commercial extract from grape-vine with high content of trans-resveratrol and other stilbenoids. The results obtained from the experiment showed that a daily administration of the extract provides negative effects on microbiota. The polyphenols metabolism, due to bacteria, works normally in the first 24h, but it seemed arrested with continuous dosages. The same evolution was observed for microbial functionality and microbial composition was also affected during the treatment. Daily intake of Vineatrol® led to a negative affection of intestinal balance. The result is very interesting, because to date, no significant studies were reported on the effects of repeated administrations of this extract.

The other two studies were performed on botanicals recovered from food by-products: an olive "pâté" from extra virgin olive oil production and a pomegranate mesocarp decoction.
Polyphenols content was determined in both materials and resulted lower than Vineatrol®. They were used for tests *in vitro*, simulating a daily intake of food supplements for 9 days.
Administration in addition of normal diets showed that the extracts did not provide significant changes to basic metabolism, functionality and microbial composition in colonic region. Moreover, polyphenols metabolization observed in the experiment was consistent with results from *in vivo* studies, confirming a normal activity of microbial communities. This result is interesting since these materials, recovered from food processing in large amount, could be used as ingredients of food, dietary supplements or nutraceutics, as delivery vehicle of polyphenols and fiber at colon level.

A second experiment was performed for administration of the same extracts, using different feed to investigate potential benefits of daily intake of these by-products also in extreme conditions. To this aim a sugar free diet with high protein content was selected
Positive effects were recorded for the administration with this feed highlighting an increase of SCFA levels. According to well-known biological properties of SCFA, it is possible to improve intestinal wellness and contrast any adverse consequence of this kind of diets, which usually leads to inflammatory states. Moreover, the high content of polyphenols, found in olive and pomegranate wastes, could help to ameliorate the overall conditions, due to the several beneficial activities recognized to phenolic compounds.

All performed experiments showed that it is important to consider many parameters.
First, the concentration of the extract is certainly a crucial aspect, since repeated doses could lead to negative effects on intestinal balance. Furthermore, the dietary

conditions are strongly related to potential activities of polyphenols and fiber included in vegetable extracts.

More information about bioavailability of phenolic compounds are still required to improve administration method and period.

Anyway, it was assessed that the in vitro system M-SHIME® can be a useful and explorative tool before experiments in human.

ANNEXES: Characterization of polysaccharides from the Wonderful and Laffan pomegranate fruit cultivars

Mohamad Khatib, Marzia Innocenti, Camilla Giuliani, [1]Amal Al-Tamimi, [2]Federico Rossi, [2]Alessandra Adessi, [3]Diana Di Gioia, [3]Giuseppe Mazzola *Nadia Mulinacci

Department of Neurofarba- Pharmaceutical and Nutraceutical Division, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, (FI) Italy.
[1]Biology Department of Ecology, Princess Noura University, Al Imam Abdullah Ibn Saud Ibn Abdul Aziz Road, Riyadh, Saudi Arabia.
[2]Department of the Sciences of Agrifood Production and the Environment, University of Florence, Piazzale delle Cascine, 18 - 50144 Firenze, Italy.
[3]Department of Agricultural Sciences , University of Bologna, Viale Fanin, 44 - 40127 Bologna, Italy.

Submitted to Carbohydrates Research

Abstract

Pomegranate (*Punica granatum* L.) fruit has been used extensively in the folk medicine of many cultures. This fruit is well known for its ellagitannin content but it also contains polysaccharides that have not yet been thoroughly investigated. Antioxidant and antiglycation activities have been reported for a polysaccharide of pomegranate peel and anticancer activity in mice for a galactomannan recovered from the fruit's rind. To date scant data have been published on the optimization of extracting the polysaccharides, the main by-products of juice production, from the mesocarp and exocarp. Furthermore, no data are available on the prebiotic properties of the polysaccharides recovered from pomegranate fruit. The aims of this study were to determine the amount of crude polysaccharides (CPS) in the mesocarp and exocarp of the Laffan and Wonderful pomegranate cultivars, to optimize the extraction process in terms of shorter time and higher yield of CPS, to determine the sugar composition and molecular shape of the main components, and to evaluate the prebiotic properties by *in vitro* testing on *Bifidobacterium* and *Lactobacillus* strains.

The maximum extractive yields in terms of crude polysaccharides (10% w/w dried matter (DM)) were achieved with a dried matter/solvent ratio of 1:40 (w/v) and through water decoction for 60 min. The two varieties showed similar profiles in terms of polysaccharides and similar amounts, with the highest concentration in the mesocarp. These CPS were able to absorb up to 99.1% of water-confirming their potential use as gelling agents in food chemistry.

To the best of the authors' knowledge, this is the first report demonstrating how CPS from the Laffan and Wonderful pomegranate mesocarp can sustain growth of beneficial and potentially probiotic bacteria such as those used in this study and, therefore, possess prebiotic properties.

Keywords: Extraction; Sugar Composition; Gelling Capacity; Prebiotic activity.

Introduction

Polysaccharides are a class of biological macromolecules with huge structural diversity that are relatively common in nature, and thus their biological properties have attracted substantial attention in medicine (Ooi & Liu, 2000; Sinha and Kumaria, 2001). Natural polysaccharides are known as thickeners, gelling agents, carriers of hydrophobic drugs and base products for preparation of nanoparticles and skin care products. They also are extensively used in the design of drug delivery systems, due to their excellent biocompatibility, aqueous solubility, and stability (Bhardwaj et al., 2000; Liu et al., 2008;Marathe, et al., 2002; Rubinstein, 2000; Deters, et al., 2001; Wang and Fang, 2004). Polysaccharides have proven to be useful candidates in the search for effective, non-toxic natural substances with pharmacological effects and represent a relatively untapped source for new drugs, which may provide novel therapeutic opportunities (Beat and Magnani, 2009). Several polysaccharides with *in vitro* antitumor effects have been isolated from plants, mushrooms, yeasts, algae, and lichens; most of them have been found to be non-toxic to normal cells and they are often able to enhance the immune system of the host (Mahady, 2001, Leung, et al. 2006) or to act as biological response modifiers. To date, several natural polysaccharides have been proven to exert antioxidant, antitumor, immunomodulatory, antimicrobial, antiulcer and hypoglycemic activities (Franz, 1989; Liu, et al., 1997; Kardošová and Machová, 2006; Schepetkin and Quinn, 2006; Ooi and Liu, 2000; Zhu et al. 2013; Xie et al.,2010).

Some polysaccharides introduced through the diet can have prebiotic activity, i.e. they can stimulate the growth of beneficial bacteria in the colon, thereby contributing to the healthy status of the gut (Marotti, et al. 2012; Di Gioia, et al. 2014b). The intestinal tract harbors a complex bacterial community which has a great impact on the nutritional and health status of the host (Laparra and Sanz, 2010). A balanced gut microbial composition confers benefits to the host, due to the modulation of metabolic and immune functions, while microbial imbalances are associated with metabolic disorders and/or disease (Tremaroli and Backhed, 2012; Di Gioia et al. 2014a). Therefore, the maintenance of a correct equilibrium

between beneficial microorganisms, mainly belonging to the *Bifidobacterium* and *Lactobacillus* genera, and potentially pathogenic strains, is crucial for host health. The presence of abundant *Bifidobacteria* and *Lactobacilli* may provide some protection against incoming enteric pathogens (Jankowska et al. 2008, Symonds et al. 2012). They are able to compete for nutrients with enteric pathogens, to adhere strongly to the intestinal mucosa, thus preventing pathogen adhesion and to stimulate the development of the mucosal immune system.

Pomegranate (*Punica granatum* L., family of Punicaceae) has been used extensively in the folk medicine of many cultures (Li et al., 2006) and has a wide range of potential clinical applications involving its antitumor, antibacterial, antidiarrheal, antifungal, and antiulcer properties (Valadares et al., 2010). Researchers have pointed out the main role played by ellagitannins as principal bioactive constituents of the different extracts obtained from the pomegranate fruit. In general, most of the studies on the pomegranate are characterized by a lack of attention toward the extraction and characterization of the polysaccharide fractions recovered from the different parts of the fruit. One study has reported that a polysaccharide from pomegranate peel shows significant antioxidant, antiglycation and tyrosinase inhibition properties (Rout and Banerjee 2007). A more recent study on galactomannan, a polysaccharide recovered from the fruit rind of *P. granatum*, shows that this molecule exerts cytotoxicity, immunomodulatory, and free radical scavenging activities in *in vitro* tests (Joseph et al., 2012), but also has anticancer activity in mice, enhancing survival and reducing the tumor either alone or in combination with doxorubicin (Sreelekha et al., 2008; Joseph et al., 2013). This study provides evidence of the non-toxic nature of this plant-derived compound, which could be used as an adjuvant or as a single agent for the treatment of cancer (Joseph et al., 2013). Other authors (Li et al., 2014) report that polysaccharides from pomegranate peel inhibit the proliferation of U-2 human osteosarcoma cancer cells by inducing apoptosis mainly through a mitochondrial signaling pathway.

Up to now, most of the reports have focused on pomegranate peel, which is reported to constitute 45-50% of the fruit's total weight. Nevertheless with this

term authors often mean the sum of the exocarp (the real peel) and mesocarp (Negi et al., 2003; Pan, et al., 2012; Saad et al., 2012; Al-Said et al., 2009; Levin, 2006) without making a real distinction between the two parts of the fruit. To date, the mesocarp and exocarp of the pomegranate, as the main by-products of juice production, have not been well investigated as a potential source of bioactive constituents, in particular polysaccharides, and no data are available on the possible prebiotic properties of these polymers.

The objectives of this study were to optimize the extraction process of polysaccharides from the mesocarp and exocarp of Laffan and Wonderful pomegranate cultivars , to determine sugar composition and the molecular shape of these polymers and to evaluate their prebiotic properties through *in vitro* testing on strains belonging to the *Bifidobacterium* and *Lactobacillus* genera.

Experimental Part

Materials

The Laffan cultivar (sour-sweet) was harvested from Rif Idlib-Syria in October 2011; the Wonderful cultivar was purchased from Ortofrutta Grosseto (Italy) in October 2013. About 5-7 kg of fresh ripe fruits of both cultivars were used as the source of exocarp and mesocarp to extract the polysaccharides.

Sample preparations and extraction processes

The exocarp and mesocarp were manually separated from fresh pomegranate fruits, then cut into small pieces and freeze-dried. To recover the polysaccharides, both parts were powdered in a grinder immediately before extraction, applying the methods described below. In this text, the term “dried matter” (DM) refers to the dried weight of the mesocarp and exocarp.

Hot water extractions were performed on coarse powdered material, which was boiled in distilled water under stirring for 30 or 60 min and by carrying out one or two successive extractive steps indicated in Table 1 (M-1 and M-2). After boiling,

each sample was cooled and centrifuged at 4500 g for 8 min at 4°C, in order to collect the supernatant containing the polysaccharides. The M-2t-p differs from M-2t only because of pre-treatment of the DM, to which water was added and then stirred at 25°C for 12 h before the hot water extraction.

Extractions were also performed (M-3r and M-3l) at room temperature, whereby the DM was soaked in distilled water (1 g DM/40 mL), then stirred for 24 h and filtered through Whatman filter paper to obtain the solution containing the polysaccharides. A modification of M-2s was made by reducing the water volume (1 g DM/25 mL) in the extractive ratio and obtaining M-4s-p. At the end a batch test was carried out applying a pretreatment before the decoction: 70% ethanol v/v was added to the powder (ratio 1 g DM/40 mL), the sample was stirred at room temperature for 24 h, then filtered through Whatman filter paper. The solid residue was treated with water (ratio 1 g DM/25) and a single decoction of 60 min was applied (M-5s-Et).

Each solution, obtained after centrifugation according to the methods summarized in Table 1 was added with 2 volumes of ethanol and kept 3 h at 0 °C, to induce the precipitation of the polysaccharides recovered after a centrifugation at 4500 rpm for 12 min at 5 °C.

Procedures to obtain crude polysaccharides (CPS) and purified polysaccharide (PPS).

Two different procedures were applied to remove part of the ellagitannins and to eliminate the proteins. All the polysaccharides shown in Tables 1 and 2 were washed with distilled water, than 2 volumes of ethanol were added and the samples kept 3 h at 0 ◦C. The precipitates were recovered after centrifugation (4500 rpm, 12 min at 5 °C), then frozen and freeze-dried to obtain the crude polysaccharides (CPS). Total recovery has been expressed as % yields of CPS on DM. The proteins were removed according to Joseph et al., (2012). Briefly CPS were dissolved in water then added with chloroform, and a liquid/liquid extraction was repeated several times until the water chloroform inter-phase became clear;

the aqueous phase containing the purified polysaccharide (PPS) was recovered and freeze-dried.

Filtration by cut-off filter of PPS

Freeze-dried PPS were re-dissolved in water, then 500 µl of this solution were transferred into an Amicon ultra-filter device (cut-off 10,000 Daltons) and centrifuged at 14,000 x g for about 15 min. The precipitate was reconstituted with the original water volume; the process was repeated up to 7 times, as indicated by the supplier, to remove about 99% of fouling, low molecular weight materials and salts, from the sample. After these cleaning steps, the filter device was placed upside down in a clean microcentrifuge tube for 2 minutes at 1,000 *x g*; 500 µl of distilled water were then added to dissolve the purified polysaccharide and the recovered solution to obtain the PPS_A sample.

Determination of the monosaccharide composition of PPS

PPS and PPS_A, derived through the purification steps of Laffan and Wonderful mesocarp and exocarp (from M-2s), were hydrolyzed according to Erbing *et al.* (1995). Briefly, a 2 M solution of trifluoroacetic acid (1 mL) was added to the PPS and PPS_A (5 mg), maintained at 120°C for 120 min, cooled on ice, then ultrafiltered at 3500 *x g* for 20min using centrifuge filter devices (Amicon ultra) having a cut-off of 3,000 Daltons, and the supernatant was dried in a rotavapor. The samples were washed twice with MilliQ-grade water, re-dissolved in 1 mL deionized water and then analyzed by ion exchange chromatography. The instrument used was a Dionex ICS-2500 ion chromatograph (Sunnyvale, CA) with an ED50 pulsed amperometric detector using a gold working electrode (Dionex, Sunnyvale, CA); the column was a Carbopac PA1 4 mm by 250 mm (Dionex, Sunnyvale, CA). The eluents used were MilliQ-grade water (solution A), 0.185 M sodium hydroxide solution (solution B), and 0.488 M sodium acetate solution (solution C). A gradient elution was used consisting of a first stage (injection time to the 7th min) with an eluent constituted by 84% solution A, 15% solution B, and

1% solution C; a second stage injection time from the 7th to 13th min) with 50% solution B and 50% solution C; and a final stage (injection time from the 13th to the 30th min) with 84% solution A, 15% solution B, and 1% solution C. The flow rate was 1 mL min^{-1}. The monosaccharides were detected according to retention time of standards purchased from Sigma-Aldrich (Milan, Italy).

Molecular shape size determination by Size Exclusion Chromatography (SEC)

PPS extracted from Laffan and Wonderful pomegranate mesocarp and exocarp, separately, were weighed and dissolved in distilled water. After homogenization, the suspension was analyzed using a Varian ProStar HPLC chromatograph (Varian, USA) equipped with a 355 refractive index (RI) detector and a column for Size Exclusion Chromatography (SEC), Biosep s4000 (Phenomenex, USA). Samples were analyzed with runs of 30 min with HPLC-grade water as eluent at a flow rate of 0.6 mL min^{-1}, using Dextran (Sigma-Aldrich, USA) at different molecular weights (MWs) (Blue dextran approx. 2000 kDa, 1100 kDa, 410 kDa, 150 kDa and 50 kDa) as standards.

In vitro evaluation of the prebiotic activity of CPS

The capability of CPS of stimulating the growth of beneficial bacteria (prebiotic activity) was assayed using two strains previously isolated from human feces: *Bifidobacterium breve* B632 (Aloisio et al. 2012) and *Lactobacillus plantarum* L12. The latter was isolated from a healthy human (unpublished results) and taxonomic characterization was performed via 16S rDNA amplification and sequencing (Gaggia et al. 2013); this strain is available at the Bologna University Scardovi Collection of *Bifidobacteria*. Both strains were stored in lyophilized form. When necessary, they were re-vitalized in de Man Rogosa Sharpe (MRS) medium (Oxoid, Basingstone, UK) supplemented with 0.05% cysteine and incubated in anaerobic conditions at 37°C for 24 h. Anaerobic conditions were

created in a capped jar using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany).

The MRS medium composition was modified to perform the growth experiment with the pomegranate polysaccharides. The modified medium (m-MRS) did not contain the carbon source (glucose), which was provided by the pomegranate polysaccharide, and had a halved amount of potential growth substrate, such as peptone, yeast extract and meat extract compared to those present in the original medium (peptone, 5 g/L; yeast extract, 2 g/L, meat extract 5 g/L were the amounts in m-MRS).

The prebiotic activity was evaluated using CPS at 0.5% (w/v) in m-MRS. A positive growth control was performed using m-MRS with 0.05% glucose and a negative control in m-MRS with no added carbon source. The medium containing CPS as the carbon source was prepared as follows: the m-MRS ingredients were weighed in a flask, dissolved in water and the medium was autoclaved at 120°C for 15 min. 0.5% (w/v) fiber or glucose at the same concentration were added to the hot medium, stirred, and sterilized again at 102°C for 10 min. This procedure allowed the fiber to dissolve in the medium and, at the same time, could prevent risk of growth of undesired microorganisms. The *B. breve* B632 and *L. plantarum* M12 strains were grown overnight in the respective media, centrifuged, washed in phosphate buffered saline (PBS) and re-suspended PBS to obtain a solution having an absorbance of 0.7 at 600 nm. This suspension was used to inoculate at 2% (v/v) the flasks containing the m-MRS medium plus the fiber, the m-MRS medium plus glucose (positive control) and the m-MRS medium with no additional carbon source (negative control). The flasks were incubated at 37°C in anaerobic conditions for 48 h and 1 mL culture was sampled from each flask for viable bacterial counts at pre-established times (0, 6, 24, 30 and 48 h of incubation). The sampled amount was mixed with 9 mL of PBS, serially diluted in the same solution and plated on agarized MRS supplemented with cysteine. Following incubation of the plates at 37°C in anaerobic conditions for 24 h, the number of colonies, corresponding to the number of viable cells, was counted.

The number of cells expressed as CFU/mL were transformed to Log_{10} value (Log CFU/mL).

Results and Discussion

Recovery of polysaccharides: preliminary evaluation

In recent decades, plant polysaccharides have attracted a great deal of attention in the biomedical field due to their wide spectra of therapeutic properties and relatively low toxicity. Nevertheless, little attention has been addressed to polysaccharides from pomegranate fruits which are mostly known as a good source of anthocyanins and ellagitannins but hardly cited for their polysaccharide content. One of the most common methods for recovering these polymers from different sources is the use of hot water, then collection of supernatant and recovery of the precipitated polysaccharides after addition of ethanol (Huie and Di, 2004; Joseph et al., 2012 ; Zhu and Liu 2013). In our study a similar procedure was applied to the mesocarp and exocarp separately, to evaluate their polysaccharide content. The first goal was to select efficient extractive procedures using the Laffan cultivar mesocarp as reference material. Later, the recovered polysaccharides were purified, analyzed to determine their sugar composition and molecular size and some of them were used for an *in vitro* evaluation of their prebiotic properties. The chart in Figure 1 summarizes the main steps of our research.

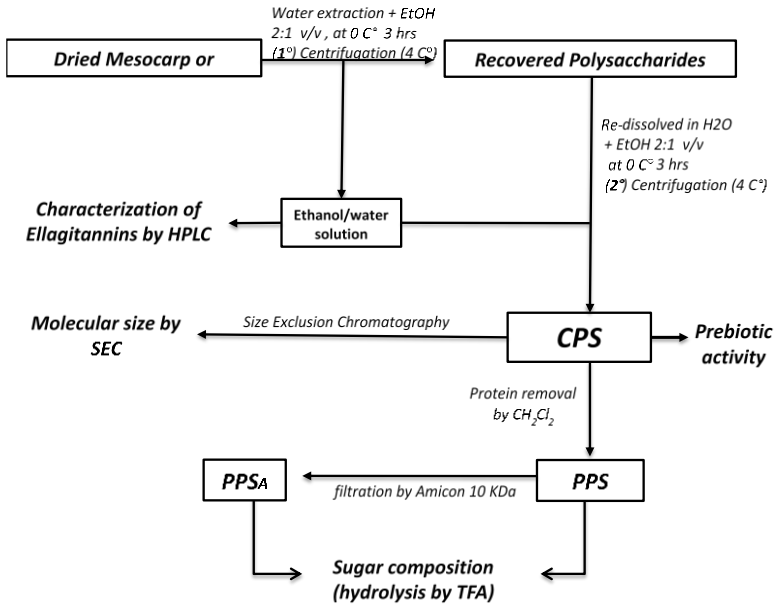


Figure 1. Chart to summarize all the analyses applied to the polysaccharides

Applied methods	DM (g)/ solvent (mL)	T (°C)	Time (min)	Yield (%)
M-1s	1/15	100	30	5
M-1t	1/15	100	30+30	8
*M-2s	1/40	100	60	10
*M-2t	1/40	100	60+ 60	9.8
*M-2t-p	1/40	100	60+ 60	9.1
*M-3r	1/40	25	720	2.0
*M-3l	1/40	25	1440	3.3
*M-4s-p	1/25	100	60	7.8
*M-5s-Et	1/25	100	60	7.15

Table 1: Methods to recover CPS from Laffan mesocarp and corresponding extractive yields (*mean values* as w/w DM); *tests were carried out in triplicate. *s =single step ; t =two consecutive steps; r= rapid* extraction; l= long extraction; p= preliminary contact with dried mesocarp before extraction at room temperature for 12 h; Et= pretreatment with ethanol 70% v/v.

In regard to extraction, three independent parameters, extraction time (h), extraction temperature (°C) and ratio DM/water, were modified during this study to increase the extractive yields and Table 1 summarizes the preliminary results obtained for the Laffan cultivar mesocarp First, 30 min or 60 min were set as extractive time in a single or two successive steps, using hot water as elective solvent (methods 1-2). In one case DM was previously extracted through a hydroalcoholic solution (M-5s-Et) and then treated with hot water according to the study of Tong et al. (2009) An aqueous extraction at room temperature was also performed (M-2t-p) as proposed by other authors (Li et al., 2007) for pomegranate. The ratio between DM and extractive solvent varied from 1:15 w/v for M-1, to 1:25 w/v for M-4s-p and M-5s-Et, with a maximum of 1:40 w/v for the M-2 and M-3. The best extraction processes were selected by evaluating the percentage yields of CPS recovered from DM.

To remove part of the impurities co-precipitated after the first ethanol addition (see Chart in Fig. 1) the recovered polysaccharides (PP) were re-dissolved in water and again treated with ethanol to obtain the CPS listed in Tables 1 and 2.

Applied methods	**Samples**	**Yield (%) CPS on DM mesocarp**	**Yield (%) CPS on DM exocarp**
M-2s	Laffan	9.80±0.28	4.47±0.50
	Wonderful	8.0±0.10	4.7±1.15
M-3l	Laffan	3.7±0.42	1.93±0.23
	Wonderful	3.33±1.15	1.99±0.02
M-4s-p	Laffan	7.80±0.28	4.20±0.20
	Wonderful	5.67±0.58	4.13±0.31
M-5s-Et	Laffan	7.15±0.21	3.93±0.12
	Wonderful	6.70±0.66	4.07±0.31

Table 2:Polysaccharides content (CPS) in mesocarp and exocarp of Wonderful and Laffan. The values are a mean of triplicates; the legend is the same of Table 1.

To verify if this stage was effective in cleaning the polysaccharides, the amount of the impurities was evaluated by weighing the dried discarded solutions recovered after the 2nd addition of ethanol. The amount of these impurities was 5.4% and 7.4% of dried mesocarp for Wonderful and Laffan, respectively, while these residues were close to 3% DM for both the cultivars in the exocarp. These results indicate that the 2nd addition of ethanol appeared necessary to obtain a more purified fraction (CPS) from the first precipitated sample (PP). Moreover, taking into account the presence of a high amount of ellagitannins (ETs) in pomegranate fruit, an HPLC/DAD analysis (Khatib, 2015) was applied to estimate the amount of ETs loss, because co-precipitated in the PP sample. To this aim, the washing solutions recovered during the precipitation of CPS were dried, re-dissolved and analyzed. Globally, it emerged that low amounts of ETs were trapped in the precipitated polysaccharides, substantially not over 1.1% and 0.4 % of DM respectively for the mesocarp and exocarp of both the cultivars.

Applying the same extractive ratio of 1:15 (w/v), the yield in CPS increased from 5% (M-1s) to 8% (M-1t) when the extraction time was extended from 30 min to 60 min. As shown in Table 1, the maximum yield of 10% was obtained with an extractive ratio of 1/40 (w/v), and by applying a single extraction of 60 min (M-2s). A successive step (M-2t) of 60 min, as well as previous contact of the DM with the water medium before boiling (M-2tp), did not increase the final recovery of CPS.

After this pre-screening, a time of 60 min was selected for decoction and applied to the mesocarp and exocarp of both cultivars to obtain the samples listed in Table 2. The mesocarp yields were up to 7.8% and 5.7% for Laffan and Wonderful cultivars, respectively. The best procedure in terms of CPS percentage was from M-2s, also showing good reproducibility of the extractive process with a maximum RSD value of 2.8%. Applying an extractive ratio of 1:25 (w/v), lower amounts of CPS (close to 7% in Table 1) were recovered. These values are in agreement with those reported by Zhu and Liu (2013) for pomegranate peel purchased from a local Chinese market and treated in a similar mode, although the authors did not clarify if the term “peel” referred to both peel and mesocarp

together. In a recent study on yield of polysaccharide from pomegranate peel, was 13.658 ± 0.133%, from pomegranate fruit purchased from a local Chinese local market and was extracted the ultrasound-assisted (Zhu et al .2015).

During this screening extraction was also carried out at room temperature as proposed by Li et al. (2007). As expected, the recovery was considerably lower and below 3.5%, even when allowing contact time between DM and water of between 12 h (M-3r) and 24 h (M-3l). In conclusion, by trying to avoid the co-precipitation of small amounts of ETs in PP (as in Figure 1), a pretreatment with ethanol 70% v/v at room temperature for 24 hours was made before the decoction (M-5s-Et). The yields in CPS were lower if compared with those derived by M-2s but this approach can be usefully applied if the objective is to efficiently recover the ellagitannins before the precipitation of polysaccharides, as previously demonstrated (Khatib , 2015).

Taking into account the results summarized in Table 1, only some methods were selected to treat the four different dried materials. Overall, similar results were obtained for the two cultivars, pointing out that the M-2s was the best procedure, of the tested methods, in terms of % yields, while M-3l carried out at room temperature was the worst one (Table 2). In agreement with other literature data (Yin and Dang, 2008; Sun Li, et al., 2010; Sun Liu, et al., 2010), the use of hot water increases polysaccharide solubility and extractability and was determinant for maximizing the recovery of CPS, but the same extraction at room temperature is always less efficient.

We verified that CPS were also localized in the exocarp of the fruit but in lower amounts than in the mesocarp, with maximum values between 4.5-4.7% for both cultivars (M-2s). As expected, the CPS percentage recovered from the exocarp had a higher variability (RSD from 11% to 25%) mainly due to the non-homogeneous thickness of the raw material that may contain different amounts of residual parts of mesocarp, which are difficult to completely remove. The decoction carried out after a previous extraction with ethanol/water (7:3v/v) provided CPS amounts close to 4% for both cultivars, and similar to those derived from M-2s. On the other hand, the lowest recovery was derived by extraction at

room temperature (M-3), in agreement with the results obtained for the mesocarp and with those reported by other authors employing an analogous extraction from the rind (Rout and Banerjee, 2007).

Sugar composition by hydrolysis

Samples were treated with trifluoroacetic acid to hydrolyze the polysaccharide strands and subsequently determine sugar composition by ion exchange chromatography according to previous methods (Erbing et al,. 1995). The composition of four purified polysaccharide fractions are reported in Table 3. On the whole, diverse monosaccharides were found, and these samples showed a very similar composition for both the cultivars. Within the hexoses galactose, glucose/mannose, and fructose; then aldopentose arabinose, pentose xylose, deoxysugar rhamnose and galacturonic acid were found (Table 3). Glucose and mannose, having very close retention times, were co-eluted in one peak on the chromatograms. In the entire analyzed sample, glucose, xylose and glucuronic acid were the most abundant sugars (Table 3).

	Molar %			
Sugars	PPS-mW	PPS-mL	PPS-eW	PPS-eL
Rhamnose	10.4	7.2	10.8	10.1
Arabinose	4.52	4.04	4.88	4.08
Galactose	5.91	7.31	7.34	7.05
Glucose	14	10.3	11.5	10.9
Xylose	11.2	7.87	9.36	9.3
Fructose	0.41	0.29	0.17	0.2
Galacturonic acid.	53.8	63.1	56	58.37

Table 3: Sugar composition of PPS obtained by acid hydrolysis (Erbing et al.. 1995). m, mesocarp; e, exocarp; L, Laffan and W, Wonderful.

Normakhmatov et al. (1999) pointed out that the main monosaccharides in the water-soluble polysaccharides from pomegranate peel varied with the growth site,

and were constituted by glucose, galactose and xylose, with larger amounts of xylose, arabinose and galactose detected in the peel of other varieties of pomegranate harvested in different growth sites. In another study the composition of a polysaccharide from pomegranate peel showed glucose at 52.8% and glucuronic acids at 33.5%. Other monosaccharides included arabinose and galactose close to 5.0%, mannose and rhamnose close to 1.6% and only 0.5% for xylose. (Jahfar et al., 2003).

From the few reports available to date on polysaccharides of pomegranate fruits, it emerges that there is a certain variability in the sugar composition that is mainly related to the variety and growth site but is also affected by the different purity grade of the polysaccharide itself, as analyzed by various authors. Notably, the two cultivars (one mainly localized in Syria and the other widely diffused throughout the western world) showed a very similar compositional profile of the polysaccharides. However, this result is not completely unexpected, and is in agreement with a previous document that hypothesized that the Wonderful variety is derived from the more ancient Laffan cultivar (Goor, 1967).

Filtration by 10,000 Dalton cut-off

In order to purify the CPS, the efficiency of ultrafiltration devices was tested on the samples obtained from mesocarp (Table 2). The recovered solid material obtained after ultrafiltration was submitted to acidic hydrolysis to evaluate the monosaccharidic composition. It emerged that the CPS samples before and after the cut-off filtration provided the same results in terms of molar percentage for all the sugars (data not shown), meaning a substantial absence of impurities represented by free oligosaccharides in the CPS. These results suggested that the samples listed in Table 2 did not need further purification by this filter device before the chemical hydrolysis.

Molecular size of polysaccharides

The CPS from mesocarp and exocarp of the two cultivars were analyzed by SEC to determine their dimension. Since these polymers may be characterized by a branched structure, often related to the presence of arabinose, galactose and xylose, the determination of the size by SEC was elaborated in terms of hydrodynamic volume, and not in terms of actual molecular weight. The fractions that comprised the CPS samples were compared to dextran standards, meaning that a 2000 kDa fraction was considered as having the same hydrodynamic volume as dextran with 2000 kDa as molecular weight (MW). The analyzed fractions throughout the text are identified as MW, although it represents an approximation. The different polymers derived from the CPS of Laffan and Wonderful cultivar mesocarp and exocarp (Figure 2) were composed of fractions with a similar MW, since no significant differences were found.

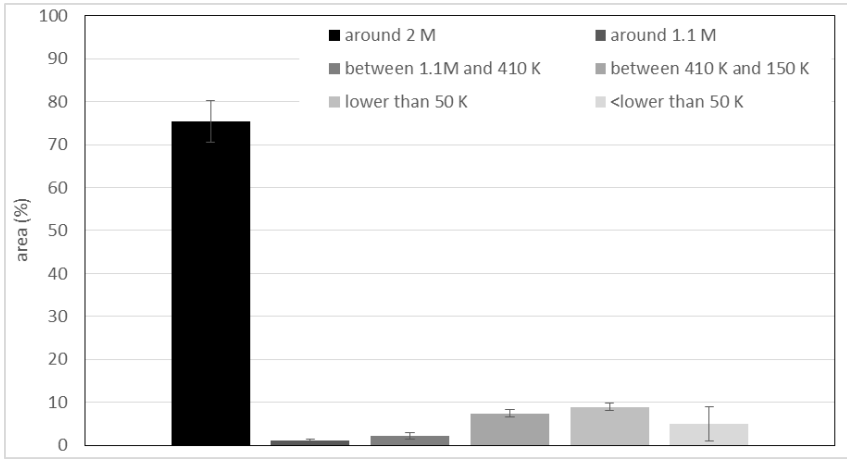


Figure 2 An average composition of the four PPS samples derived from mesocarp and exocarp of Laffan and Wonderful cultivars (see Table 3).

The polymers deriving from the Laffan and Wonderful cultivar mesocarp and exocarp were all characterized by a predominant fraction of about 2000 kDa, accounting for 75.4% of the total. The remaining 24.6% were comprised of five minor fractions, the most represented being: i) a fraction corresponding to a MW smaller than 410 kDa, and larger than 150 kDa (accounting for 7.4% of the total);

ii) a fraction having a MW smaller than 50 kDa, accounting for the 8.9% of the total. As expected, more variability was observed for the fractions having small MWs (much smaller than 50 kDa).

Few reports are available to date on polysaccharide structure recovered from pomegranate fruit. A first report described a glucofructan extracted from the peel, having a molecular weight of 31 kDa and separated on a Sephadex G100 column (Khodzhaeva et al., 1984). Soni described a polysaccharide extracted from the rind of a non-specified variety, with a MW of 110 kDa determined by gel filtration on a Sephadex G200 and characterized by comparison with different dextrans (Jahfar et al., 2003). More recently, a glucomannan was extracted from the rind of a ripened pomegranate fruit and, by applying a similar analytical approach to that described by Sreelekha (2008), the authors determined its MW as 110 kDa (Joseph et al 2012).

The present study determines for the first time the MW distribution of pomegranate polymers obtained from Laffan and Wonderful cultivars by using SEC. We demonstrate that CPS samples have similar MW distribution, in the same proportion for both cultivars and in the two parts of the fruit. The almost complete overlapping in the SEC profiles of the two cultivars is again in agreement with a previous work that suggested the Wonderful is derived from the more ancient cultivar, Laffan (Goor, 1967).

Even if the most abundant fraction has a hydrodynamic volume corresponding to a dextran molecule of 2000 kDa, it must be taken into account that, in the present analysis, the possible branching of the polysaccharides can result in a hydrodynamic volume larger than the estimated MW.

Gelling capacity of CPS

The water-holding capacity of plant polysaccharides is determined by their chemical and structural properties and also by the pH and osmolality of the surrounding fluids (Eastwood et al.,1976). When expressed as water retained per gram of dried material, a range of 1.5 g water per gram of fiber for maize, to 23.7 g water per gram of fiber for lettuce has been reported **(**Eastwood et al.,1976;

Connell, et al.,1974). It is well known that purified plant fibers such as carrageenan can form gels containing 99% water per gram of dried material (Eastwood et al.,1973).

To the best of our knowledge, there are no data on the water absorption capacity of polysaccharides from pomegranate available to date. In this work a preliminary test was carried out on some representative CPS samples shown in Table 4, pointing out that the percentage of water adsorbed ranged from 98.6 to 99.1% of dried material.

Samples	Amount of CPS (g)	% H_2O
CPS-mL	0.3	99.2
CPS-mW	0.3	99
CPS-eW	0.3	98.6

Table 4. Gelling capacity evaluated as amount of adsorbed water by 300 mg of each CPS; data expressed as % on DM. ***e= exocarp; m= Mesocarp; W = Wonderful; L= Laffan***

This confirms that these polymers are potential gelling agents in other fields such as food applications. These preliminary data help to improve our knowledge of the physical properties of polysaccharides recovered as typical by-products of the pomegranate fruit, and open future perspectives for adding value to this food waste.

In vitro evaluation of prebiotic properties

In this study the ability of *B. breve* B632 and *L. plantarum* L12 strains to use crude polysaccharides from pomegranate exocarp and mesocarp as the carbon source was investigated and compared to growth on glucose, *i.e.* an easily fermentable carbon source. Figure 3 shows that both strains could grow well on the CPS of Laffan and Wonderful mesocarp, being comparable to that of an easily fermentable carbon source such as glucose. Growth at 24 h on the Laffan variety was only 0.6 and 0.1 Log CFU/mL lower than that on glucose for *L. plantarum* L12 and *B. breve* B632, respectively. Growth at 24 h on the Wonderful variety

was 1.0 and 0.2 Log CFU/ml lower than that on glucose for the same strains. After the 24th h of incubation, both strains grown on glucose enter the stationary phase, whereas a small decrease in cell survival was observed on CPS as the carbon source.

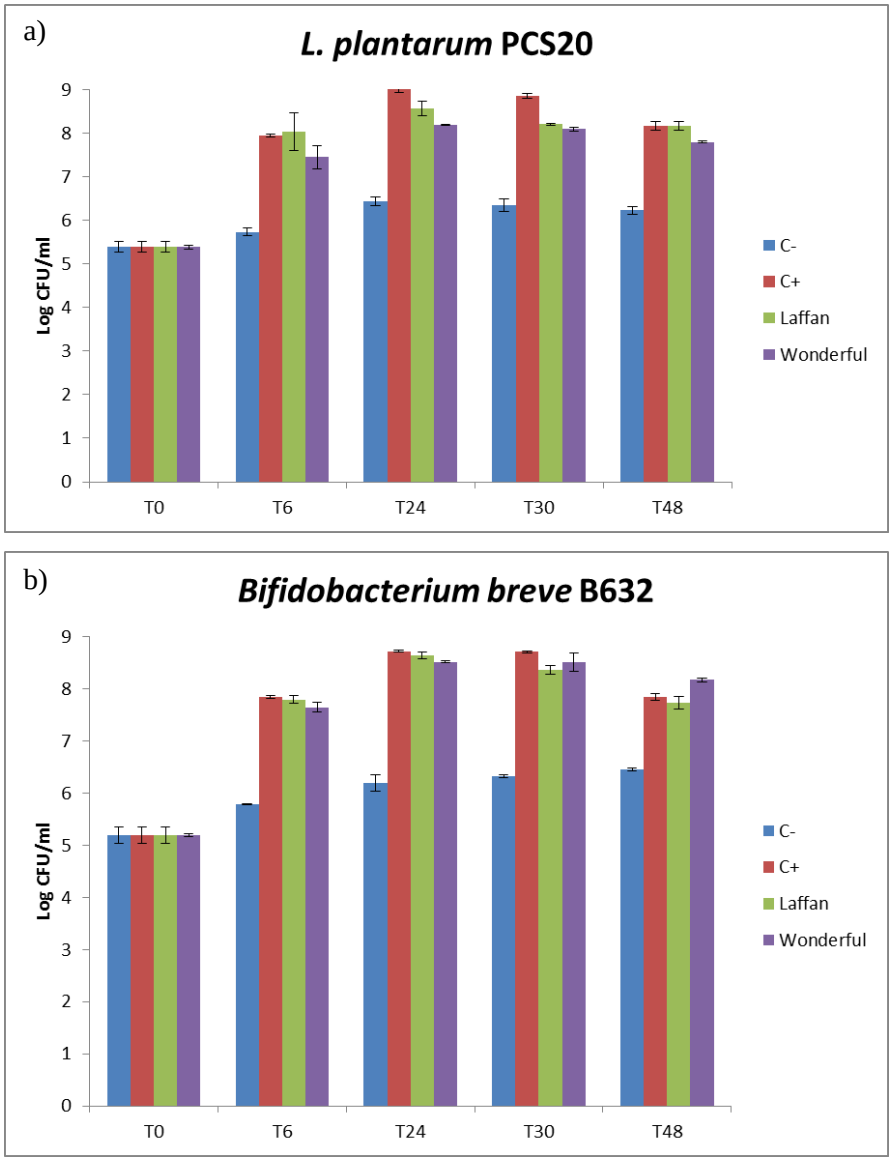


Figure 3 . Evaluation of prebiotic properties of CPS from mesocarp of Laffan and Wonderful on the (a) *L.planctarum*PCS20 and (b) *Bifidobacterium breve* B632.

C-: growth on m-MRS with no added carbon source; C+: growth on m-MRS with 0.5 % glucose

However, the results shown in Figure 3 clearly indicate that CPS or the metabolites of their degradation are not toxic for the assayed strains and, on the contrary, are good growth substrates for them. Growth on the medium with no added carbon source reached only 1 Log CFU/mL increase at the 24th h compared to the beginning of incubation, thus showing that the m-MRS medium used in the experiments is a good one for performing prebiotic activity tests.

Conclusions

This study for the first time determines the MW distribution of pomegranate polymers obtained from Laffan and Wonderful cultivars by using SEC. It has been demonstrated that CPS samples have a similar MW distribution for both the cultivars and the same distribution in the two parts of the fruit (mesocarp and exocarp). The almost complete overlapping of SEC profiles is in agreement with previous data that suggest that the Wonderful cultivar is derived from the more ancient cultivar, Laffan.

The maximum extractive yield in terms of crude polysaccharides was 10% on dried mesocarp, achieved through water decoction for 60 min. The two varieties showed similar amounts, with the highest concentration in the mesocarp. In agreement with the literature, the use of hot water increases polysaccharide solubility and extractability and maximizes the recovery of CPS, while the same extraction at room temperature is always less efficient. These CPS were able to absorb up to 99.1% of water, confirming a potential use as gelling agents in food chemistry. On the whole, the prebiotic activity tests allow us to conclude that CPS from Laffan and Wonderful pomegranate cultivar mesocarp can sustain growth of beneficial and potentially probiotic bacteria such as those used in this study and, therefore, possess prebiotic properties. Growth of the *Lactobacillus* strain on the Laffan variety was higher than on the Wonderful one, whereas growth of the *Bifidobacterium* strain was very similar on the CPS from both varieties. These data on the polysaccharides of pomegranate help to open future perspectives for adding value to this typical waste recovered after fruit processing.

References

Aloisio, I.,Santini, C., Biavati, B., Dinelli, G., Cencič, A., Chingwaru, W., Mogna, L., Di Gioia, D**.** (2012). Characterization of *Bifidobacterium* spp. strains for the treatment of enteric disorders in newborns. *Applied Microbiology and Biotechnology*, 96, 1561–1576

Al-Said, F. A., Opara, L. U., and Al-Yahyai, R. A. (2009). Physico-chemical and textural quality attributes of pomegranate cultivars (*Punica granatum* L.) grown in the Sultanate of Oman. *Journal of Food Engineering*, 90(1), 129-134.

Beat, E., and Magnani, J. L. (2009). From carbohydrate leads to glycomimetic drugs. *Nature Reviews*, 8, 661–678.

Bhardwaj, T. R., Kanwar, M., Lal, R., & Gupta, A. (2000). Natural gums and modified natural gums as sustained-release carriers. *Drug Development and Industrial Pharmacy*, 26, 1025–1038.

Connell Mc, A. A., Eastwood, M. A. & Mitchell, W. D. (1974). Physical characteristics of vegetable foodstuffs that could influence bowel function. *Journal of the Science of Food and Agriculture*, 25, 1457-1464.

Deters, A., Dauer, A., Schnetz, E., Fartasch, M., Hensel, A. (2001). High molecular compounds (polysaccharides and proanthocyanidins) from *Hamamelis virginiana* bark: Influence on human skin keratinocyte proliferation and differentiation and influence on irritated skin. *Phytochemistry*, 58: 949-958.

Di Gioia, D., Aloisio, I., Mazzola G., Biavati, B. (2014a). Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. *Appl Microbiol Biotechnol*, 98,563–577

Di Gioia, D., Strahsburger, E., Lopez de Lacey, A. M., Bregola, V., Marotti, I., Aloisio, I., Biavati B., Dinelli G. (2014b) Flavonoid bioconversion *in Bifidobacterium pseudocatenulatum* B7003: A potential probiotic strain for functional food development. *Journal of Functional Foods*, 7, 671-679.

Eastwood, M. A., & Mitchell,W. D. (1976). Physical properties of fiber: A biological evaluation. In:Fiber in Human Nutrition, edited by G. A. Spiller and R. J. Amen. New York: Plenum Press, p.109.

Eastwood, M. A. (1973).Vegetable fibre: its physical properties. *Proceedings of the Nutrition Society*, 32, 137-143.

Erbing, B., Jansson, P. E., Widmalm, G., & Nimmich, W. (1995). Structure of the capsular polysaccharide from the Klebsiella K8 reference strain 1015. *Carbohydrate Research*, 273, 197–205

Franz, G. (1989). Polysaccharides in pharmacy: Current applications and future concepts. *Planta Medica*, 55, 493–497.

Gaggìa, F., Baffoni, L., Di Gioia, D., Accorsi, M., Bosi, S., Marotti, I., Biavati, B. and Dinelli, G., (2013). Inoculation with effective microorganisms of *Lolium perenne* L.: evaluation of plant growth parameters and endophytic colonization of roots. *New Biotechnology*, 30, 695-704.

Goor, A. (1967). The history of the pomegranate in the Holy Land. *Economic Botany*, 21(3), 215-230.

Huie, C. W., & Di, X. (2004). Chromatographic and electrophoretic methods for Lingzhi pharmacologically active components. *Journal of Chromatography* B,812(2), 241–257.

Jahfar M,. Vijayan K.K., & Azadi, P. (2003). Studies on a polysaccharide from the fruit rind of Punica granatum. *Research Journal of Chemistry and Environment*, 7(1), 43–50.

Jankowska, A., Laubitz D., Antushevich, H., Zabielski, R., Grzesiuk, E. (2008). Competition of Lactobacillus paracasei with Salmonella enterica for adhesion to Caco-2 cells. *J Biomed Biotechnol*, 59, 795-810.

Joseph, M. M., Aravind, S. R., Varghese, S., Mini, S., & Sreelekha, T. T. (2012). Evaluation of antioxidant, antitumor and immunomodulatory properties of polysaccharide isolated from fruit rind of Punica granatum. *Mol Med Report*, *5*(2), 489-496.

Joseph, Manu M., S.R. Aravinda, Suraj K. Georgeb, Sheeja Varghesea, T.T. (2013). Sreelekhaa, A galactomannan polysaccharide from *Punica granatum*

imparts in vitro and in vivo anticancer activity. *Carbohydrate Polymers, 98*(2), 1466-1475.

Kardošová, A., Machová, E. (2006). Antioxidant activity of medicinal plant polysaccharides, *Fitoterapia*, 77,367-373.

Khodzhaeva, M. A., Yuldasheva, N. P., Khasanov, M., Kondratenko, E. S., & Umarov, A. U. (1984). Polysaccharides of the wastes from Punica granatum. *Chemistry of Natural Compounds*, 20(5), 615-616.

Khatib, M. (2015). Bioactive compounds into edible syrian plants: pomegranate and capper .PhD Thesis University of Florence , Italy.

Laparra, J.M., Sanz, Y. (2010). Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacol. Res*, 61, 219–25.

Leung, M. Y. K., Liu, C., Koon, J. C. M., & Fung, K. P. (2006). Polysaccharide biological response modifiers. *Immunology Letters*, 105, 101–111.

Levin, G.M. (2006). Pomegranate roads: *a Soviet botanist's exile from Eden*. pp. 15–183. B.L. Baer (ed.), Floreat Press, Forestville, CA.

Liu, F., Ooi, V., & Chang, S.T. (1997). Free radical scavenging activities of mushroom polysaccharide extracts. *Life Science*, 60, 763–771.

Li, J., Zhang, F., & Wang, S. (2014). A polysaccharide from pomegranate peels induces the apoptosis of human osteosarcoma cells via the mitochondrial apoptotic pathway. *TumorBiology*, 1-8.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., & Cheng, S. (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry*, 96, 254–260.

Li, X.M., Li, X.L., Zhou, A.G. (2007): Evaluation of antioxidant activity of the polysaccharides extracted from Lycium barbarum fruits in vitro, *European Polymer Journal* 43, 488-497.

Liu, Z., Jiao, Y., Wang, Y., Zhou, C., & Zhang, Z. (2008). Polysaccharide-based nanoparticles as drug delivery systems. *Advanced Drug Delivery Reviews*, 60,1650–1662.

Mahady, G. B. (2001). Global harmonization of herbal health claims. *Journal of Nutrition*, 131, 1120–1123.

Marathe, R. M., Annapure, U. S., Singhal, R. S., & Kulkarni, P. R. (2002). Gelling behavior of polyose from tamarind kernel polysaccharide. *Food Hydrocolloids*, 16, 423–426.

Marotti I., Bregola V., Aloisio I., Di Gioia D., Bosi S., Di Silvestro R., Quinn R., Dinelli G. (2012). Prebiotic effect of soluble fibers from modern and old durum-type wheat varieties on *Lactobacillus* and *Bifidobacterium* strains. *Journal of the Science of Food and Agriculture*, 92, 2133-2140

Negi, P. S., G. K. Jayaprakasha, and Jena, B. S. (2003).Antioxidant and antimutagenic activities of pomegranate peel extracts. *Food chemistry* 80(3), 393-397.

Normakhmatov, R., Rakhmanberdyeva, R. K., & Rakhimov, D. A. (1999).Polysaccharides of the fruit of Punica granatum. *Chemistry of natural compounds*, 35(1), 96-97.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry*, 7, 715–729.

Pan, Z., Qu, W., Ma, H., Atungulu, G. G., & McHugh, T. H. (2012). Continuous and pulsed ultrasound-assisted extractions of antioxidants from pomegranate peel. *Ultrasonics sonochemistry*, 19(2), 365-372.

Rout, S., & Banerjee, R. (2007). Free radical scavenging, anti-glycation and tyrosinase inhibition properties of a polysaccharide fraction isolated from the rind from Punica granatum. *Bioresource technology*, 98(16), 3159-3163

Rubinstein, A. (2000). Natural polysaccharide as targeting tools of drugs to the human colon. *Drug Delivery Research*, 50, 435–439.

Saad, H., Charrier-El Bouhtoury, F., Pizzi, A., Rode, K., Charrier, B., & Ayed, N. (2012). Characterization of pomegranate peels tannin extractives. *Industrial Crops and Products*, *40*, 239-246.

Schepetkin IA., Quinn, MT. (2006). Botanical polysaccharides:macrophage immunomodulation and therapeutic potential. *Int Immunopharmacol*, 6, 317–333.

Sinha, V. R., & Kumaria, R. (2001). Polysaccharides in colon-specific drug delivery. *International Journal of Pharmaceutics*, 224, 19–38.

Sreelekha, T. T., Vijayan, K. K., & Prabha, B. (2008). Antimitotic polysaccharide from *Punica granatum.* In P. Pushpangadan, V. George, & K. K. Janardhanan (Eds.), Ethnopharmacology – Recent advances (19) (pp. 231–245). Delhi, India: *Day a Publishing House*.

Sun, Y., Li, T., Yan, J., & Liu, J. (2010). Technology optimization for polysaccharides (POP) extraction from the fruiting bodies of Pleurotus ostreatus by Box–Benhken statistical design. *Carbohydrate Polymers*, 80, 242–247.

Sun, Y., Liu, J., & Kennedy, J. F. (2010). Application of response surface methodology for optimization of polysaccharides production parameters from the roots of Codonopsis pilosula by a central composite design. *Carbohydrate Polymers*, 80,949–953.

Symonds EL, O'Mahony C, Lapthorne S, O'Mahony D, Sharry JM, O'Mahony L, Shanahan F. (2012). *Bifidobacterium infantis* 35624 protects against salmonella-induced reductions in digestive enzyme activity in mice by attenuation of the host inflammatory response. *Clin Transl Gastroenterol* 3:e15

Tremaroli, V., Bäckhed, F. (2012).Functional interactions between the gut microbiota and host metabolism. *Nature* 489, 242–9.

Tong, H., Xia, F., Feng, K., Sun, G., Gao, X., Sun, L., & Sun, X. (2009). Structural characterization and in vitro antitumor activity of a novel polysaccharide isolated from the fruiting bodies of Pleurotus ostreatus. *Bioresource Technology*, 100(4), 1682-1686.

Valadares, M. C., Pereira, E. R. T., Benfica, P. L., & Paula, J. R. (2010). Assessment of mutagenic and antimutagenic effects of Punica granatum in mice. *Brazilian Journal of Pharmaceutical Sciences*, *46*(1), 121-127.

Wang, QJ., Fang, YZ. (2004). Analysis of sugars in traditional Chinese drugs. *J Chromatogr*. B, 812, 309-324.

Xie, J. H., Xie, M. Y., Nie, S. P., Shen, M. Y., Wang, Y. X., & Li, C. (2010). Isolation, chemical composition and antioxidant activities of a water-soluble

polysaccharide from Cyclocarya paliurus (Batal.) Iljinskaja. *Food Chemistry*, *119*(4), 1626-1632.

Yin, G., & Dang, Y. (2008). Optimization of extraction technology of the Lycium barbarum polysaccahrides by Box–Behnken statistical design. *Carbohydrate Polymers*, 74, 603–610.

Zhu, Caiping, and Xiaolin Liu. (2013). Optimization of extraction process of crude polysaccharides from Pomegranate peel by response surface methodology. *Carbohydrate polymers*, 92(2),1197-1202.

Zhu, KX., Nie, SP., Li, C, et al. (2013). A newly identified polysaccharide from *Ganoderma atrum* attenuates hyperglycemia and hyperlipidemia. *Int J Biol Macromol*. 2013;57:142–150.

Zhu, C. P., Zhai, X. C., Li, L. Q., Wu, X. X., & Li, B. (2015). Response surface optimization of ultrasound-assisted polysaccharides extraction from pomegranate peel. *Food chemistry*, 177, 139-146.

Scientific acknowledgements

University of Florence

Professor Nadia Mulinacci

Professor Marzia Innocenti

Maria Bellumori

Mohammad Kathib

Andrea Ghiori

All Phytolab's staff

Ghent University

Professor Tom Van de Wiele

Massimo Marzorati

Ramiro Vilchez-Vargas

Emma Hernandez-Sanabria

Jana De Bodt

Tim Lacoere

All Labmetians

Scientific pubblications

- Camilla Giuliani.
  Oral communication: “Phenolic compounds as inhibitors of the amyloid aggregation of Hen Egg White Lysozyme: a multidisciplinary approach”
  PhDDay 4, May 16 2013, Firenze

- Camilla Giuliani; Marzia Innocenti; Fabrizio Melani; Valentina Noccioli; Donatella degl’Innocenti; Nadia Mulinacci.
  Oral communication: “Phenolic compounds as inhibitors of the amyloid aggregation of Hen Egg White Lysozyme: a multidisciplinary approach.”
  NPCF 7, May 29-31 2013, Savigliano (CN)

- Camilla Giuliani, Massimo Marzorati, Tom Van de Wiele, Mohammad Khatib , Marzia Innocenti, Nadia Mulinacci
  Oral communication: “Microbiota modulation by olive oil and pomegranate by-products evaluated in in vitro simulation technology M-SHIME”
  CHIMALI 2014, 6-10 2014, Firenze

- Mohammad Khatib, Selene Alcamisi, Camilla Giuliani, Marzia Innocenti, Nadia Mulinacci
  Poster: “Recovery of ellagitannins from mesocarp of pomegranate fruit of the Syrian Laffan cultivar”
  CHIMALI 2014, July 6-10 2014, Firenze

- Mohammad Khatib, Selene Alcamisi, Camilla Giuliani, Marzia Innocenti, Nadia Mulinacci
  Poster: “Recovery of crude polysaccharides from peel and mesocarp of pomegranate: the Wonderful and Laffan cultivars”
  CHIMALI 2014, July 6-10 2014, Firenze

- Camilla Giuliani, Andrea Ghiori, Marzia Innocenti, Massimo Marzorati, Tom Van de Wiele, Nadia Mulinacci

Poster: “Vineatrol® metabolism by gut microbial community in in vitro simulator M-SHIME®”

3rd MS Food Day, October 7-9 2015, Foggia

- Marzia Innocenti, Mohamad Khatib, Camilla Giuliani, Diana di Gioia, Nadia Mulinacci

  Poster: “Wastes from pomegranate fruit processing: a good source of ellagitannins and polysaccharides”

  EUROFOODCHEM 2015, October 13-16 2015, Madrid

- Camilla Giuliani, Massimo Marzorati, Tom Van de Wiele, Marzia Innocenti, Nadia Mulinacci

  Poster: “Microbiota modulation by olive oil and pomegranate by-products evaluated in in vitro simulation technology M-SHIME®”

  EUROFOODCHEM 2015, October 13-16 2015, Madrid