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SCIENZE E TECNOLOGIE VEGETALI,
MICROBICHE E GENETICHE

CICLO XXVII

COORDINATORE Prof. Paolo Capretti

Investigation on microbiota of extra virgin olive oil extraction process

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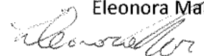

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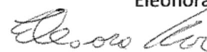

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Keywords: Microbiota, Olio d'oliva, Difetti sensoriali, Lieviti, Identificazione molecolare.

Riassunto

Scopo: Lo scopo del lavoro è stato quello di indagare sul microbiota presente nel processo di estrazione dell'olio extra vergine di oliva e, in particolare, studiare l'impatto della popolazione di lievito sulla qualità dell'olio.

Metodi e Risultati: Le popolazioni di lievito presenti nel processo di estrazione dell'olio extra vergine di oliva sono risultate numericamente significative. La concentrazione dei lieviti è risultata positivamente o negativamente correlata con alcune componenti aromatiche degli oli ottenuti. Sono state poi identificate diciotto specie di lievito attraverso il sequenziamento dei geni dell' rRNA e/o attraverso l'analisi dei profili di restrizione della regione ITS. Inoltre è stata messa a punto una metodica molecolare rapida e riproducibile per l'identificazione delle specie di lievito presenti nel processo. Per testare le capacità enzimatiche dei lieviti isolati dal processo ed il loro impatto sulla qualità dell'olio durante la conservazione, i lieviti isolati sono stati testati per l'attività β-glucosidasica, cellulasica, poligalacturonasica, perossidasica e lipasica. Infine, per dimostrare l'influenza sulla qualità dell'olio, tre ceppi di lievito con attività enzimatiche diverse, e potenzialmente in grado di modificare chimicamente l'olio, sono stati inoculati separatamente in paste frante e olio filtrato. I risultati di questa sperimentazione hanno dimostrato come i lieviti siano in grado di incidere negativamente sulla composizione chimica dell'olio confermando quanto osservato nei processi reali.

Conclusioni: La presenza di diverse specie di lievito suggerisce un fenomeno di contaminazione dell'impianto di estrazione dell'olio che porta a selezionare alcune specie di lievito piuttosto che altre. Gran parte di questi lieviti, in base alle loro attività enzimatiche, possono incidere positivamente, ma soprattutto negativamente sulla qualità dell'olio.

Significato e impatto dello Studio: Il presente studio rappresenta la prima indagine riguardo la contaminazione da parte dei lieviti di un impianto per l'estrazione dell'olio extravergine d'oliva e contribuisce alla comprensione del ruolo dei lieviti nella definizione della qualità di questo prodotto.

Lavori correlati alla tesi

- Guerrini S., Mari E., Vincenzini M., 2013. *Microrganismi nel processo di estrazione*. Capitolo 4 in "PROGETTO OLEOSALUSISTEM, validazione di protocolli per la produzione di oli ad elevato valore nutrizionale ed a ridotto impatto ambientale". Pag: 43-52.
- Guerrini S., Mari E., Migliorini M., Cherubini C., Trapani S., Zanoni B., Vincenzini M. 2015. *Investigation on microbiology of olive oil extraction process*. Italian Journal of Food Sciences, 27, 108-119.

Manoscritti sottomessi

- Mari E., Guerrini S., Granchi L., Vincenzini M. *Yeast microbiota in the olive oil extractive process: a three-year study at an industrial scale*. World Journal of Microbiology and Biotechnology.

Comunicazioni a convegni

- Mari E., Guerrini S., Mancini O., Vincenzini M. "*Identification and enumeration of the yeast populations occurring in the extraction process of extra virgin olive oil produced in Tuscany*" at the Congress of SIMTREA (Società Italiana Microbiologia Agraria, Alimentare, Ambientale) Bari 26-29 Giugno 2012.
- Trapani S., Guerrini S., Mari E., Migliorini M., Cherubini C., GianniG., Zanoni B., Vincenzini M. **"***Extra virgin olive oil: microbial ecology of the extractive processes and its effect on the aromatic composition of the final products"* at the 10th Euro Fed Lipid Congress "Fats, Oils and Lipids: from Science and Technology to Health", Krakow (Poland) 23-26 September 2012.
- Granchi L., Mari E., Guerrini S., Vincenzini M. "*Enzymatic capabilities of oil-born yeasts and their impact on olive oil quality during its storage"* at the 32nd International Specialized Symposium on Yeasts "Yeasts biodiversity and biotechnology in the twenty-first century", Perugia, 13-17 September 2015.
- Granchi L., Mari E., Guerrini S., Vincenzini M. "*A survey on yeast species occurring in the olive oil extraction process*" at the 3rd International

Conference on Microbial Diversity “The Challenge of Complexity”, Perugia, 27-29 October 2015.

Keywords: Microbiota, Olive oil, Sensory defects, Yeasts, Molecular identification

Abstract

Aim: The aim of this work was to investigate the microbiota occurring in extra virgin olive oil extraction process and, in particular the impact of the yeast population on the olive oil quality.

Methods and Results: The yeast populations occurring in extra virgin olive oil extraction process demonstrated to be numerically significant. The yeast concentrations were positively or negatively related to some aromatic compounds of oil. Eighteen dominant yeast species were identified sequencing rRNA genes and/or their flanking ITS regions and a reproducible and rapid molecular method for differentiating the yeast species of the oleic ecosystem was also provided. To assess the enzymatic capabilities of oil-born yeasts and their impact on olive oil quality during its storage, yeast isolates were assayed for β-glucosidase, cellulase, polygalacturonase, peroxidase and lipase activities. Finally, three strains belonging to three different yeast species, with different enzymatic activities, were separately inoculated in crushed pastes and filtered olive oil to investigate their influence on the oil quality. The results demonstrated that oil-born yeasts may negatively affect the chemical composition of olive oil confirming the results obtained with real extraction processes.

Conclusion: The occurrence of the various yeast species in olive oil extraction process suggest a phenomenon of contamination of the plant for oil extraction that selects some yeast species at the expense of others. Most of these yeasts have enzymatic activities that can change both positively but mostly negatively the quality of the oil.

Significance and Impact of the Study: This study concern the first investigation regarding the contamination by yeasts of a plant for the extraction of extra virgin olive oil and contributes to the understanding of the role of yeasts in the definition of the olive oil quality.

Papers related to the Thesis

- Guerrini S., Mari E., Vincenzini M., 2013. *Microrganismi nel processo di estrazione*. Capitolo 4 in "PROGETTO OLEOSALUSISTEM, validazione di protocolli per la produzione di oli ad elevato valore nutrizionale ed a ridotto impatto ambientale". Pag: 43-52.
- Guerrini S., Mari E., Migliorini M., Cherubini C., Trapani S., Zanoni B., Vincenzini M. 2015. *Investigation on microbiology of olive oil extraction process*. Italian Journal of Food Sciences, 27, 108-119.

Manuscripts submitted

- Mari E., Guerrini S., Granchi L., Vincenzini M. *Yeast microbiota in the olive oil extractive process: a three-year study at an industrial scale*. World Journal of Microbiology and Biotechnology.

Conference communications

- Mari E., Guerrini S., Mancini O., Vincenzini M. "*Identification and enumeration of the yeast populations occurring in the extraction process of extra virgin olive oil produced in Tuscany*" at the Congress of SIMTREA (Società Italiana Microbiologia Agraria, Alimentare, Ambientale) Bari 26-29 Giugno 2012.
- Trapani S., Guerrini S., Mari E., Migliorini M., Cherubini C., GianniG., Zanoni B., Vincenzini M**.** **"***Extra virgin olive oil: microbial ecology of the extractive processes and its effect on the aromatic composition of the final products"* at the 10th Euro Fed Lipid Congress "Fats, Oils and Lipids: from Science and Technology to Health", Krakow (Poland) 23-26 September 2012.
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Conference on Microbial Diversity “The Challenge of Complexity”, Perugia, 27-29 October 2015.

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1 INTRODUCTION

1.1 The extraction process of extra virgin olive oil

Virgin olive oil is the oil obtained from the fruit of the olive tree (*Olea europaea*) either by mechanical or other physical means under particularly thermal conditions, that do not lead to alterations in the oil, and which has not undergone any treatment other than washing, decantation, centrifugation and filtration. It constitutes a key staple in the Mediterranean diet due to its nutritional, therapeutic (Psaltopoulou et al., 2004) and sensory properties (Servili et al., 2004). Strategic choice of plant engineering systems and of processing technologies should be made to influence the enzymatic activities who modulate the nutritional and the sensory quality of the extra virgin olive oil (Clodoveo et al., 2014). Figure 1 shows the flowchart of the extraction process of extra virgin olive oil.

EXTRACTION PROCESS

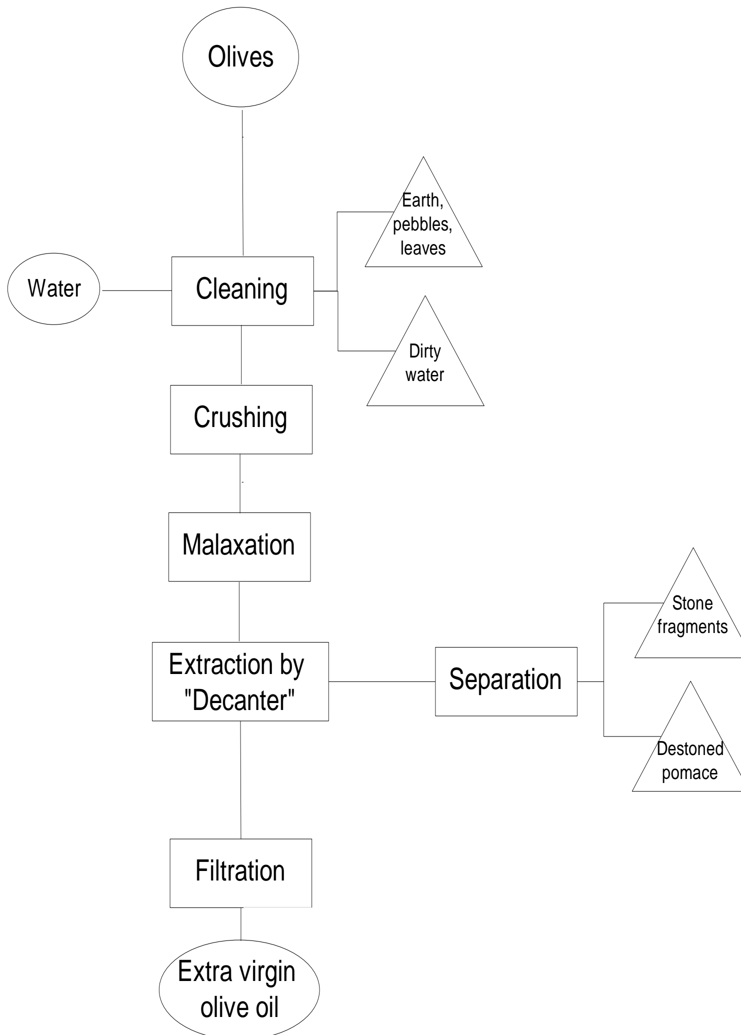


Figure 1: Extraction process of extra virgin olive oil

HARVESTING TIME, HARVESTING TECHNIQUES AND OLIVE STORAGE

The harvesting time, the harvesting methods and the post harvesting storage are the main factor in establishing the final quality of extra virgin olive oil (Clodoveo et al., 2014).

Chosing the right time is useful to obtain the largest quantity and the highest quality of oil (Dag et al., 2011). In order to calibrate the choice of the fruit picking time and the most suitable technology to extract

the extra virgin olive oil, with the desired sensory and nutritional characteristics, it is important to know the influence of the ripening stage on enzyme activities and related phenol composition and concentration, depending on the cultivar characteristics**.** Moreover, during fruit ripening enzyme activity may change, varying the composition of fruits (Clodoveo et al., 2014).

A study on the kinetic and molecular properties of polyphenol oxidase in olive fruits and its relationship with the oleuropein concentration during fruit ripening, found that oleuropein concentration of olive fruit, and consequently in virgin olive oil, depended on *β*-glucosidase, polyphenol oxidase and peroxidase activity (Ortega-Garcia et al., 2008).

El Riachy et al. (2011) observed that in the first period of growth phase, the oleuropein concentration reached higher levels. Indeed, during the green maturation, it declined with the physiological development of the fruit probably due to the increase of hydrolytic enzymes activity. This phenomenon is accompanied by the demethyloleuropein and elenolic acid glucoside increment. During black maturation the decline of oleuropeine continued rapidly, suggesting a likely function of β-glucosidase in this metabolism confirmed by the appearance of oleuropein derivatives. The presence of isomer of oleuropein aglycone and its dialdehydic form of elenolic acid in the olive fruit is important because they may subsequently be released into oil during the mechanical extraction process, and determining appearance, the flavor and the health- promoting properties of the resulting extra virgin olive oil (El Riachy et al., 2011).

Olive peroxidase activity was found to increase during the maturation process (Garcia-Rodriguez et al., 2011), contributing to the phenolic oxidation that takes place during the industrial process of obtaining olive oil (Luaces et al., 2007).

During fruit ripening, the enzyme activity responsible for the virgin olive oil aroma may change. In fact, Kalua et al.,(2007) reported that the enzyme that produces volatile aldehydes and oxoacids (hydroperoxide lyase) was identified in green olive fruits harvested in the initial developmental stages and it slightly decreased at maturity. Also alcohol dehydrogenase activity declined during the ripening process leading to a reduction in the content of C6 alcohols in the aroma of virgin olive oil as the fruit ripeness increased (Salas and Sanchez 1998).

The choice of the harvesting method and its influence on virgin olive oil quality is related to fruit integrity (Clodoveo et al., 2014). If a particular method causes bruises on the fruit surface as a result of its mechanical impact or compression, olive respiration and the susceptibility to decay at a faster rate will increase. The oil extracted from these damaged olives can be high in acidity, low in stability and poor in polyphenols (Garcia et al., 1996). Moreover it could develop off-flavors due to the enzymatic activities favored by the breakdown of the cells and the contact between enzymes and substrates, which were initially compartmented differently. Therefore, hand picking appears to be the best method for preventing fruit damage (Jimenez-Jimenez et al., 2013), even if olive manual harvest is quite expensive. In order to decrease harvest costs, mechanical harvesting has been introduced with the burden of an increase of fruit injury (Kader and Rolle 2004) that might cause a gradual disintegration of the cell structure (Koprivnjak et al., 2000).

Prolonged storage of fruits in uncontrolled conditions produces volatile compounds that are responsible for off-flavors (Kiritsakis, 1998; Koprivnjak et al., 2000) due to the activity of endogenous or microbial enzymes that can find the optimal conditions for their activity.

WASHING AND LEAF REMOVAL

The olives pass over a vibrating screen with a blower that removes leaves and other debris to preserve the extraction plant from damages caused by stones and to avoid off-flavors deriving from the presence of leaves or other foreign bodies. After the

grading step, the olives can be also washed, especially if they have been harvested from the soil or have had spray residues, even if most of the olive washing machines are usually equipped with a water recycling system. This method, if the processing water is not frequently renovated, can promote a high microbial concentration in the recycled water and their fermentation activity, that might compromise the sensory quality of the final product.

CRUSHING

The first step to extract virgin olive oil from fruits consists of crushing the drupes. The choice of the type of crusher is critical to determine the quantity and the quality of the final product because it can influence various properties of the resultant olive paste (Clodoveo 2012). Depending on the different mechanical actions, the main aspects that can change are temperature, granulometry of fragments, exposition to the atmospheric oxygen, and differential crushing of olive tissues. These differences are critical for the release and start of endogenous enzymatic activities as polyphenol oxidase, peroxidase, and *β*-glucosidases (Servili et al., 2012). The virgin olive oil aroma is determined by the activity and properties of the enzymes involved in the LOX pathway that acts immediately after drupe crushing (Sanchez-Ortiz et al., 2012). This enzymatic pool is sensitive in particular to the temperature, as this parameter can affect the level and the activity of enzymes involved in the pathway (Angerosa and Basti, 2001; Angerosa, 2002). If the olive paste temperature rises up to 30 °C, the structure of such enzyme will begin to denature, interrupting the cascade pathway and the synthesis of the aromatic compounds, causing a decrease of volatile contents in virgin olive oil; in particular of concentration of C6 esters, which are very important contributors of delicate green perceptions, and of cis-3-hexen-1-ol, which gives pleasant real green sensations (Angerosa et al., 2001).

The industrial equipments employed to crush the olives are the traditional stone mill, the hammer or disk crusher, and finally the innovative de-stoner (Amirante et al., 2010a).

The stone mill includes 2 to 3 stone wheels, which rotate in circles on a block of granite to crush the olives into paste. It is very expensive and, it does not cut the skin and releases less chlorophyll. However, during the prolonged rotation, large drops of oil are formed**,** and, in some conditions, the malaxing step is unnecessary. Moreover, the energy released during breaking is low and olive paste isn't heated with protective effect on the pathway of LOX (Padilla et al., 2009). The exposition of the olive paste to the atmospheric oxygen can promote the oxidation of polyphenols, but simultaneously a decrease in the bitter and pungent taste of the product (Amirante et al., 2006).

The hammer-crushing machine is a continuous machine with high throughput. It is composed by a four-lobe rotor with wear-resistant metal plates that crush the olives against a stationary grid. The dimension of the olive fragments are regulated by the dimension of the grid holes. The hammer-crusher, cutting deeply the skin, extracts more phenols, so the resultant oil has longer shelf-life than the oil obtained from employing the stone mill (Clodoveo et al., 2014).The hammer-crusher produces some disadvantages: due to the violent mechanical action, it may form an emulsion which impedes oil-water separation, causing a more intense fragmentation of the olive pits and determining a substantial increase in temperature of the olive paste, thus compromising the activity of the LOX pathway (Amirante et al., 2010a). The disk crusher too is a continuous machine with high throughput (Amirante et al, 2010a). Olives fed into the crusher are flung away from the center and crushed as they meet the toothed disc. It is less expensive than the stone mill, but it does not tolerate debris such as rocks and grit. It is possible to have precise regulation of olive paste particle size setting the distance between the disks, but it is not easily adjustable during the working of the machine (Clodoveo et al., 2014). The use of the disk-crusher avoids the olive paste overheating if confronted with the hamer-crusher and the stone mill, minimizing the risk of oxidation. However, also this crusher may form an emulsion, which impede oil–water separation, but they are less abundant if compared to the hammer-crusher action. The use of the disk crusher may also affect the sensory

characteristics: the oil can have a stronger, spicy taste, but less bitter than one obtained by the hammer-crusher, which may be an advantage for “mild taste” or “sweet” olives (Clodoveo et al., 2014).

A relatively new approach to olive-crushing is based on differentiated crushing of the constituent parts of the fruit, such as the skin, pulp and seed. The de-stoner crushes only the pulp tissues (Amirante et al., 2006; Dugo et al., 2007; Servili et al., 2007; Rodrıguez et al., 2008) and the resulting oil has higher phenol content than those obtained by other crushing systems (Amirante et al., 2006; Servili et al., 2007). The use of the de-stoner can improve the working capacity of the mill plant excluding about a quarter of the residual solid waste before the extraction process. Moreover, the seeds, after the recovering, have a high economic appeal because of their residual value for the cosmetic and pharmaceutical industries (Amirante et al., 2010a). Also the de-stoned pomace is easier to use as an animal feed. The absence of stone fragments causes a change in olive paste viscosity, therefore, in order to ensure good virgin olive oil extraction yield, the de-stoned olive paste requires long mixing times and a third-generation decanter to separate the oil from the olive paste (Amirante and Catalano 2000). Mechanical extraction of the olive oil from de-stoned paste emphasizes nutritional and sensory characteristics of the product not only because of the phenolic fraction but also to the volatile compounds produced by the LOX pathway (Clodoveo et al., 2014). However, this technology is not widespread because the de-stoning technology produces minor quantities of higher quality virgin olive oil.

MALAXATION

The malaxer machine consists of a stainless steel tank containing the olive paste, and a malaxing central-screw stirring the paste slowly and continuously, at monitored temperature. During the malaxation process milling process, pectic, cellulosic, and hemicellulosic enzymes are set free, increasing the oil yield (Obergfoll, 1997). These endogenous wall-degrading enzymes are also able to break the oil-water emulsions changing the rheological properties of the paste and to increase the minor compound

concentration (Clodoveo et al., 2014). During malaxation of olive paste, a complex bioprocess occurs (Clodoveo, 2012), modifying deeply the quality and composition of the final product in particular the phenol and volatile contents (Ranalli et al., 2001; Servili et al., 2003). Temperature, time and atmosphere composition inside the malaxer are the main process parameters to control for modulating the endogenous enzymatic activities (Clodoveo, 2012).

An increase in temperature (from 25 to 35 °C) can reduce the enzymatic oxidative reaction causing an increase in both total phenolics and the pungent phenolic oleocanthal (Esposto et al., 2013). However, the increase in temperature lead to an increment of esters and cis-3-hexen-1-ol and an accumulation of hexan-1-ol and trans-2-hexen-1-ol, considered a far from pleasant odor (Angerosa et al., 2001; Kalua et al., 2007). The duration of malaxation can influence aromatic, volatile and phenolic profile (Servili et al., 2003; Gomez-Rico et al., 2009). In fact, an increase in the duration of malaxation lead to an increment of C6 and C5 carbonyl compounds, especially of hexanal, which represents an important contributor to the olive oil flavor (Amirante et al., 2006). Moreover, it favors the activity of *β*-glucosidases which produce the aglycon molecules from the glycosides (Clodoveo et al., 2014) and determine a reduction of the 3,4-DHPEA-EDA and 3,4-DHPEA-EA concentrations, and reduce the oxidative degradation of the 4-HPEA-EDA (Obied et al., 2008).

The control of the atmosphere composition of the headspace of the tank could regulate the oxidative enzymatic activities (Servili et al., 2003; Clodoveo 2012). In the early malaxers, characterized by a cradle shape and a nonhermetic closure, the olive paste was exposed to a great amount of oxygen, which could promote oxidative reaction, while the volatile compounds were vaporized in the ambient atmosphere (Clodoveo et al., 2014). To ensure a perfect control of the atmosphere, which get in contact with the olive paste, an hermetic sealing was added. Even if expensive, an inert gas such as nitrogen or argon was also employed. This reduces the activity of the oxidase enzymes, preserving the polyphenolic substances and inhibits the synthesis of volatile compounds (Clodoveo et al., 2014). If the oxidative enzymes activity is

inhibited under N2, an extension of the duration of malaxing is possible without detrimental effect on virgin olive oil quality. Parenti et al., (2006a, 2006b) suggested to take advantage of the phenomenon of carbon dioxide emission coupled with the oxygen depletion during malaxation under sealed conditions. This technique is more advantageous than the saturation of the malaxation chamber with inert gas (N_2) as a partial oxidation of the fatty acid chains is necessary (especially in the initial part of malaxation) for the development of volatile compounds constituting the aroma through the LOX pathway (Servili et al., 2003).

SEPARATION OF OIL

Tree different systems are used to separate the oil from solid and liquid phases of olive paste: pressure, percolation, or centrifugation (Amirante et al., 2010b).

The pressure extraction system is considered an obsolete technique. It could be a valid form of producing high-quality olive oil only if after each extraction the disks are properly cleaned from the remains of paste, in order to avoid the development of unpleasant odor notes arising from endogenous or microbial enzymatic activities (Clodoveo et al., 2014).

The modern method of olive oil extraction uses two types of industrial decanters to separate all the phases by centrifugation: a 2- or a 3-phase centrifugal decanter (Amirante et al., 2000, 2001). In the 2-phase decanter, paste is separated into a liquid phase and a solid phase, while in the 3-phase centrifugal decanter, the paste is divided into oil, vegetation water, and solids (olive pomace). The main difference between the 2 typologies of machine is the amount of water added to dilute the olive paste: the 2-phase process has low water consumption and low waste water production and the oils obtained exhibited a higher content of polyphenols, induction time values, and sensory score (Di Giovacchino et al., 2001).

The industrial machinery used for the percolation method is known as “Sinolea.” This method is based on the different surface tensions of the vegetation water and the oil. It is an expensive method and the extraction yield obtained is low if not combined

with the horizontal centrifuge. However, the quality of the virgin olive oil is very high because no water is added. Indeed, oil obtained through percolation (1st extraction), has a higher content of phenols, o-diphenols, hydroxytyrosol, tyrosol aglycones, and tocopherols than oils obtained through centrifugation (2nd extraction) (Ranalli et al., 1999).

VERTICAL CENTRIFUGATION

The oil phases are further clarified in an automated discharge vertical centrifuge (disk centrifuge) with lukewarm tap water added that separates the residual water and the solid impurities in order to obtain a clear oil (Clodoveo et al., 2014). However, the addition of water reduces the hydrophilic phenol content, the amount of C6 and C5 volatile compounds and a strong oxygenation of the virgin olive oil, that can lead to a noticeable shortening of the oil shelf-life as a consequence of accelerated oxidation (Giovacchino et al., 1994; Parenti et al., 2007; Masella et al., 2012). A technical solution for reducing the oil oxygenation appears to be virgin olive oil vertical centrifugation under inert gas that causes a strong reduction of the oil oxygenation in terms of reduced dissolved oxygen concentration and oxidative indexes (peroxide values and K232) (Masella et al., 2012).

FILTRATION

There is a dispute between the researchers about the not filtered virgin olive oil and its stability along the time (Fregapane et al., 2006; Lozano-Sanchez et al., 2010). This kind of oil contains polyphenols, phospholipids and sugars that during filtration could be loose favoring a reduction of oxidative stability. However, veiled virgin olive oil could contain too hydrolytic and oxidative enzymes, such as lipase, LOX, and polyphenol oxidase that favor enzymatic reactions (Clodoveo et al., 2014). Moreover, the filtration of VOO can avoid the fermentation of sugars or proteins producing volatile compounds responsible for an unpleasant muddy odor by butyric fermentation.

STORAGE

The virgin olive oil extracted should be stored in stainless steel and maintained at a constant temperature between 10 and 18 °C before bottling. The main cause of oil deterioration during storage is the oxidative rancidity caused by the reaction occurs between unsaturated fatty acids and oxygen (Frankel 1991, (Morello et al., 2004a, 2004). The fatty acid composition and the antioxidant compound concentration, (carotenoids, tocopherols, and phenolic compounds) are the two compositional factors able to determine the virgin olive oil susceptibility to oxidation (Psomiadou and Tsimidou, 2002). Phenolic compounds are responsible for the bitter and pungent taste of oil and are involved in the resistance to oxidation (Clodoveo et al., 2014). The phenolic compounds tend to decrease during the storage, while the lignans seem to be the most stable. On the contrary, the secoiridoid derivatives, 3,4-DHPEA-EDA, pHPEA-EDA and 3,4-DHPEA-EA are characterized by a more active participation in the oxidative processes (Clodoveo et al., 2014). When VOO is improperly stored unpleasant odor or taste arising from the rancidity process could occur (Frankel, 2005). Volatile substances as hexanal, octane and other C8 and C9 compounds, are formed through nonenzymatic oxidation during virgin oil storage. This process is favored by high temperatures, oxygen, light, and pro-oxidants. In order to increase oil shelf life the use of stripping nitrogen to remove the dissolved oxygen from the oil immediately after production has been suggested (Masella et al., 2010).

1.2 National and European regulation

NATIONAL REGULATIONS

The first form of oil regulation goes back to 1890. The Royal Decree Law. 7045 was an initial attempt to resolve issues concerning the authenticity and quality of the oil and fats. Eighteen years later, with the Law No. 136 of 5 April 1908, there was an initial classification of the oil, with the distinction between “genuine olive oil” and “olive oil mixed”. The Law. 562 of 18 March 1926 had as its objective the prevention of fraud in the preparation and trade of substances in agricultural use and agricultural products.

The olive oil was defined as "*the product obtained by processing olive (Olea europaea) without the addition of foreign substances or other oils* "and is distinct from '" mixed oil "and by'" seed oil ". That law authorized "*the sale and the trade, for edible use, of olive oil deodorized, deacidified or refined, provided that no foreign substances is added to correct the color or other properties*" meanwhile the low prohibited the sale "*to edible use, of pomace oil , of rancid oils or oils significantly defective or altered* ". The Royal Decree. 1361 of 1926 defined the parameters for the edible oil, as the total acidity and the absence of "disgusting smells". Subsequently, the Royal Decree Law. 2316 of 1929 required the use of the name "*seeds oil*" and the Law no. 378 of 16 March 1931 allowed the sale of oil extracted from olive pomaces for edible use, with the forced designation of " *edible pomace oils*". In 1936, the Royal Decree Law. 1986 defined "*an official classification of olive oils*" based on the acidity (% of oleic acid) of oils and for the first time the term “*virgin*” was used. Finally, with the Law no. 1407 of 1960, for the first time the expression "*extra virgin*" was used to indicate quality olive oil.

EUROPEAN REGULATIONS

The Treaty of Brussels, in 1966 introduced the Reg. EEC No. 136, which gives life to Single Market Organization (CMO) in oils and fats. In the annex, the virgin olive oil is defined as "*the natural olive oil obtained by mechanical, including pressure, excluding any mixture with other oils or olive oil obtained by another process* ". In this Regulation, the percentage of acidity was the only analytical parameter used for classificating the various types of olive oil. Afterwards, the Reg. EEC No. 1058 of 18 May 1977, considered a discriminatory parameter, not only the percentage of acidity, but also the extinction coefficient at 270 nm. From this moment various regulations were issued; they introduced other parameters, in addition to those already defined, and modified the limits. In Figure 2 is shown the evolution of Community legislation concerning the characteristics of olive oil and the relevant methods of analysis; these

regulations have been reviewed and modified or replaced to protect the quality of olive oil.

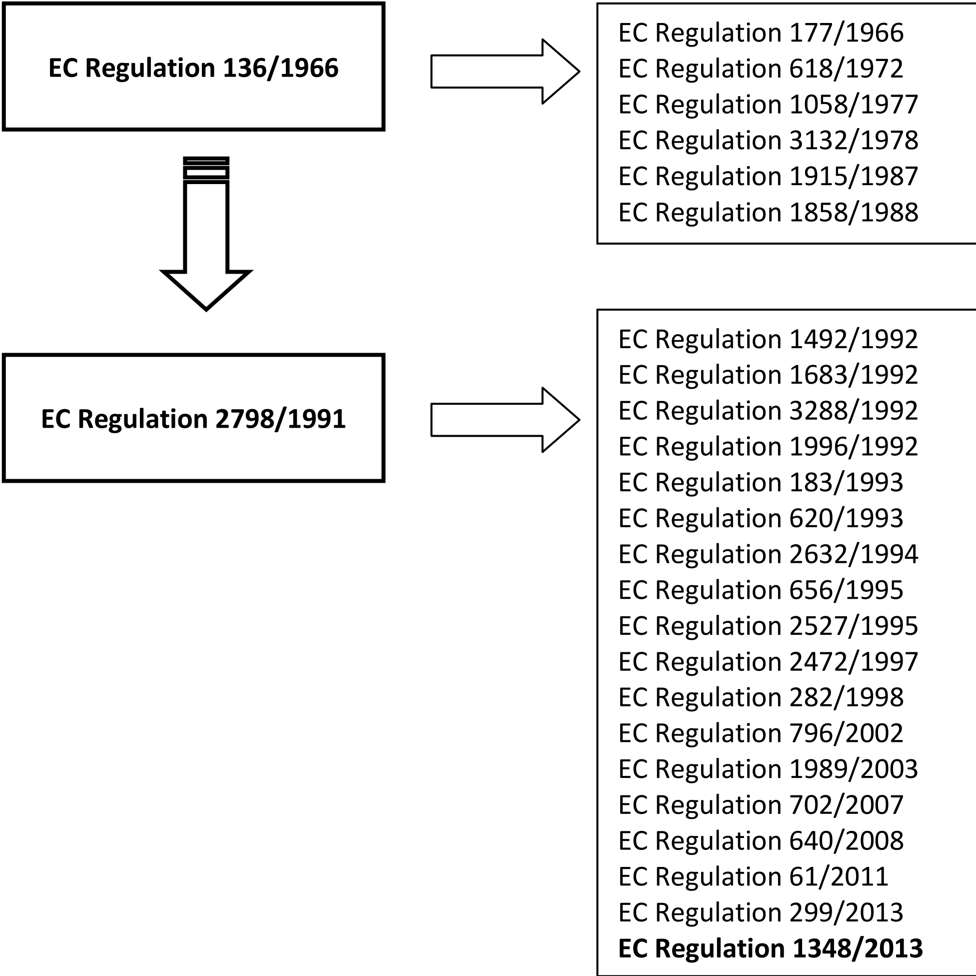


Figure 2: Evolution of Community legislation.

The EC Regulation. 2568 of 11 July 1991 marked an important innovation compared to the previous regulations: it reorganized the Community rules of olive oils, conformed the various analytical methods, bringing them all in a single text and, finally, introduced the sensory analysis of oils a discriminatory parameter. Subsequently, other analytical parameters were introduced to discriminate olive oils, as the content in waxes, in stigmastadienes and evaluation of triacylglycerols. In Table 1 are reported the various classes of olive oil as divided by European legislation (EC Regulation 1348/2013, Annex I).

Table 1: Analytical and sensory properties of olive oils (EC Regulation 1348/2013, Annex I)

Category	Fatty acid ethyl esters (FAEEs) mg/kg (*)	Acidity (%) (*)	Peroxide index mEq O_2/kg (*)	Waxes mg/kg (**)	2-glyceril monopalmitate (%)	Stigmastadienes mg/kg (1)	Difference: ECN42 (HPLC) and ECN42 (2) (theoretical calculation)	K_{232} (*)	K_{268} or K_{270} (*)	Delta-K (*)	Organoleptic evaluation Median of defect (Md) (*)	Organoleptic evaluation Fruity median (Mf) (*)
1. Extra virgin olive oil	FAEEs ≤ 40 (2013-2014 crop year) (3) FAEEs ≤ 35 (2014-2015 crop year) FAEEs ≤ 30 (after 2015 crop years)	≤ 0,8	≤ 20	$C_{42} + C_{44} + C_{46} \leq 150$	≤ 0,9 if total palmitic acid % ≤ 14 %	≤ 0,05	≤ |0,2|	≤ 2,50	≤ 0,22	≤ 0,01	Md = 0	Mf > 0
					≤ 1,0 if total palmitic acid % > 14 %							
2. Virgin olive oil	—	≤ 2,0	≤ 20	$C_{42} + C_{44} + C_{46} \leq 150$	≤ 0,9 if total palmitic acid % ≤ 14 %	≤ 0,05	≤ |0,2|	≤ 2,60	≤ 0,25	≤ 0,01	Md ≤ 3,5	Mf > 0
					≤ 1,0 if total palmitic acid % > 14 %							
3. Lampante olive oil	—	> 2,0	—	$C_{40} + C_{42} + C_{44} + C_{46} \leq 300$ (4)	≤ 0,9 if total palmitic acid % ≤ 14 %	≤ 0,50	≤ |0,3|	—	—	—	Md > 3,5 (5)	—
					≤ 1,1 if total palmitic acid % > 14 %							
4. Refined olive oil	—	≤ 0,3	≤ 5	$C_{40} + C_{42} + C_{44} + C_{46} \leq 350$	≤ 0,9 if total palmitic acid % ≤ 14 %	—	≤ |0,3|	—	≤ 1,10	≤ 0,16	—	—
					≤ 1,1 if total palmitic acid % > 14 %							

Category	Fatty acid ethyl esters (FAEEs) mg/kg (*)	Acidity (%) (*)	Peroxide index mEq O_2/kg (*)	Waxes mg/kg (**)	2-glyceril monopalmitate (%)	Stigmastadienes mg/kg [1]	Difference: ECN42 (HPLC) and ECN42 [2] (theoretical calculation)	K_{232} (*)	K_{268} or K_{270} (*)	Delta-K (*)	Organoleptic evaluation Median of defect (Md) (*)	Organoleptic evaluation Fruity median (Mf) (*)
5. Olive oil composed of refined and virgin olive oils	—	≤ 1,0	≤ 15	$C_{40} + C_{42} + C_{44} + C_{46} \leq 350$	≤ 0,9 if total palmitic acid % ≤ 14 %	—	≤ |0,3|	—	≤ 0,90	≤ 0,15	—	—
5. Olive oil composed of refined and virgin olive oils					≤ 1,0 if total palmitic acid % > 14 %							
6. Crude olive-pomace	—	—	—	$C_{40} + C_{42} + C_{44} + C_{46} > 350$ [6]	≤ 1,4	—	≤ |0,6|	—	—	—	—	—
7. Refined olive-pomace oil	—	≤ 0,3	≤ 5	$C_{40} + C_{42} + C_{44} + C_{46} > 350$	≤ 1,4	—	≤ |0,5|	—	≤ 2,00	≤ 0,20	—	—
8. Olive-pomace oil	—	≤ 1,0	≤ 15	$C_{40} + C_{42} + C_{44} + C_{46} > 350$	≤ 1,2	—	≤ |0,5|	—	≤ 1,70	≤ 0,18	—	—

[1] Total isomers which could (or could not) be separated by capillary column.
[2] The olive oil has to be in conformity with the method set out in Annex XXa.
[3] This limit applies to olive oils produced as from 1st March 2014
[4] Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be lampante olive oil if the total aliphatic alcohol content is less than or equal to 350 mg/kg or if the erythrodiol and uvaol content is less than or equal to 3,5 %.
[5] Or where the median of defect is above 3,5 or the median of defect is less than or equal to 3,5 and the fruity median is equal to 0.
[6] Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be crude olive-pomace oil if the total aliphatic alcohol content is above 350 mg/kg and if the erythrodiol and uvaol content is greater than 3,5 %.

Category	Fatty acid composition [1]						Total transoleic isomers (%)	Total translinoleic + translinolenic isomers (%)	Sterols composition						Total sterols (mg/kg)	Erythrodiol and uvaol (%) (**)
	Myristic (%)	Linolenic (%)	Arachidic (%)	Eicosenoic (%)	Behenic (%)	Lignoceric (%)			Cholesterol (%)	Brassicasterol (%)	Campesterol [2] (%)	Stigmasterol (%)	App β-sitosterol (%) [3]	Delta-7-stigmastenol [2] (%)		
1. Extra virgin olive oil	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,20	≤ 0,20	≤ 0,05	≤ 0,05	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
2. Virgin olive oil	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,20	≤ 0,20	≤ 0,05	≤ 0,05	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
3. Lampante olive oil	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,20	≤ 0,20	≤ 0,10	≤ 0,10	≤ 0,5	≤ 0,1	≤ 4,0	—	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5 [4]
4. Refined olive oil	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,20	≤ 0,20	≤ 0,20	≤ 0,30	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
5. Olive oil composed of refined and virgin olive oils	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,20	≤ 0,20	≤ 0,20	≤ 0,30	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
6. Crude olive-pomace oil	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,30	≤ 0,20	≤ 0,20	≤ 0,10	≤ 0,5	≤ 0,2	≤ 4,0	—	≥ 93,0	≤ 0,5	≥ 2 500	> 4,5 [5]
7. Refined olive-pomace oil	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,30	≤ 0,20	≤ 0,40	≤ 0,35	≤ 0,5	≤ 0,2	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 800	> 4,5
8. Olive-pomace oil	≤ 0,03	≤ 1,00	≤ 0,60	≤ 0,40	≤ 0,30	≤ 0,20	≤ 0,40	≤ 0,35	≤ 0,5	≤ 0,2	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 600	> 4,5

[1] Other fatty acids content (%): palmitic: 7,50-20,00; palmitoleic: 0,30-3,50; heptadecanoic: ≤ 0,30; heptadecenoic: ≤ 0,30; stearic: 0,50-5,00; oleic: 55,00-83,00; linoleic: 3,50-21,00.
[2] See the Appendix to this Annex.
[3] App β-sitosterol: Delta-5,23-stigmastadienol+chlerosterol+beta-sitosterol+sitostanol+delta-5-avenasterol+delta-5,24-stigmastadienol.
[4] Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be lampante olive oil if the total aliphatic alcohol content is less than or equal to 350 mg/kg or if the erythrodiol and uvaol content is less than or equal to 3,5 %.
[5] Oils with a wax content of between 300 mg/kg and 350 mg/kg are considered to be crude olive-pomace oil if the total aliphatic alcohol content is above 350 mg/kg and if the erythrodiol and uvaol content is greater than 3,5 %.

Notes:

(a) The results of the analyses must be expressed to the same number of decimal places as used for each characteristic. The last digit must be increased by one unit if the following digit is greater than 4.

(b) If just a single characteristic does not match the values stated, the category of an oil can be changed or the oil declared impure for the purposes of this Regulation.

(c) If a characteristic is marked with an asterisk (*), referring to the quality of the oil, this means the following: - for lampante olive oil, it is possible for both the relevant limits to be different from the stated values at the same time, - for virgin olive oils, if at least one of these limits is different from the stated values, the category of the oil will be changed, although they will still be classified in one of the categories of virgin olive oil.

(d) If a characteristic is marked with two asterisks (**), this means that for all types of olive-pomace oil, it is possible for both the relevant limits to be different from the stated values at the same time.

At present, there are two other EU regulations governing the olive oil sector. One of these is the Reg. EC 1513/01, which, in Annex I describes and defines the classification of olive oils as:

1. VIRGIN OLIVE OILS

Oils obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alteration in the oil, which have not undergone any treatment other than washing, decantation, centrifugation or filtration, to the exclusion of oils obtained using solvents or using adjuvant having a chemical or biochemical action, or by re-esterification process and any mixture with oils of other kinds

Virgin olive oils are exclusively classified and described as follows:

> <u>(a) Extra virgin olive oil</u>
>
> Virgin olive oil having a maximum free acidity, in terms of oleic acid, of 0,8 g per 100 g, the other characteristics of which comply with those laid down for this category.
>
> <u>(b) Virgin olive oil</u>
>
> Virgin olive oil having a maximum free acidity, in terms of oleic acid, of 2 g per 100 g, the other characteristics of which comply with those laid down for this category.
>
> <u>(c) Lampante olive oil</u>
>
> Virgin olive oil having a free acidity, in terms of oleic acid, of more than 2 g per 100 g, and/or the other characteristics of which comply with those laid down for this category.

2. REFINED OLIVE OIL

Olive oil obtained by refining virgin olive oil, having a free acidity content expressed as oleic acid, of no more than 0,3 g per 100 g, and the other characteristics of which comply with those laid down for this category.

3. OLIVE OIL — COMPOSED OF REFINED OLIVE OILS AND VIRGIN OLIVE OILS

Olive oil obtained by blending refined olive oil and virgin olive oil other than lampante oil, having a free acidity content expressed as oleic acid, of not more than 1 g per 100 g, and the other characteristics of which comply with those laid down for this category.

4. CRUDE OLIVE-POMACE OIL

Oil obtained from olive pomace by treatment with solvents or by physical means or oil corresponding to lampante olive oil, except for certain specified characteristics, excluding oil obtained by means of re-esterification and mixtures with other types of oils, and the other characteristics of which comply with those laid down for this category.

5. REFINED OLIVE-POMACE OIL

Oil obtained by refining crude olive-pomace oil, having a free acidity content expressed as oleic acid, of not more than 0,3 g per 100 g, and the other characteristics of which comply with those laid down for this category.

6. OLIVE-POMACE OIL

Oil obtained by blending refined olive-pomace oil and virgin olive oil other than lampante oil, having a free acidity content expressed as oleic acid, of not more than 1 g per 100 g, and the other characteristics of which comply with those laid down for this category.

The last important Regulation is the Reg. EC 1335/2013, amending Reg. EC 29/2012, which establishes rules regarding the marketing and labeling of oils and food products claiming to contain olive oil.

PROTECTED DESIGNATIONS OF ORIGIN (PDO) AND PROTECTED GEOGRAPHICAL INDICATIONS (PGI)

Citizens and consumers in the Union increasingly demand quality as well as traditional products. They are also concerned to maintain the diversity of the agricultural production in the Union. This generates a demand for agricultural products or foodstuffs with identifiable specific characteristics, in particular those linked to their

geographical origin (Reg. EC 1151/2012). For this reason the European Community introduced the PROTECTED DESIGNATIONS OF ORIGIN (PDO) AND PROTECTED GEOGRAPHICAL INDICATIONS (PGI) regulated by Regulation EC 2081/92, replaced by Regulation EC 510/2006 and finally by Regulation EC 1151/2012. Article 5 of the current Regulation defines:

'designation of origin' is a name which identifies a product:

(a) originating in a specific place, region or, in exceptional cases, a country;

(b) whose quality or characteristics are essentially or exclusively due to a particular geographical environment with its inherent natural and human factors; and

(c) the production steps of which all take place in the defined geographical area.

'geographical indication' is a name which identifies a product:

(a) originating in a specific place, region or country;

(b) whose given quality, reputation or other characteristic is essentially attributable to its geographical origin; and

(c) at least one of the production steps of which take place in the defined geographical area.

The PDO and PGI products must comply with a specification that includes very precise and detailed elements, as described in art. 7 of the current regulation. Before having the recognition, an entry application should be sent to the European Commission, which includes, according to Article 8:

(a) the name and address of the applicant group and of the authorities or, if available, bodies verifying compliance with the provisions of the product specification;

(b) the product specification provided for in Article 7;

(c) a single document setting out the following:

(i) the main points of the product specification: the name, a description of the product, including, where appropriate, specific rules concerning packaging and labelling, and a concise definition of the geographical area;

(ii) a description of the link between the product and the geographical environment or geographical origin referred to in Article 5(1) or (2), as the case may be, including,

where appropriate, the specific elements of the product description or production method justifying the link.

From the date of the submission of the application, the Member State of the applicant gives a national protection, until the final decision of the Commission. If the application is accepted, it can be applied as PDO or PGI after twenty days from the date of publication in the Official Journal of the European Union. As regards the industry of extra virgin olive oil, we find 43 quality products: one PGI (the Tuscan extra virgin olive oil), while all others are PDO (www.istat.it). In Tuscany there are, besides the PGI Tuscan extra virgin olive oil, 4 PDO certifications: Extra Virgin Olive Oil Chianti Classico, Extra Virgin Olive Oil Terre di Siena, Lucca Extra Virgin Olive Oil and Extra Virgin Olive Oil of Seggiano (Migliorini et al., 2009).

1.3 Extra virgin olive oil quality

The evaluation of food quality is based on the degree of consumer satisfaction. According to the UNI EN ISO 9000/2015:

"*The adjective* **quality** *applies to objects and refers to the degree to which a set of inherent characteristics fulfills a set of requirements. The* **quality of an object** *can be determined by comparing a set of inherent characteristics against a set of requirements. If those characteristics meet all requirements, high or excellent quality is achieved but if those characteristics do not meet all requirements, a low or poor level of quality is achieved. So the quality of an object depends on a set of characteristics and a set of requirements and how well the former complies with the latter*".

The customer expectations which should provide an extra virgin olive oil must be related to the characteristics of:

- Safety: the product should not be dangerous for the consumer health;
- Genuineness: the product has been produced according to good manufacturing practice;
- Nutritional: the product is healthy;
- Hedonistic: the product has appreciated features;

- Service: the product should be storable.

Expectations have a mostly generic connotation, so they must be translated into measurable characteristics, defined as product specifications, chemical, physical, biological or sensory. Specifications are selected in reference to laws, voluntary standards or records. In Table 2 is shown the summary table of extra virgin olive oil quality.

Table 2: Summary table of extra virgin olive oil quality

Customer expectations	Product specifications	Bibliographical reference	Methods of analysis	Values of compliance
Safety	phytosanitary residues halogenated solvents (mg/kg)	D.M. 19/5/00 a.m. Reg.(EC)1989/03	GC or HPLC Reg. (EC)1348/2013	legal limits ≤0,2
Genuiness	Acidity(%ac.oleico) N° peroxide K232 K270 Delta-K Organolaptic negative attributes Other specifications	Reg. (EC)1348/2013 Reg. (EC)1348/2013 " " " Reg. (EC)1348/2013 Reg. (EC)1348/2013	Reg. (EC)1348/2013 Reg. (EC)1348/2013 " " " Panel test (Reg. (EC)1348/2013) Reg. (EC)1348/2013	≤ 0,8 ≤20 ≤2,5 ≤0,22 ≤0,01 MD=0 legal limits
Nutritional	Antioxidant activity Phenolic compounds (mg/kg); Secoiridoids Lignans Tocoferoli	Visioli *et al.*,2002 Servilli and Montedoro, 2002 Wagner and Elmadfa, 2000	In vivo and in vitro methods HPLC HPLC	Volontary standard Volontary standard Volontary standard
Hedonistic	Sensory characteristic: Aspect: color turbidity Aroma: fruity taste: bitter spicy "Flavour": grass dried fruit	Reg. (EC)1348/2013 Morales et *al*., 2005	Panel test or colorimetry Panel test or nefelometry Panel test Reg. (EC)1348/2013 Panel test Reg. (EC)1348/2013 Panel test Reg. (EC)1348/2013	Volontary standard MF>0 Volontary standard Volontary standard
Service	Resistance to degradation	Zanoni et al., 2005	Accelerated preservation test Rancimat test	Volontary standard

NUTRITIONAL PROPRIERTIES

The composition of oils is the result of many contributions related to the production and processing of olives. The production area (environment and climate), the features and cultivation systems contribute to the genesis of an olive oil quality.

The extra virgin olive oil consists of about 98% of triglycerides and the remaining 2% of minor components. Triglycerides are chemical compounds formed by a structure of glycerol, to which are bonded three fatty acids, which may be saturated or unsaturated, depending from the absence or not of double bonds along the hydrocarbon chain. The fatty acid composition is determined by the variety of olive and is strongly influenced by the production area that gives some important features to the product. An oil rich in polyunsaturated fatty acids is better from a nutritional point of view, has a more fluid aspect, solidifies less easily at low temperatures but has a shorter shelf life because it is more susceptible to oxidation (Harwood et al., 2000). The peculiarity of the extra virgin olive oil consists in the clear predominance of unsaturated acids compared to the content of saturated, particularly of oleic acid, which being monounsaturated, represents a good compromise between the advantages of unsaturated fatty acids and high oxidation resistance. Of particular importance are also linoleic and linolenic acids. These two unsaturated fatty acids, in fact, are called essential and should be taken thought the diet because the human organism is not able to synthesize them. Moreover, the minor components have an important role to define the particularity of the extra virgin olive oil. These components, although representing no more than 2% of the oil, include more than 200 compounds; among these, phenols and tocopherols, pigments and aromatic substances are of great importance. Contrary to the fatty acid composition, which remains the same from the fruit to the oil, the composition and the relative distribution of the phenolic constituents depend not only on the raw material, but also on the processing technologies and oil storage. The phenols and tocopherols, in particular α-tocopherol (vitamin E), are considered the most important antioxidants naturally found in extra virgin olive oil; they intervene in the prevention of oxidation of

the oil during storage, inactivating chemical reactions between oxygen and oil fatty acids, which lead to the formation of free radicals (Servili et al., 2004; Servili et al.,2009). Secoiridoids derivatives and lignans, have antioxidant activities and healthy properties (Servili et al., 2004; Servili et al.,2009; Cicerale et al., 2010; Obied et al., 2012; Carrera-Gonzales et al., 2013). Moreover, phenolic fraction have a strong impact on bitterness, astringency and pungency; in particular the dialdehydic form of decarboximethyl ligstroside aglycone (*p*-HPEA-EDA) is responsible for the strong "pungent" attribute, while oleuropeina aglycone (3,4-DHPEA-EA) and ligstroside aglycone (p-HPEA-EA) represent the impact components for the "bitter" note (Servili et al., 2014). Also the dialdehydic form of decarboximethyl oleuropein aglycone (3,4-DHPEA-EDA) contributes to the sensation of bitter but with a marginal role for the "pungent" note (Andrewes et al., 2003).

The pigments (chlorophyll and carotenoids) determine the color of the olive oil. The chlorophyll gives a green color, its content depends on the variety and ripeness of the olives but also the technology and transformation temperatures (Mancini, 2012). Chlorophylls may act as pro oxidizing agents, favoring oxidation when the oil is exposed to light. Carotenoids give the oil a yellow color and, unlike the chlorophylls, do not accelerate the oxidation process; on the contrary, the principal carotenoid, the β-carotene, appears to protect the oil from the harmful action of light.

Even though they are minor components of extra virgin olive oil, volatile compounds have a critical role on liking and legal conformity of product. They are so numerous that no clear pathways of biosynthesis and transformation have been so far evidenced (Zanoni, 2014). However, volatile compounds can be correlated with common sensory attributes, both positive and negative (Di Giacinto et al., 2010; Morales et al., 2005). Hexyl acetate, Cis-3-hexenyl acetate, Trans-2-hexenyl acetate, Hexanal, Cis-3-Hexenal, Trans-2-Hexenal, 2.4 Hexadienal, 1-Penten-3-ol, Trans-2-Hexenol, Hexanol, Cis-3-Hexenol, Cis-2- Pentenol, 1-Penten-3-one, are intermediate of LOX pathway and they are considered (Di Giacinto et al., 2010; Kotti et al., 2011; Aparicio et al., 2012) to be responsible for olive oil "fruity", "grassy" and other positive attribute. Trans-2-

heptenal, trans-2-decenal, 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol, 1-octen-3-one and 2-octanone are related to olive oil defects. Indeed, as reported by Morales et al., 2005, these compounds have been associated with "musty", "winey–vinegary", "fusty" and "rancid" defect.

SENSORY EVALUATION

The International Olive Council (IOC) and the European Community (EC) determine the quality of olive oil on chemical parameters, including free fatty acids, peroxide, UV extinction coefficient of (K232 and K270) and sensory evaluations. Based on this analysis, the oil may be classified as extra virgin, virgin or lampante (Zullo et al., 2013). The sensory codified methodology for virgin olive oils is known as the "COI *Panel test*". Such an approach is based on the judgments of a panel of technicians, conducted by a *panel* leader, who has sufficient knowledge and skills to prepare sessions of sensory analysis, motivate judgment, process data, interpret results and draft the report (Mazzalupo et al., 2012). The sensory evaluations were conducted by a *panel* of trained tasters (EN ISO / IEC 2005). The *panel* generally consists of a group of 8 to 12 persons, selected and trained to identify and measure the intensity of the different positive and negative sensations perceived.

According to the Reg. 1348/2013 the positive attributes are defined as:

<u>Fruity</u>: Set of olfactory sensations characteristic of the oil which depends on the variety and comes from sound, fresh olives, either ripe or unripe. It is perceived directly and/or through the back of the nose.

<u>Bitter</u>: Characteristic primary taste of oil obtained from green olives or olives turning colour. It is perceived in the circumvallate papillae on the "V" region of the tongue.

<u>Pungent</u>: Biting tactile sensation characteristic of oils produced at the start of the crop year, primarily from olives that are still unripe. It can be perceived throughout the whole of the mouth cavity, particularly in the throat.

However, the main negative attributes are defined as:

Fusty/muddy sediment: Characteristic flavour of oil obtained from olives piled or stored in such conditions as to have undergone an advanced stage of anaerobic fermentation, or of oil which has been left in contact with the sediment that settles in underground tanks and vats and which has also undergone a process of anaerobic fermentation.

Musty-humid-earthy: Characteristic flavour of oils obtained from fruit in which large numbers of fungi and yeasts have developed as a result of its being stored in humid conditions for several days or of oil obtained from olives that have been collected with earth or mud on them and which have not been washed.

Winey-vinegary-acid-sour: Characteristic flavour of certain oils reminiscent of wine or vinegar. This flavour is mainly due to a process of aerobic fermentation in the olives or in olive paste left on pressing mats which have not been properly cleaned and leads to the formation of acetic acid, ethyl acetate and ethanol.

Rancid: Flavour of oils which have undergone an intense process of oxidation.

Frostbitten olives (wet wood): Characteristic flavour of oils extracted from olives which have been injured by frost while on the tree.

All other negative attributes, such as heated, burnt, hay-wood, rough, greasy, vegetable water, brine, metallic, esparto, grubby and cucumber are described in Annex V of Reg. EC 1348/2013.

Sensory assessment is carried out according to codified rules, in a specific tasting room, using controlled conditions to minimize external influences, using a proper tasting glass and adopting both a specific vocabulary and a profile sheet that includes positive and negative sensory attributes. Collection of the results and statistical elaboration must be standardized (Mazzalupo et al., 2012). Subsequently, the *panel* leader analyzes the responses through a program that can calculate the median, expressed through a decimal coefficient, and the robust coefficient of variation, used for checking the reliability of the panel assessors, as described in Appendix EEC Reg. 1348 / 2013.

The oil is graded by comparing the median value of the defects and the median for the fruity attribute, as:

(a) Extra virgin olive oil: the median of the defects is 0 and the median of the fruity attribute is above 0;

(b) Virgin olive oil: the median of the defects is above 0 but not more than 3,5 and the median of the fruity attribute is above 0;

(c) Lampante olive oil: the median of defect is above 3,5 or the median of the defect is less than or equal to 3,5 and the fruity median is equal to 0.

CRITICAL ASPECTS OF THE PRODUCTION PROCESS

The quality of extra virgin can improve or worsen after the extraction process transformation phenomena due to compounds present already in the olives.

As shown in Figure 3, free radicals and hydroperoxides can be formed by auto-oxidation and photo-oxidation (i.e., *radical oxidation*) of triglycerides. Hydroperoxides are very unstable and break down to alkoxy free radicals, which decompose to aldehydes, alcohols and ketones (Fig. 3). These compounds (as hexanal and nonanal) are volatile and responsible for oil rancid defect (Aparicio et al., 2012; Frankel, 1991; Hamilton, 1983). Absence of water and long holding times at critical temperatures are known to favor non-enzymatic auto-oxidation and photo-oxidation, which are typically defined as slow reactions (Zanoni, 2014).

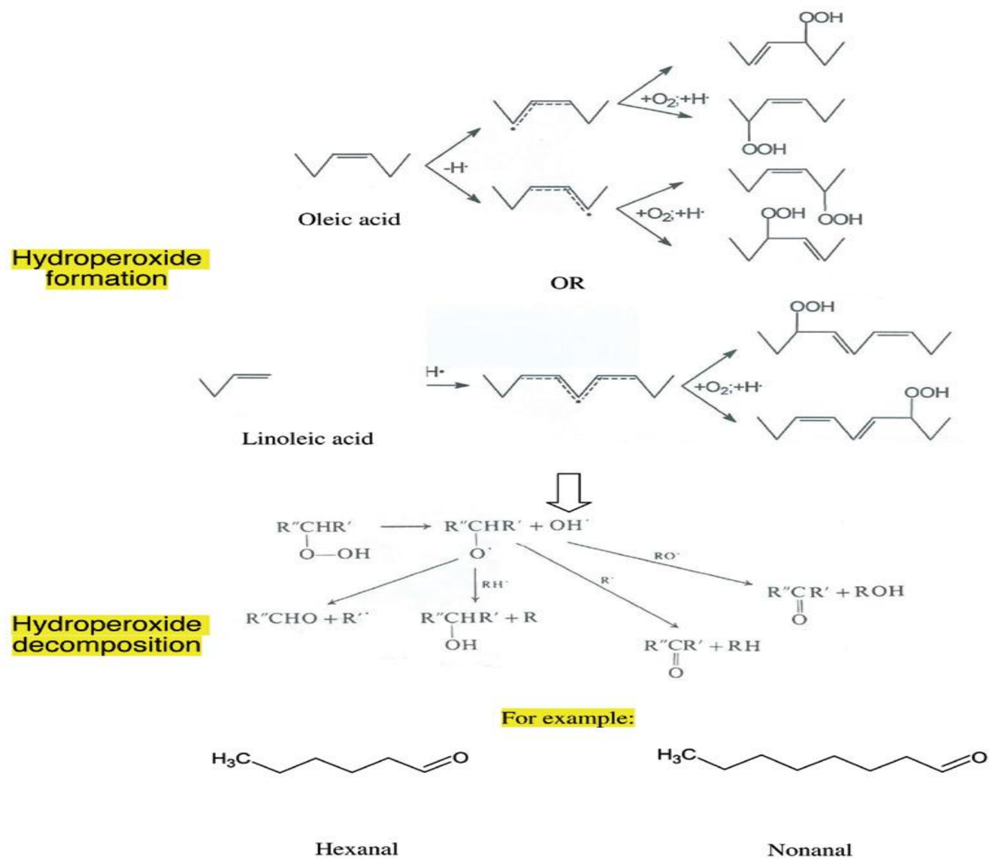


Figure 3: Radical oxidation of triglyceride. Adapted by Frankel (1991) and Hamilton (1983).

Another kind of transformation is the enzymatic oxidation, also called "*LOX pathway*": in this case by the polyunsaturated free fatty acids, through a series of enzymes, including lipoxygenase, are formed aldehydes, alcohols and esters, responsible of the sensory positive attributes of oil, such as, for example, the fruity (Angerosa et al.,

2001). These reactions are favorite by presence of water, fruit surface scratches and olive crushing; moreover, optimal temperature conditions make fast such reactions .
These transformations take place during the process of production of the oil in various stages: lipase activity takes place during the maturation of the olives, enzymatic oxidation occurs during the process of oil extraction and finally the radical oxidation occurs during the oil conservation. Transformation phenomena of triglycerides take place during the process of production of the oil in various stages (Fig. 4): lipase activity occurring during the process operations from olive transport to washing step; enzyme oxidation occurring during the production process of oil (from olive crushing to olive oil separation); radical oxidation occurring during the oil storage and distribution (Zanoni, 2014).

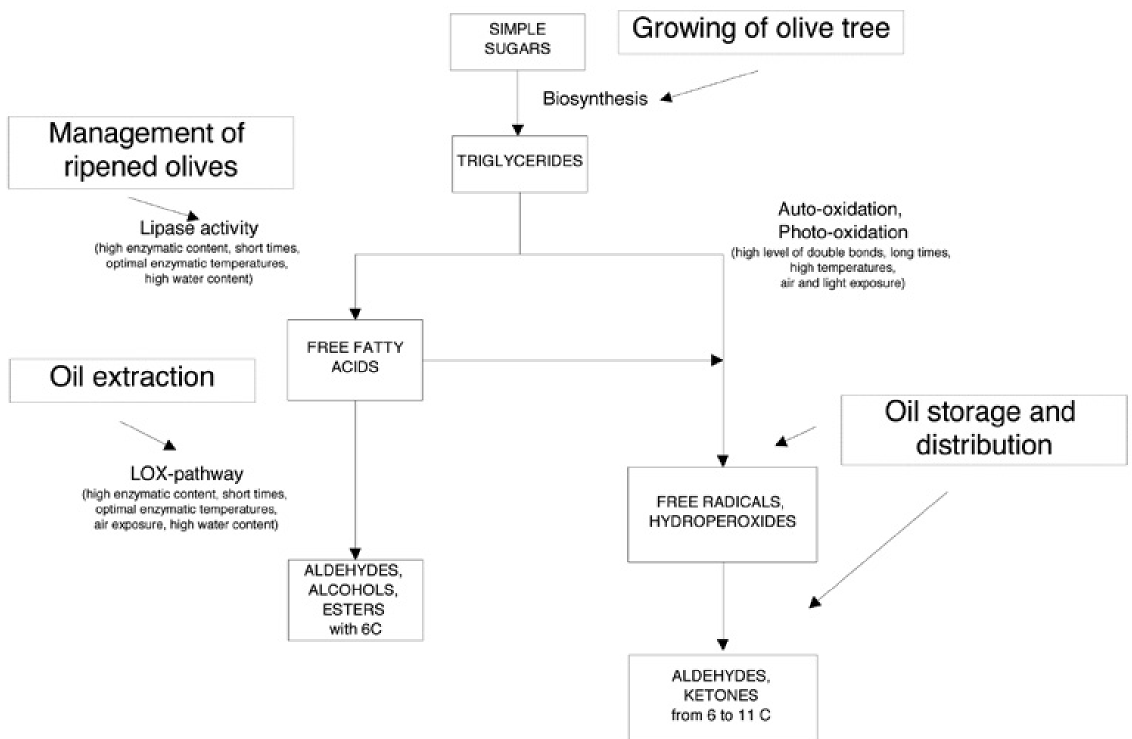


Figure 4: Transformation pathway of triglycerides (Zanoni, 2014)

Even the phenolic component may be subject to changes, which will also impact on various sensory properties, first of all the spicy and bitter. Oleuropein and ligstroside

are thought to be subjected to degradation, resulting in hydrolytic and oxidative changes of both an enzymatic and non-enzymatic nature (Artajo *et al.*, 2007; Guterriez-Rosalez *et al.*, 2010; Kalua *et al.*, 2006, Servili *et al.*, 2004). Hydrolytic degradation pathway (Fig. 5) causes fast formation of aglycons (3,4-DHPEA-EA - oleuropein aglycone; p-HPEA-EA - ligstroside aglycone) that can undergo isomerization, followed by rearrangement into open dialdehydic forms (Zanoni, 2014). Dialdehydic forms in turn decarboxylate into respective aglycons (3,4-DHPEA-EDA – dialdehydic form of decarboximethyl oleuropein aglycone; p-HPEA-EDA - dialdehydic form of decarboximethyl ligstroside aglycone). Finally hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HPEA) compounds are slowly formed by hydrolysis of the ester linkage (Zanoni, 2014).

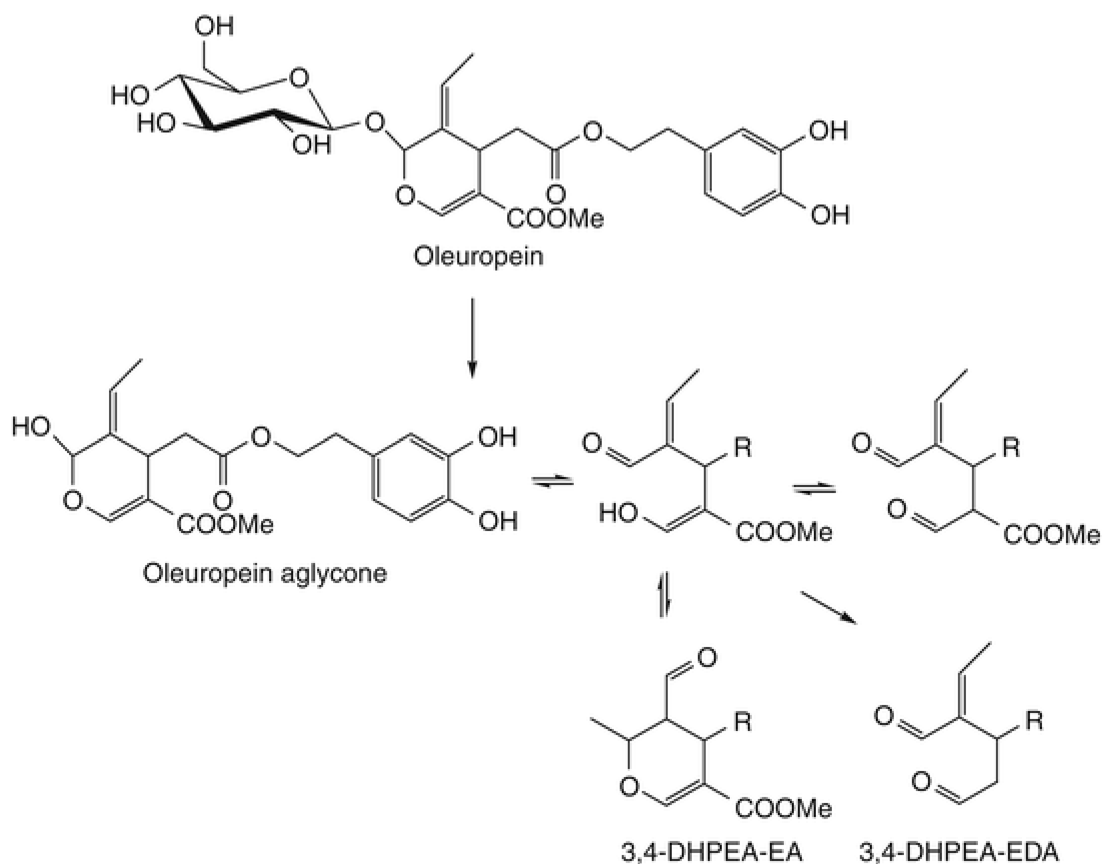


Figure 5: Hydrolytic degradation pathway of oleuropein (Bulotta et al., 2013)

In the enzymatic degradation pathway, polyphenoloxidases and peroxidases in olive paste, in the presence of oxygen and high water content, catalyze the oxidation of phenolic compounds to corresponding quinones; causing browning of the olive paste in processing. The non enzymatic degradation pathway is connected to termination reactions of radical auto-oxidation of triglycerides to peroxides and derivatives. In this reaction the release of hydrogen atoms by phenolic compounds can inhibit the formation of hydroperoxide radicals, that cause a degradation of phenolic compounds and can bring about changes in both intensity of bitter and pungent sensory descriptors and degree of antioxidant power of oil (Zanoni, 2014). The result is the decrease of shelf-life and nutritional value of product. An exception is represented by β-glucosidase activity that increases the amount of phenolic compounds in olive oil, as aglicons are more soluble in oil than oleuropein and ligstroside (Zanoni, 2014).

Biosynthesis of secoiridoids and their first enzymatic hydrolysis to form aglicons occurs from selection of olive cultivar to harvesting of olives. A remarkable presence of 3,4-DHPEA and p-HPEA also in ripe olives (Vinha et al., 2005) should be regarded as a result of biosynthetic pathway of secoiridoids as well as formation of anthocyanins (El Riachi et al., 2011). During oil extraction process, especially during olive crushing and malaxation, enzymatic degradation prevails, as well as the first steps of hydrolytic degradation, leading to the formation of dialdehydic forms of decarboxymethyl aglycons (Zanoni, 2014). On the contrary, during oil storage and distribution, prevail the non-enzymatic oxidative degradation as well as 3,4-DHPEA and p-HPEA forming hydrolytic degradation (Zanoni, 2014). It should be noted that enzymatic activities have both an endogenous and exogenous nature as a result of contamination by moulds, bacteria and yeasts (Vichi et al., 2011). In Figure 6 is shown the transformation pathway of oleuropein and ligstroside as described by Zanoni (2014).

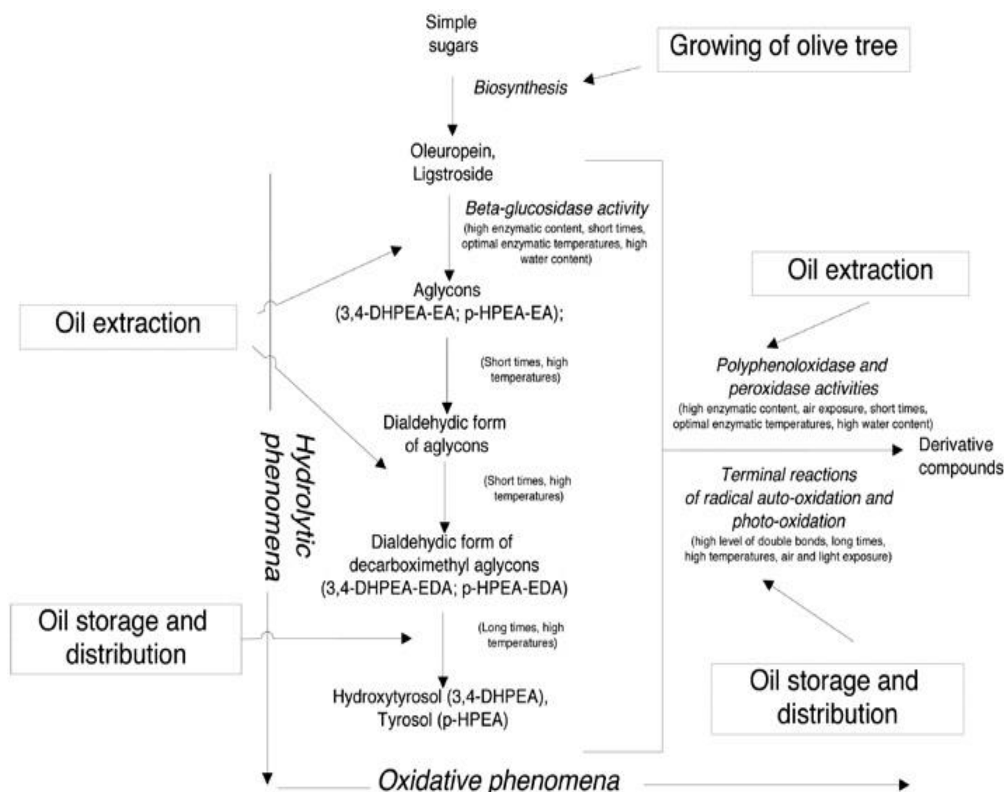


Figure 6: Transformation pathway of secoiridoids (Zanoni, 2014)

Taking into consideration volatile components, fusty, musty and winey-vinegary defects have been shown to be influenced by operating conditions performed during process operations from olive transport to washing step and the oil extraction process. Microbial contamination and activity are very important to develop the above negative attributes. Instead, rancid negative attribute may significantly occur during the oil storage and distribution. Fruity attribute is expressed by LOX pathway, it is then influenced by the oil extraction process conditions (Zanoni, 2014).

Therefore, the control of the whole process of production of extra virgin olive oil is important to obtain a quality product. Such control may be focused on the monitoring of all phases at two levels: the first checks that the various operating conditions, such as residence time and temperature, are the best ones for obtaining a product, while

the second controls the effects of the operations during the process. For the monitoring can be used a basic level or a high level, such as the choice of implementing chemical-physical analyzes of the main parameters or of all parameters that characterize an oil (Zanoni, 2014).

The transformation phenomena of the main components of olive oil are planned to be monitored by critical steps of process (i.e., CCPs) as showed in table 3. Two levels of control are identified: a basic and a high level. Basic level control includes chemical and sensory markers, which are commonly measured to control conformity of both process extraction and extra virgin olive oil, while high level control markers are direct expression of monitoring phenomena. Some table cells are blank, as the relevant simple markers have not yet been identified in the literature (Zanoni, 2014). In the last few years, many studies showed that, in addition to the chemical composition, also the microbial ecology, and its relative metabolic activity, can affect the properties of the oil. Indeed, Ciafardini et al. (2002) had demonstrated that, together with the suspended material in the extra-virgin olive oil, there are numerous micro-organisms, primarily yeasts, that confer certain organoleptic attributes and sensory, both positive and negative (Morales et al, 2005; Vichi et al., 2011; Zullo et al., 2013). Therefore, the microbiological control of the olive oil extraction process might just be the solution for the optimization of the process of production of an extra virgin quality.

Table 3: Process control of extra virgin olive oil chain (Zanoni, 2014).

What? Monitoring phenomena	Where? Critical Control Points (CCPs)	How? Basic level control markers	How? High level control markers	Why? Effects on EVOO quality
Biosynthesis of TGs	Olive harvesting	Visual inspection for olive ripening indices	Olive oil content	Yield
Enzymatic hydrolysis of TGs	Olive storage	Visual inspection for olive sanitary conditions	Olive mould count	Acidity level Shelf-life stability
	Olive oil separation	Olive oil acidity		
LOX-pathway of TGs	Olive oil separation	Olive oil taste	Sum of C6 volatile compounds content from C18:3 free fatty acid	Fruity level
Radical oxidation of TGs	Olive oil storage	Olive oil taste Peroxide value UV spectroscopic indices	Sum of hexanal and nonanal volatile compounds content	Rancid level Peroxide value level UV spectroscopic indices level Antioxidant power
Mass transfer of TGs	Olive paste malaxation	Visual inspection for olive oil coalescence	Microscopic or rheological measurements of olive oil coalescence	Yield
	Olive oil separation	Visual inspection for efficacy of separation	Material balances	
Biosynthesis of PhCs	Olive harvesting	Visual inspection for olive ripening indices	Total phenolic compounds content Oleuropein content	Bitter/Pungency level Antioxidant power Shelf-life stability
Hydrolytic phenomena of PhCs	Olive oil separation	-	3,4-DHPEA-EDA content	Antioxidant power Shelf-life stability
	Olive oil storage	-	3,4-DHPEA content	
Oxidative phenomena of PhCs	Olive paste malaxation	Olive paste color	3,4-DHPEA-EDA content	Bitter/Pungency level Antioxidant power Shelf-life stability
	Olive oil storage	Total phenolic compounds content		
Mass transfer of PhCs	Olive paste malaxation	-	Total phenolic compounds content 3,4-DHPEA-EDA content	Bitter/Pungency level Antioxidant power Shelf-life stability
	Olive oil separation	Olive oil taste Total phenolic compounds content	3,4-DHPEA-EDA content Material balances	
Microbial contamination and activity	Olive storage	Visual inspection for olive sanitary conditions	Olive mould and yeast count	Fusty, Musty and Winey-vinegary level
	Olive oil separation	Olive oil taste	Sum of volatile compounds correlated with negative attributes	

TGs - Triglycerides; PhCs - Secoiridoids; EVOO - Extra Virgin Olive Oil.

1.4 Microbiota of olives and extra virgin olive oil

The issue of the microbial contamination of the oil is of recent interest in food security. Only in the last few years scientific articles have been published on this particular aspect of extra virgin olive oil ,highlighting the interest of not only microbiologists, but also oil producers, because a "safe" oil is synonymous of higher quality and therefore of competitiveness in the global market. The minimum quantity of water (microdrops) naturally present in suspension after the extraction process, even though it represents an excellent substrate for the contamination, has dimensions of the order of microns, greatly limiting microbial growth in terms of number and availability of nutrients (Ciafardini and Zullo, 2002a). However, the presence of a "feature" microbial contamination within the extra-virgin olive oil is not completely synonymous of poor quality oil: in fact, it can be affected positively or negatively, with phenomena desirable or not, strictly dependent on the quality and quantity of the microbiota present.

OLIVES MICROBIOTA

Olives, in addition to being used to produce oil, are also consumed as table olives after a fermentative process. In the numerous research made on this products the role of yeasts have been widely described (Arroyo-Lopez et al., 2006 and 2008). Gonzalez-Sancho (1965) in an article about the fermentation process of green olives, described the presence of a characteristic microflora on the surface of the fruit composed by the genera *Candida, Hansenula, Pichia, Torulopsis* and *Saccharomyces*. From that moment, many researchers were interested in the subject: in 1967, Balatsouras found in Greek olive *cultivars* yeasts belonged to the genera *Trichosporon, Candida, Pichia, Kloeckera, Torulopsis and Debaryomyces*; in 1973 Florenzano et al. investigated the presence of yeasts on the surface of the ripe olives; afterwards, Pelegatti in 1978 analyzed the microorganisms present on the fruit of 12 different *cultivars* of Italian olive tree, isolating 56 yeast isolates. and, moreover, Borcalli et al. (1993), identified on Turkish olives different species of *Debaryomyces*. Duran-Quintana et al. (1986) described that

the olives fermented in the air showed the presence of *Candida saitoana, Debaryomices hansenii, Pichia membraniefaciens* and *Williopsis saturnus var. mrakii*. In 2006 Arroyo-Lopez et al. applied the method for molecular identification of the different species of yeast in table olives. This study led to the identification of new species that had never been described before in table olives : *Issatchenkia occidentalis, Geotrichum candidum* and *Hanseniaspora guilliermondii*. Still Arroyo-Lopez et al. in 2008 investigated the role of yeasts in the deterioration of olives, and isolated even species such as *C. boidinii, Debaryomyces hansenii, P. abnormal, P. membranifaciens, Rhodotorula glutinis* and *S. cerevisiae*.

More recent studies investigated on the lipolytic activity *of S. cerevisiae*, *P.galeiformis* and *P.membranifaciens* (Rodríguez-Gómez et al., 2010) and the correlation between microbiological, chemical and sensory features of *C. parapsilosis, P. guilliermondii e P. kluyveri* (Aponte et al., 2010). In 2011, Bautista-Gallego et al. explored the role of yeast in relation to their metabolic properties, desirable or not, during the process of fermentation of the olives. In this study, the yeast species of *C.diddensiae, S. cerevisiae, P. membranifaciens, C. tropicalis and P. galeiformis* emerged among the species of interest. Finally in 2012, the study of Alves et al., has shown that the fermentation activity of *Citeromyces matritensis*, *Zygotorulaspora mrakii* and *S. cerevisiae* in the final stage of the process was associated with problems of deterioration during storage of the fermented olives.

The bacteria are present in large quantities on the olives, especially in the damaged ones. Their presence can damage oil, if they produce volatile compounds (Morales et al., 2005). *Enterobacteriaceae*: *Aerobacter* and *Escherichi* were found in the initial part of the olives storage, while high amounts of bacteria of the genus *Pseudomonas, Clostridium* and *Serratia* were found after prolonged storage (Angerosa et al., 1996; Rodriguez de la Borbolla, 1958). Compounds found in the oil, as lactic acid and acetic acid, were associated to the presence of bacteria belonging to the *Lactobacillus* genus (Angerosa et al, .1996; Rodriguez de la Borbolla, 1958).

Particular attention should be dedicate to the presence of mould. In olive fruits stored in piles, under high humidity conditions, (Angerosa, Lanza, & Marsilio, 1996; Rodriguez de la Borbolla, 1958) the presence of several species of genus *Aspergillus*, together with ascomycetes, *Penicillium notatum*, have been reported as being among the most abundant *deuteromycetes* (Morales et al., 2005). Other fungi (*Alternaria, Fusarium, Rhizopus*) have also been detected although they were less abundant (Morales et al., 2005). These microorganisms have the ability to oxidize free fatty acids, producing malodorous volatile compounds such as methyl ketones (2-heptanone, 2-nonanone) (Morales et al., 2005) and the production of mycotoxins (Zinedine et al, 2009).

EXTRA VIRGIN OLIVE OIL MICROBIOTA

The microbiota of the olive oil comes from the epidermis of the drupe, migrating from its surface to the oil during the steps of crushing and kneading. Studies on the microbiota of the oil are recent and confirm the presence of a microbial population similar to that present on the surface of the drupe, independently by the kind of the oil extraction process. Most of the research consider oils from the Mediterranean region (Italy, Spain and Greece), the States of greater production, consumption and export of extra virgin olive oil.

Ciafardini and Zullo have been the first to study the microbial populations in olive oil. In 2002 they conducted a research for identifying lactic acid bacteria, yeasts and molds in oil extracted from olives belonging to the *cultivar* "leccino", during the process of sedimentation at low temperatures. The results reported that yeasts were consistently present in the initial phase and during storage, moulds belonging to the genus *Aspergillus* were present only occasionally, while bacteria were not found. Moreover, it was observed that newly produced olive oil contained numerous solid particles and microdrops of olive vegetation water containing, trapped within, a high number of microorganisms that remain during the entire period of olive oil preservation (Fig. 7) (Ciafardini and Zullo, 2002a). Microbiological analyses carried out on the sediments demonstrated a high number of yeasts especially in the samples taken during the first

10 days of decantation of the olive oil (Ciafardini and Zullo, 2002b). Yeast isolates identified in the extravirgin olive oil during these first studies belonged to the species *S. cerevisiae* and *Candida wickerhamii*; afterwards, in the following years, also *Williopsis californica, Candida boidinii* (Ciafardini, 2003; Ciafardini et al., 2004; Ciafardini et al., 2006b;) *Pichia mexicana* and *Pichia minuta* (Zullo and Ciafardini, 2008) were identified.

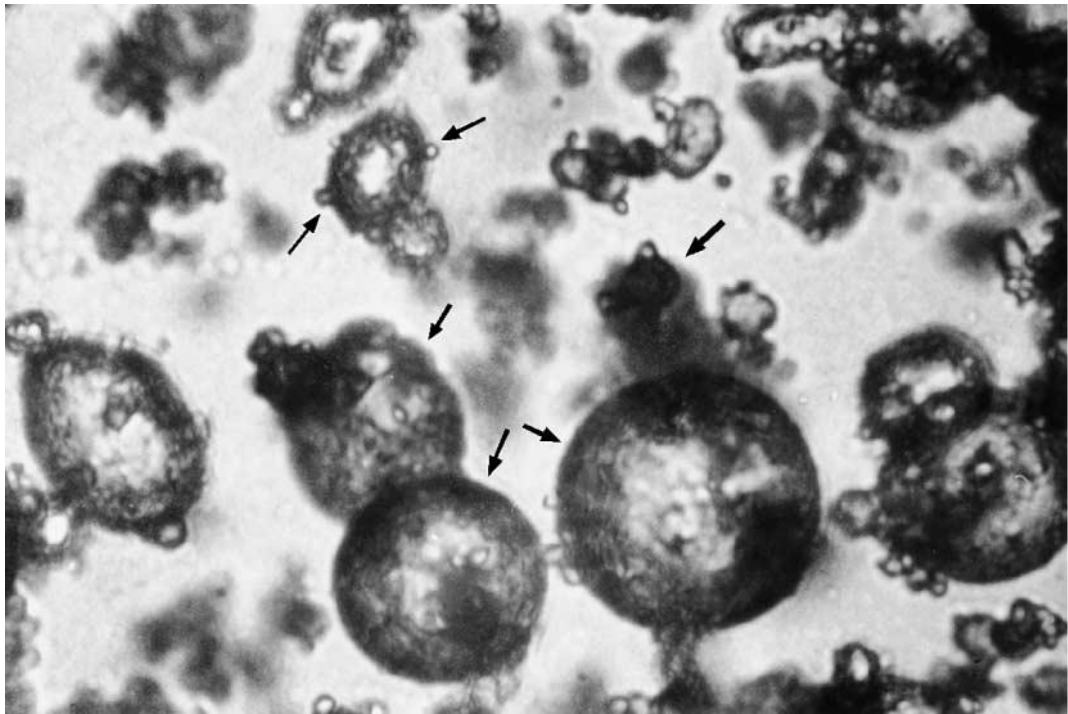


Figure 7: Microdrops of vegetation water and solid particles observed with a light microscope at 600 magnification in the newly produced olive oil. The arrows show the microorganisms and the solid particles entrapped in the microdrops of vegetation water suspended in the olive oil (Ciafardini and Zullo, 2002a).

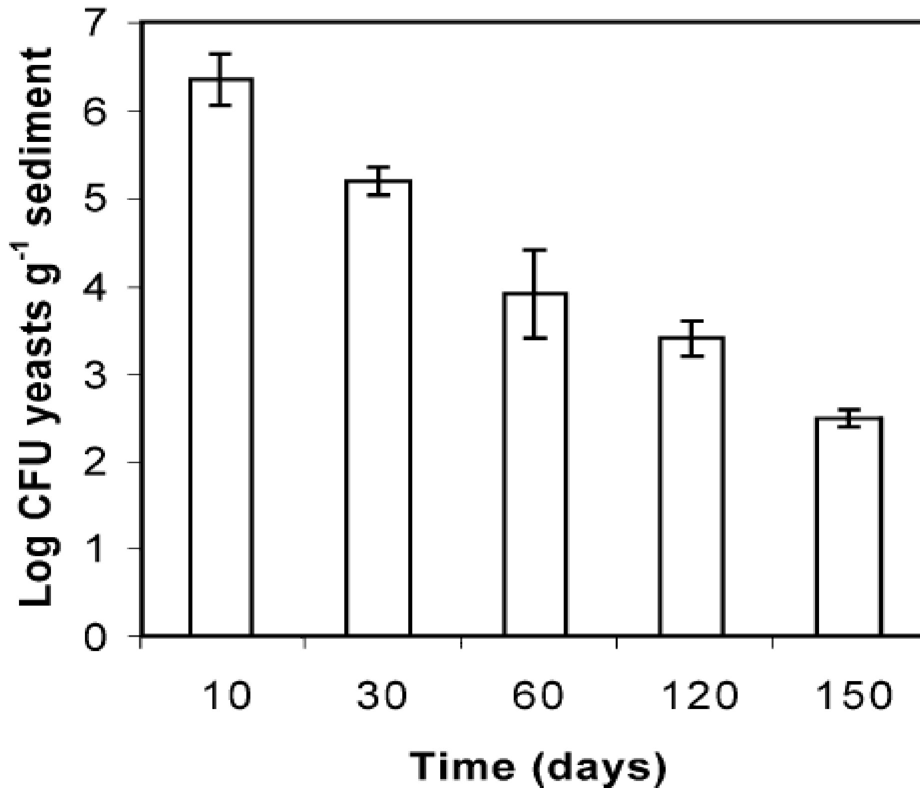


Figure 8: Yeast found in the sediments of olive oil during storage (Ciafardini and Zullo, 2002b).

Other studies have found in the freshly produced olive oil, the presence of dimorphic yeast, considered to be opportunistic pathogens for humans (Koidis et al., 2008; Zullo end Ciafardini, 2008; Zullo et al., 2010). Two species were found to be opportunistic pathogens for humans: *Candida parapsilosis* and *Candida guillermondi*, while *Candida diddensiae* is a dimorphic yeasts considered not pathogenic to human. Nevertheless, many researches carried out by other authors demonstrate that both the two opportunistic pathogenic species and *C. diddensiae* are widespread on the carposphere of the olive fruits since they were found in the waters of seasoned table olive fermentation (Hernández et al., 2007, 2008; Hurtado et al., 2008) or in olive flies *Bactrocera oleae larvae* (Chakri et al., 2007).

An important contribution to the study of the olive oil microbiota was made by Koidis et al. in 2008, which observed the presence of lactic acid bacteria, yeasts and moulds

in the cloudy olive oil during a storage period of three months. In this study, the yeast population (composed by the species *C. guilliermondii, C. parapsilosis, Candida lusitaniae, Candida famata , Candida. albicans,* and *Rhodotorula mucilaginosa)* was not the most dominant microbial population; bacteria (Lactic acid bacteria) and moulds (*Helicosporium*, *Alternaria*, *Penicillium* and *Aspergillus* genera) were also observed in this study. The different results between the studies of Ciafardini and Zullo (2002a) and Kodis et al.(2008) were probably due to the different environment in the olive tree (air, soil, *etc.*) and to the contamination of the olive oil from the conditions in the olive mill (Kodis et al., 2008).

In 2012 Cadez et al. had isolated, identified and described, in virgin olive oil and its by-products, two new species: *Candida adriatica* and *Candida molendinolei*, while, in 2013, another new species, *Yamadazyma terventina*, was described in extra virgin olive oil, during the storage phase (Ciafardini et al., 2013).

Only a few studies have focused on the microbiota of the olive oil extraction process. In the study of Giannoutsou et al *(*2004) three selected isolates from olive oil pomace were identified as being most closely related to *Saccharomyces sp., Candida boidinii* and *Geotrichum candidum*. Moreover, in 2010 Romo-Sanchez had studied the yeast biodiversity of fresh olive (*Olea europaea*) fruits, olive paste (crush olives) and olive pomace from Arbequina and Cornicabra varieties. In this study fourteen different species of yeasts were identified, they belonged to seven different genera: *Zygosaccharomyces, Pichia, Lachancea, Kluyveromyces, Saccharomyces, Candida, Torulaspora*. As shown in Fig. 9, *Pichia caribbica, Zygosaccharomyces fermentati (Lachancea fermentati)* and *Pichia holstii (Nakazawaea holstii)* were the most commonly isolated species, followed by *Pichia mississippiensis, Lachancea sp., Kluyveromyces thermotolerans* and *Saccharomyces rosinii* (Romo-Sanchez et al., 2010).

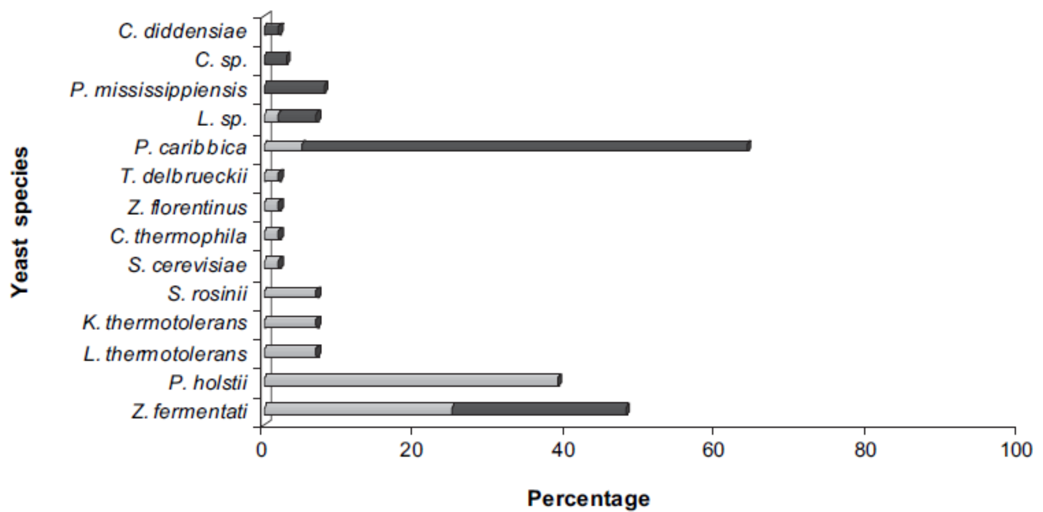


Figure 9: Percentage of yeast species isolated in olive fruit, olive paste and olive pomace from Arbequina ■ and Cornicabra □ varieties.

MICROBIAL CONTAMINATION AND SENSORY PROPERTIES OF EXTRA VIRGIN OLIVE OIL

The extra virgin olive oil is usually consumed after a storage period that can range from few weeks to a several months. During this period a significant improvement of the chemical-physical and sensorial properties of the product is observed. Until a few years ago, studies had focused mainly on the description of the nutritional and chemical properties of oil, while the enzyme activity observed was attributed exclusively to the endogenous enzymes present in the fruit (Montedoro et al., 1993; Botia et al., 2001). Subsequently, investigating the role of microbial contamination present in extra virgin olive oil, it became obvious that the enzymatic activity responsible for the chemical changes of the oil could be caused by the microorganisms. The most investigated microbial activities is related to the metabolism of the yeasts, which are the microbial population more frequently isolated in the olive oil. Indeed, the yeasts seem to play a key role in the storage phase, influencing positively or negatively, the organoleptic and nutritional properties of the product.

In fact, during the storage of newly produced olive oil the disappearance of the bitter taste occurs; it is due to the enzymatic hydrolysis of the bitter-tasting secoiridoid compound known as oleuropein by the ß-glucosidase activity present in the olives. The study of Ciafardini and Zullo (2002b) demonstrated that oleuropein present in olive oil can be also hydrolysed by ß-glucosidase from the yeasts *Saccharomyces cerevisiae* and *Candida wickerhamii*.

Unfortunately, the yeasts enzymatic activity may also be responsible for undesirable phenomena that take place in the oil. Ciafardini and Zullo in 2006 and 2008 had demonstrated the presence of lipase-positive yeasts (the strains belonged to *S.cerevisiae*, *W. californica, C.wickerhamii and C.parapsilosis*) in some samples of extra virgin olive oil. This enzymatic activity can lower the quality of the oil through the hydrolysis of the triglycerides, increasing both the diglycerides and the acidity due to production of free fatty acids. These chemical modifications may affect the classification of the oil, according to the parameters of law, and change in taste and odor (defect of rancidity). The lipolytic activity of some lipase-producer strains (belonging to the species *Candida adriatica, Candida diddensiae* and *Yamadazyma terventina*)can be modulated by the water and the polyphenol content of olive oil. In fact, when the lipase-producer strains were inoculated in olive oil characterized by high water content and low polyphenol concentration, a substantial increase in free fatty acid was observed (Ciafardini and Zullo, 2015). The typical phenolic compounds of olive oil represent an important factor able to condition the viability and the lipolytic activity of the lipase-producer yeasts (Ciafardini and Zullo, 2015). In 2013 Zullo et al. demonstrated the influence which some yeast strains, belonging to *Candida adriatica, Candida diddensiae* and *Candida wickerhamii* species, have had on the olive oil sensory characteristics during its storage. After four months of storage, the inoculated oils were still classified as extra virgin, according to the mean of the five analytical indices analyzed (free fatty acids, peroxide value, K232, K270 and DK). The sensory attributes of the treated olive oils instead, varied according to the composition of the volatile and non volatile carbonyl compounds produced with the

treatments. The analyses of volatile and non volatile carbonyl compounds, according to the yeast inoculated, showed in the samples of oil treated with *C. adriatica*, *C. wickerhamii* and some strains of *C. diddensiae*, a lower concentration of C6 volatile carbonyl compounds and polyphenols, responsible for positive oil attributes. “Muddy-sediment”, “rancid” or both defects were found in olive oil samples treated with *C. adriatica* DAPES 1933, *C. wickerhamii* DAPES 1885 and *C. diddensiae* DAPES 1912 and 1913 strains. On the contrary, olive oil samples treated with *C. diddensiae* DAPES 1918 and 1922 after four months of storage were defect-free, and still categorized as extra virgin, according to the requirements of both chemical and sensory quality indices of European Community Regulations (Zullo et al., 2013).

The study of Romo-Sanchez et al. (2010) showed the biotechnological properties of 108 isolates from olive oil extraction process. ß-glucosidase, ß -glucanase, carboxymethylcellulase, polygalacturonase, peroxidase and lipase activity was evaluated. The results pointed out that none of the isolates showed lipase activity, a few number showed cellulase and polygalacturonase activities and the majority of them presented ß -glucanase, ß -glucosidase and peroxidase activities.

The study of the olive oil microbiota and its enzymatic activity is very important, in order to better understand the origin of olive oil sensory defects (Vichi et al., 2011).

REFERENCES

Alves M., Gonçalves T., Quintas C., (2012). Microbial quality and yeast population dynamics in cracked green table olives fermentations. Food Control, 23, 363-368.

Andrewes P, Busch JL, de Joode T, Groenewegen A, Alexandre H. 2003. Sensory properties of virgin olive oil polyphenols: Identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. Journal of Agricultural and Food Chemistry, 51, 1415–1420

Amirante R., Catalano P. 2000. PH—postharvest technology: fluid dynamic analysis of the solid–liquid separation process by centrifugation. Journal of Agricultural Engineering Research, 77, 193–201.

Amirante R., Cini E., Montel G.L., Pasqualone A. 2001. Influence of mixing and extraction parameters on virgin olive oil quality. Grasas Aceites, 52, 198–201.

Amirante P., Clodoveo M.L., Dugo G., Leone A., Tamborrino A. 2006. Advance technology in virgin olive oil production from traditional and de-stoned pastes: influence of the introduction of A, heat exchanger on oil quality. Food Chemistry, 98, 797–805.

Amirante P., Clodoveo M.L., Leone A., Tamborrino A., Paice A. 2010a. Influence of the crushing system: phenol content in virgin olive oil produced from whole and de-stoned pastes. In: Health and Disease Prevention, London, England: Academic Press Ltd, Elsevier Science Ltd. 69–76.

Amirante P., Clodoveo M.L., Leone A., Tamborrino A., Patel V.B. 2010b. Influence of different centrifugal extraction systems on antioxidant content and stability of virgin olive oil olives and olive oil. In: Health and Disease Prevention, London, England: Academic Press Ltd, Elsevier Science Ltd. 85–93.

Angerosa F. 2002. Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. European Journal of Lipid Science *and* Technology, 104, 639–660.

Angerosa F., Basti C. 2001. Olive oil volatile compounds from the lipoxygenase pathway in relation to fruit ripeness. Italian Journal of Food Science, 13, 421–428.

Angerosa F., Mostallino R., Basti C., Vito R. 2001. Influence of malaxation temperature and time on the quality of virgin olive oils. Food Chemistry, 721, 19–28.

Angerosa F., Lanza B., Marsilio V. 1996. Biogenesis of “fusty” defect in virgin olive oils. Grasas y Aceites, 47, 142-150.

Aparicio R., Morales M.T., Garcia-Gonzales D.G. 2012. Towards new analyses of aroma and volatiles to understand sensory perception of olive oil. European Journal of Lipid Science and Technology, 114, 1114-1125.

Aponte M., Ventorino V., Blaiotta G., Volpe G., Farina V., Avellone G., Lanza M.C., Moschetti G. 2010. Study of green Sicilian table olive fermentations through microbiological, chemical and sensory analyses. Food Microbiology, 27, 162–170.

Arroyo-Lopez, F.N., Duran-Quintana, M.C., Ruiz-Barba, J.L., Querol, A., Garrido-Fernandez, A. 2006. Use of molecular methods for the identification of yeast associated with table olives. Food Microbiology, 23, 791-769.

Arroyo-López F.N., Querol A., Bautista-Gallego J., Garrido-Fernández A., 2008. Role of yeasts in table olive production. Food Microbiology, 23, 791–796.

Artajo L.S., Romero M.P., Saurez M. and Motilva M.J. 2007. Partition of phenolic compounds during the virgin olive oil industrial extraction process. European Food Research and Technology, 225, 617.

Balatsouras G.D. 1967. Processing the naturally ripe back olives. In:Preoceeding of the International Olive Oil Seminar. International olive oil council. Perugia-Spolete, Italy. 491, 510.

Bautista-Gallego J., Rodríguez-Gómez F., Barrio E., Querol A., Garrido-Fernández A., Arroyo-López F.N. 2011. Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. International Journal of Food Microbiology, 147, 89–96.

Borcalli M., Orzay G., Alperden I., Ozsan E., Erdek Y. 1993. Changes in the chemical and microbiological composition of two varieties of olive during fermentation. Grasas y Aceites, 44, 253- 260.

Botıa J., Ortuno M.A., Benavente-Garcia O., Baidez A.G., Frıas J., Marcos D., Del Rıo J.A. 2001. Modulation of the biosynthesis of some phenolic compounds in Olea europea L. fruits: their influence on olive oil quality. Journal of Agricultural and Food Chemistry 49, 355– 358.

Bulotta S., Oliverio M., Russo D., Procopio A. 2013. Biological Activity of Oleuropein and its Derivatives. Natural Products, 3605-3638

Čadež, N., Raspor, P., Turchetti, B., Cardinali, G., Ciafardini, G., Veneziani, G., Peter, G., 2012. *Candida adriatica* sp. nov. and *Candida molendinolei* sp. nov., two novel yeast species isolated from olive oil and its by-products. International Journal of *Systematic and Evolutionary* Microbiology, 62, 2296-2302.

Carrera-Gonzales M.P, Ramìrez-Esposito M.J., Mayas M.D., Martìnez-Martos J.M. 2013. Protective role of oleuropein and its metabolita hydroxytyrosol on cancer. Trends in Food Science & Technology, 31, 92–99.

Chakri M., El Haidani A., El Mzibri M., Haggoud A., Iraqui H.M., Houari A., Ibnsouda K.S. 2007 Yeast strains from the endogenous microfloras of the olive flies *Bactrocera oleae larvae*

which could degrade the olive oil mill wastewaters polyphenols. Annals of Microbiology, 57, 143-148

Ciafardini G. 2003. Presenza di microrganismi nell'olio extra vergine d'oliva. L'Informatore Agrario, 44, 39-42.

Ciafardini G., Cioccia G., Peca G., Zullo B.A. 2004.Transfer of selected yeasts to oil through olive inoculation. Italian Journal of Food Science, 1, 1–7.

Ciafardini, G., Zullo, B.A., 2002a. Survival of microorganisms in extra virgin olive oil during storage. Food Microbiology, 19, 105-109.

Ciafardini G., Zullo B.A. 2002b. Microbiological activity in stored olive oil. International Journal of Food Microbiology, 75, 111–118.

Ciafardini G., Zullo B.A., Iride A. 2006a. Lipase production by yeasts from extra virgin olive oil. Food Microbiology, 23, 60–67.

Ciafardini G., Zullo B.A., Cioccia G., Iride A. 2006b. Lipolytic activity of Williopsis californica and Saccharomyces cerevisiae in extra virgin olive oil. International .Journal of Food Microbiology, 107, 27–32.

Ciafardini G., Zullo B.A., Antonielli L., Corte L., Roscini L., Cardinali G., 2013. *Yamadazyma terventina* sp.nov., a yeast species of the *Yamadazyma* clade from Italian olive oils. International Journal of Systematic and Evolutionary *Microbiology*, 63, 372-376.

Ciafardini G., Zullo B.A. 2015 Effect of lipolytic activity of *Candida adriatica, Candida diddensiae* and *Yamadazyma terventina* on the acidity of extra-virgin olive oil with a different polyphenol and water content. Food Microbiology, 47, 12-20.

Cicerale S., Lucas L., Keast R. 2010. Biological activities of phenolic compounds present in virgin olive oil International Journal of Molecular Sciences, 11, 458–479.

Clodoveo M.L. 2012. Malaxation: influence on virgin olive oil quality Past present and future—an overview. Trends in Food Science and Technology, 25, 13–23.

Clodoveo M.L., Hbaieb R.H., Kotti F., Scarascia Mugnozza G., and Gargouri M. 2014. Mechanical Strategies to Increase Nutritional and Sensory Quality of Virgin Olive Oil by Modulating the Endogenous Enzyme Activities Comprehensive Reviews in Food Science and Food Safety, 13, 135-154.

Dag A., Kerem Z., Yogev N., Zipori I., Lavee S., Ben-David E. 2011. Influence of time of harvest and maturity index on olive oil yield and quality. Scientia Horticulturae, 127, 358–366.

Di Giacinto L., Di Loreto G., Di Natale C., Gianni G., Guasti S., Migliorini M., Pellegrino M., Perri E. and Santonico M. 2010 Caratterizzazione analitica degli attributi sensoriali degli oli vergini di oliva - AROMOLIO. Camera di Commercio di Firenze, Nuova Grafica Fiorentina, Firenze.

Di Giovacchino L. Solinas M., Miccoli M. 1994. Effect of extraction systems on the quality of virgin olive oil. Journal of the American Oil Chemists' Society, 71, 1189–1194.

Di Giovacchino L., Costantini N., Serraiocco A., Surricchio G., Basti C. 2001. Natural antioxidants and volatile compounds of virgin olive oils obtained by two or three-phases centrifugal decanters. European Journal of Lipid Science and Technology, 103, 279–285.

Dugo G., Pellicano T.M., Pera L., Lo Turco V.L., Tamborrino A., Clodoveo M.L. 2007. Determination of inorganic anions in commercial seed oils and in virgin olive oils produced from de-stoned olives and traditional extraction methods using suppressed ion exchange chromatography (IEC). Food Chemistry, 102, 599–605.

Duran- Quintana, M. C., Garcìa Garcìa, P., Garrido Fernandez, A., (1986). Fermentacìon en medio aeròbico de aceitunas negras maduras en salmuera con inyecciòn alternante de aire. Grasas y Aceites, 37, 242-249.

El Riachy M., Priego-Capote F., Leòn L., Rallo L., de Castro L., Dolores M. 2011. Hydrophilic antioxidants of virgin olive oil Part 2: biosynthesis and biotransformation of phenolic compounds in virgin olive oil as affected by agronomic and processing factors. European Journal of Lipid Science and Technology, 113, 692–707.

Esposto S., Veneziani G., Taticchi A., Selvaggini R., Urbani S., Maio I.D., Sordini B., Minnocci A., Sebastiani L., Servili M. 2009. Monitoring of virgin olive oil volatile compounds evolution during olive malaxation by an array of metal oxide sensors. Food Chemistry, 113, 345–350.

European Community, Commission Regulation 2568/91, 1991. On the characteristics of olive oil and olive residue oil and on the relevant methods of analysis. Official Journal of the European Communities, July 11, L248, 1-83.

European Community, Commission Regulation 2472/97, 1997. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, December 11, L341, 25-39.

European Community, Commission Regulation 796/2002, 2002. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, May 6, L128, 8-28.

European Community, Commission Regulation 1989/2003, 2003. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, November 6, L295, 57-77.

European Community, Commission Regulation 640/2008, 2008. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, July 4, L178, 11-16.

European Community, Commission Regulation 61/2011, 2011. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, January 24, L23, 1-14.

European Community, Commission Regulation 1348/2013, 2013. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, December 16, L338, 31-67.

Frankel E.N. 1991. Review recent advances in lipid oxidation. Journal of the Science of Food and Agriculture, 54, 495–511.

Fregapane G., Lavelli V., Leòn S., Kapuralin J., Desamparados S.M. 2006. Effect of filtration on virgin olive oil stability during storage. European Journal of Lipid Science and Technology, 108, 134–142.

Garcìa J.M., Gutièrrez F., Castellano J.M., Perdiguero S., Morilla A., Albi M.A. 1996. Influence of storage temperature on fruit ripening and olive oil quality. Journal of Agricultural and Food Chemistry, 44, 264–267.

Garcìa-Rodrìguez R., Romero-Segura C., Sanz C., S´anchez-Ortiz A, P´erez A.G. 2011. Role of polyphenol oxidase and peroxidase in shaping the phenolic profile of virgin olive oil. Food Research International, 44, 629–35.

Giannoutsou, E.P., Meintanis, C., Karagouni, A.D. 2004. Identification of yeast strains isolated from a two-phase decanter system olive oil waste and investigation of their ability for its fermentation. Bioresource Technology, 93, 301-306.

Gòmez-Rico A., Inarejos-Garcia A.M., Salvador M.D., Fregapane G. 2009. Effect of malaxation conditions on phenol and volatile profiles in olive paste and the corresponding virgin olive oils (Olea europaea L, Cv Cornicabra). Journal of Agricultural and Food Chemistry, 57, 3587–3595.

Gonzalez-Cancho F. 1965. Yeast ecology in French cider and black olive natural fermentation. Grasas y Aceites, 16, 230-234.

Gutierrez-Rosales G., Romero M.P., Casanovas M., Motilva M.J. and Minguez-Mosquera M.I. 2010. Metabolites involved in oleoeuropein accumulation and degradation in fruits of *Olea europaea* L*.: Hojiblanca* and *Arbequina* varieties. Journal of Agricultural and Food Chemistry, 8, 12924.

Hamilton R.J. 1983. The chemistry of rancidity in foods. In:"Rancidity". J.C. Allen and R.J. Hamilton (Eds.) p. 1. ElsevierScience and Technology, Paris.

Harwood J., Aparicio R. 2000. Handbook of olive oil : analysis and properties. An Aspen publication.

Hernández A., Martín A., Córdoba M. G., Benito M. J., Aranda E., Pérez-Nevado F. 2008. Determination of killer activity in yeasts isolated from the elaboration of seasoned green table olives. International Journal of Food Microbiology, 121, 178–188.

Hernández A., Martín A., Aranda E., Pérez-Nevado F, Córdoba M. G. 2007. Identification and characterization of yeast isolated from the elaboration of seasoned green table olives. Food Microbiology, 24, 346–351.

Hurtado, A., Reguant, C., Esteve-Zarzoso, B., 2008. Microbial population dynamics during the processing of Arbequina table olives. Food Research International, 41, 738-744.

Jimenez-Jimenez F., Castro-Garcia S., Blanco-Roldan G.L., Gonzàlez-Sànchez E.J., Gil-Ribes J.A. 2013. Isolation of table olive damage causes and bruise time evolution during fruit detachment with trunk shaker. Spanish Journal Of Agricultural Research 11, 65–71

Kader A.A., Rolle R.S. 2004. The role of post-harvest management in assuring the quality and safety of horticultural produce. Vol 152. Rome: FAO.

Kalua C.M., Bedgood D.R., Bishop A.G. and Prenzler, P.D. 2006. Changes in volatile and phenolic compounds with malaxation time and temperature during virgin olive oil production. Journal of Agricultural and Food Chemistry, 54, 7641.

Kalua C.M., Allen M.S., Bedgood Jr D.R., Bishop A.G., Prenzler P.D., Robards K. 2007. Olive oil volatile compounds flavour development and quality: a critical review. Food Chemistry 100, 273–286.

Kiritsakis A.K. 1998. Flavor components of olive oil—a review. Journal of the American Oil Chemists, 75, 673–681.

Koidis A., Triantafillou E., Boskou D. 2008. Endogenous microflora in turbid virgin olive oils and the physicochemical characteristics of these oils. European Journal of Lipid Science and Tecnology, 110, 164-171.

Koprivnjak O., Procida G., Zelinotti T. 2000. Changes in the volatile components of virgin olive oil during fruit storage in aqueous media. Food Chemistry, 70, 377–384

Kotti F., Cerretani L., Gargouri M., Chiavaro, E., Bendini A. 2011.Evaluation of volatile fraction of commercial virgin olive oils from Tunisia and Italy: relation with olfactory attributes. Journal of Food Biochemistry, 35, 681–698.

Lozano-Sanchez J., Cerretani L., Bendini A., Segura-Carretero A., Fernàndez-Gutierrez A. 2010. Filtration process of extra virgin olive oil: effect on minor components oxidative stability and sensorial and physicochemical characteristics. Trends in Food Science and Technology, 21, 201–211.

Luaces P., Sanz C., Pèrez A.G. 2007. Thermal stability of lipoxygenase and hydroperoxide lyase from olive fruit and repercussion on olive oil aroma biosynthesis. Journal of Agricultural and Food Chemistry, 55, 6309–6313.

Masella P., Parenti A., Spugnoli P., Calamai L. 2010. Nitrogen stripping to remove dissolved oxygen from extra virgin olive oil. European Journal of Lipid Science and Technology, 112, 1389–1392

Masella P., Parenti A., Spugnoli P., Calamai L. 2012. Vertical centrifugation of virgin olive oil under inert gas. European Journal of Lipid Science and Technology, 114, 1094–1096.

Muzzalupo I., Pellegrino M., Perri E. 2012. Sensory Analysis of Virgin Olive Oils In Tech, Chapter 11, 223-228

Migliorini M., Marinelli C., Cherubini C., 2009. Oli extra vergini di oliva di Firenze. Selezione 2009. Camera di commercio di Firenze.

Montedoro, G.F.; Servili M.; Baldioli, M.; Selvaggini, R.; Miniati E.; Macchioni A. 1993. Simple and Hydrolyzable Compounds in Virgin Olive Oil. 3. Spectroscopic Characterizations of the Secoiridoids Derivatives. *Journal of Agricultural and Food Chemestry*, 41, 2228-2234.

Morales M.T., Luna G. and Aparicio R. 2005. Comparative study of virgin olive oil sensory defects. Food Chemistry, 91: 91, 293–301.

Morello J.R., Romero M.P., Motilva M.J. 2004a. Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from Arbequina, Farga, and Morrut cultivars. Journal of Agricultural and Food Chemistry, 52, 6002–6009.

Morello J.R., Motilva M.J., Tovar M.J., Romero M.P. 2004b. Changes in commercial virgin olive oil (cv Arbequina) during storage with special emphasis on the phenolic fraction. Food Chemistry 85, 357–364

Obied H.K., Prenzler P.D., Omar S.H., 2012. Pharmacology of Olive Biophenols. In: Fishbein JC and Heilman JM. eds.Advances in molecular toxicology, Vol. 6.

Obied H.K., Prenzler P.D., Ryan D., Servili M., Taticchi A., Esposto S., Robards K. 2008. Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from Olea europaea L. Natural Product Reports, 25, 1167–1179.

Ortega-Garcìa F., Blanco S., Peinado M.A., Peragòn J. 2008. Polyphenol oxidase and its relationship with oleuropein concentration in fruits and leaves of olive (Olea europaea) cv'Picual'trees during fruit ripening. Tree Physiology, 28, 45-54.

Padilla M.N., Hernàndez M.L., Sanz C., Martìnez-Rivas J.M. 2009. Functional characterization of two 13-lipoxygenase genes from olive fruit in relation to the biosynthesis of volatile compounds of virgin olive oil. Journal of Agricultural and Food Chemistry, 57, 9097–9107.

Parenti A., Spugnoli P., Masella P., Calamai L. 2006a. Carbon dioxide emission from olive oil pastes during the transformation process: Technological spin offs. European Food Research and Technology, 222, 521–526.

Parenti A., Spugnoli P., Masella P., Calamai L., Pantani O.L. 2006b. Improving olive oil quality using CO2 evolved from olive pastes during processing. European Journal of Lipid Science and Technology, 108, 904–912.

Parenti A., Spugnoli P., Masella P., Calamai L. 2007. Influence of the extraction process on dissolved oxygen in olive oil. European Journal of Lipid Science and Technology, 109, 1180–1185.

Pelagatti O. 1978. Sulla microflora lattica e blastomicetica associata alle drupe di alcune cultivars di Olea europea. *L.* annali dell'Istituto sperimentale per L'elaiotecnica, 7, 177-192.

Psaltopoulou T., Naska A., Orfanos P., Trichopoulos D., Mountokalakis T., Trichopoulou A. 2004. Olive oil, the Mediterranean diet, and arterial blood pressure: the Greek European Prospective Investigation into Cancer and Nutrition (EPIC) study. American Journal of Clinical Nutrition, 80, 1012–1018.

Psomiadou E., Tsimidou M. 2002. Stability of virgin olive oil 1 Autoxidation studies. Journal of Agricultural and Food Chemistry, 50, 716–721.

Ranalli A., Ferrante M.L., De Mattia G., Costantini N. 1999. Analytical evaluation of virgin olive oil of first and second extraction. Journal of Agricultural and Food Chemistry, 47, 417–424.

Ranalli A., Contento S., Schiavone C., Simone N. 2001. Malaxing temperature affects volatile and phenol composition as well as other analytical features of virgin olive oil. European Journal of Lipid Science and Technology, 103, 228–238.

Rodríguez de la Borbolla J.M., (1958). Conservación de aceitunas de molino. Madrid, Spain: Sindicato Nacional del Olivo.

Rodrìguez G., Lama A., Rodrìguez R., Jimènez A., Guillèn R., Fernàndez-Bolanos J. 2008. Olive stone an attractive source of bioactive and valuable compounds. Bioresource Technology, 99, 5261–5269.

Rodríguez-Gómez F., Arroyo-López F.N., López-López A., Bautista-Gallego J., Garrido-Fernández A. 2010. Lipolytic activity of the yeast species associated with the fermentation/storage phase of ripe olive processing. Food Microbiology, 27, 604-612.

Romo-Sánchez S., Alves-Baffi M., Arévalo-Villenaa M., Úbeda-Iranzoa J., Briones-Pérez A., 2010. Yeast biodiversity from oleic ecosystems: Study of their biotechnological properties. Food Microbiology 27, 487-492.

Salas J.J., Sànchez J. 1998. Hydroperoxide lyase from olive (olea europaea) fruits in advances in plant lipid research. In: The Proceedings of the 13th International Symposium on Plant Lipids Held at Sevilla Spain July 1998 (Vol 53 P, 300) Univ. de Sevilla.

Sànchez-Ortiz A., Romero-Segura C., Sanz C., Pèrez A.G. 2012. Synthesis of volatile compounds of virgin olive oil is limited by the lipoxygenase activity load during the oil extraction process. Journal of Agricultural and Food Chemistry, 60, 812–822.

Servili M., Montedoro G.F. 2002**.** Contribution of phenolic compounds to virgin olive oil quality. European Journal of Lipid Science And Technology, 104, 602-613.

Servili M., Selvaggini R., Taticchi A., Esposto S., Montedoro G. 2003. Volatile compounds and phenolic composition of virgin olive oil: optimization of temperature and time of exposure of olive pastes to air contact during the mechanical extraction process. Journal of Agricultural and Food Chemistry, 51, 7980–7988.

Servili M., Selvaggini R., Esposto S., Taticchi A., Montedoro G., Morozzi G. 2004. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. Journal of Chromatography A, 1054, 113–127.

Servili M., Taticchi A., Esposto S., Urbani S., Selvaggini R., Montedoro G. 2007. Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. Journal of Agricultural and Food Chemistry, 55, 7028–7035.

Servili M., Esposto S., Fabiani R., Urbani S., Taticchi A., Mariucci F., Selvaggini R., Montedoro G.F. 2009. Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure. Inflammopharmacology. 17, 76-84.

Servili M., Taticchi A., Esposto S., Sordini B., Urbani S. 2012. Technological aspects of olive oil production. In: Muzzalupo I, editor. Olive germplasm: the olive cultivation, table olive and olive oil industry in Italy. In Tech. Available from: http://www.intechopen.com/books/olive-germplasm-theolive-cultivation-table-olive-and-olive-oil-industry-in-italy/technologicalaspects-of-olive-oil-production.

Servili M. 2014. The phenolic compounds: a commercial argument in the economic war to come on the quality of olive oil? Oil Seeds and Fats Crops and Lipids, 21(5) D509

Visioli F., Galli C., Galli G., Caruso D. 2002. Biological activities and metabolic fate of olive oil phenols. European Journal of Lipid Science and Technology, 104, 677–684.

Vichi S., Romero A., Tous J. and Caixach J. 2011. The activity of healthy olive microbiota during virgin olive oil extraction influences oil chemical composition. Journal of Agricultural and Food Chemistry, 59, 4705-4714.

Vinha A.F., Ferreres F., Silva B.M., Valentao, P., Goncalves A., Pereira J.A., Oliveira M.B., Seabra R.M. and Andrade P.B. 2005. Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): influences of cultivar and geographical origin. Food Chemistry, 89, 561.

Wagner K.H and Elmadfa I. 2000. Effects of tocopherols and their mixtures on the oxidative stability of olive oil and linseed oil under heating. European Journal of Lipid Science and Technology, 102, 624–629.

Zanoni B. 2014. Which processing markers are recommended for measuring and monitoring the transformation pathways of main components of olive oil? Italian Journal of Food Science, 26, 1-11.

Zinedine A., Mañes J. 2009. Occurrence and legislation of mycotoxins in food and feed from Morocco. Food Control, 20, 334-344.

Zullo B.A. and Ciafardini G. 2008. Lypolitic yeast distribution in commercial extra virgin olive oil. Food Microbiology, 25, 970-977.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2010. Distribution of dimorphic yeast species in commercial extra virgin olive oil. Food Microbiology. 27, 1035-1042.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2013. Effects of some oil-born yeasts on the sensory characteristics of Italian virgin olive oil during its storage. Food Microbiol. 36, 70-78.

2. AIMS AND STRUCTURE OF THE THESIS

The aim was to investigate the microbiota occurring in olive oil extraction process and, in particular the impact of the yeast population on the olive oil quality. It is important to remark that there are no information about the microorganisms occurrence in the different phases of the extraction process of extra virgin olive oils. Moreover, the yeasts present on the olive carposphere, during the crushing of the olives, migrate into the oil together with the solid particles of the fruit and the micro-drops of vegetation water. Some yeast species may remain metabolically active during olive oil storage and thus modify olive oil characteristics. Indeed, enzymatic activities of yeasts and moulds, which have been isolated from either olives or extra virgin olive oil, included β-glucosidase, β-glucanase, polyphenoloxidases, peroxidase, lipase and cellulase activities which can either improve or worsen the oil quality.

Therefore, the study was focused on the enumeration and isolation of microorganisms from 35 extraction processes of three consecutive crop seasons in the same oil mill located in Tuscany (Chapter 3.1). Moreover, yeast isolates occurring in the different phases of the extraction process were isolated and identified (Chapter 3.2). To fulfill this aim, a reproducible molecular method for differentiating the yeast species from olive oil environment was provided (Chapter 3.2). Furthermore, the aromatic and polyphenolic compounds of oils from the studied extraction process were analyzed and correlations studies between these compounds and yeast concentrations in the different phases of the process were carried out (Chapter 3.3). Finally, the enzymatic capabilities of the yeast isolates belonging to the species which were most frequently isolated from the different extractive processes, were investigated and their abilities to modify the chemical composition of the olive oil was assessed (Chapter 3.4).

The structure of the results is the following:

a) Investigation on the microbiota of the olive oil extraction process (Chapter 3.1).

b) Yeast species biodiversity of olive oil extraction process (Chapter 3.2).

c) Chemical analysis of the oils (Chapter 3.3).

d) Investigation on the metabolic behavior of the yeast (Chapter 3.4).

Investigation on the microbiota of the olive oil extraction process (Chapter 3.1)

The study was carried out sampling olives (washed and not washed), pastes (after crushing and after kneading), oils (after centrifugation in a two-phase decanter and after filtration) and pomaces obtained during 35 extra virgin olive oil extraction processes. These processes were carried out in the same oil mill located in Tuscany (Italy) during different days of the harvesting time in three consecutive years. Microorganism were counted on different media: MYPG agar for yeasts, Sabouraud agar for moulds and PCA agar for bacteria.

Yeast species biodiversity of olive oil extraction process (Chapter 3.2)

The yeast isolates occurring in washed olives, pastes (after crushing and after kneading), oils (after centrifugation in a two-phase decanter and after filtration) and pomaces (Chapter 3.1) were identified by combining various molecular methods:

1. PCR-RFLP analysis of the rDNA-ITS (Internal Transcribed Spacer) region, using *Cfo*I, *Hae*III and *Hinf*I as restriction endonucleases;
2. sequencing of specific rDNA regions (the D1/D2 domain of 26S rDNA gene);
3. and phenotypic characterization using the yeast identification system ID 32 C system (Biomerieux, Marcy-l'Etoile, France).

Finally, a RAPD–PCR method for a rapid yeasts identification was develop.

Chemical analysis of oils (Chapter 3.3)

The aromatic and polyphenolic compounds of oils of the studied extraction processes (Chapter 3.1) were quantified. The volatile compound content was determined using HS-SPME-GC-MS technique. Extraction, identification and determination of phenolic compounds were performed in agreement with IOC Official Method (IOC, 2009) by HPLC analysis. The tocopherol content was determined according to ISO 9936:2006 (ISO, 2006) using liquid chromatography. Quantitative analysis was carried out using the external standard method. Correlations studies between aromatic and polyphenolic compounds and yeast concentrations in the different phases of the

process (crushed pastes, kneaded pastes, oil from decanter and pomaces) were carried out by calculating both Pearson and Spearman coefficients. Finally, Principal Component Analysis (PCA) was used to classify samples by Statistica 7.0 software package.

Investigation on the metabolic behavior of the yeast (Chapter 3.4)

The enzymatic capabilities of the yeast isolates belonging to species which were most frequently isolated from the extractive process was assayed (Chapter 3.1 and 3.2). 117 yeast isolates from different samples were screened for relevant enzymatic activities in the processing of olives and oils, and of potential interest in terms of product quality. The screened enzymatic activities were: cellulase, polygalacturonase, ß-glucosidase, lipase and peroxidase; the used substrates were, respectively, carboxymethylcellulose (CMC), polygalacturonic acid, cellobiose, $CaCl_2$/Tween 80 and H_2O_2. Moreover, three strains, with different enzymatic activities, were inoculated as axenic cultures in crushed pastes (collected from another mill situated in Tuscany) and commercial filtered olive oil to investigate their influence on the oil quality. The oils obtained from crushed pastes after 1 hour of incubation and the oils after two months of storage were analyzed (acidity level, peroxide value, total polyphenols, yeast concentrations) and statistically compared with the control (oil or pastes incubated without yeast inoculation). Yeasts in pastes and inoculated oil were quantified on MYPG agar; the content of free fatty acids was performed by titration with an ethanol solution of 0.1 N sodium hydroxide; the number of peroxides was carried out by titration with standardized sodium thiosulphate solution; total polyphenols were determined by colorimetric method with Folin-Ciocalteau reagent. Finally, the volatile compounds content and the oil fatty acid composition of the oils after two months of storage was performed.

3. RESULTS

3.1 INVESTIGATION ON THE MICROBIOTA OF THE OLIVE OIL EXTRACTIVE PROCESS

Abstract

The yeasts found in the oil derive from the olive carposphere which, during the crushing of the olives, migrate into the oil together with the solid particles of the fruit and the micro-drops of vegetation water (Ciafardini and Zullo, 2002a). Some yeast species do not survive a long time whereas others persist and become the typical microflora of each oil. Considering the lack of information on the microorganisms occurrence in the different steps of the extraction process of extra virgin olive oils, the aim of the study was to assess the microbiota occurring in washed and not washed olives, pastes, oil (after centrifugation in a two-phase decanter and after filtration) and pomaces coming from 35 extra virgin olive oils extraction processes. These processes were carried out in the same manufacture located in Tuscany during different days of the harvest time in three consecutive crop seasons (2011, 2012 and 2013). Yeasts, moulds and bacteria were quantified respectively on MYPG, Sabouraud and PCA agar. The microbial concentrations in the samples analyzed in the three years ranged between values below 10 and above 10^5 CFU/g or mL. Correlation studies showed that yeast densities in the pastes and in the oils before and after the filtration were unrelated, suggesting a role of the environment in the oil contaminations. Moreover, the yeast concentration in the pomaces resulted statistically higher than in pastes and oil from decanter, suggesting a possible accumulation of yeasts during the subsequent centrifugations of the kneaded pastes in the two-phase decanter. According to their metabolic capability, that can both improve or worsen the oil quality (Zullo et al., 2010), the yeast population occurred in the extraction process could be a source of oil contamination.

3.1.1 Introduction

Non-Saccharomyces yeasts, lactic acid bacteria (LAB) and filamentous fungi are the spontaneous microbiota of olives (Rodriguez de la Borbolla, 1958). Previous studies on

olives in brine have reported the growth of certain yeast species (Arroyo-López et al., 2006; Coton et al., 2006; Hurtado et al., 2008), that reaches around 10^4 e 10^6 CFU/ml (Garrido Fernández et al., 1997). the olive oil production is also important and there are few references in the literature about yeast biodiversity present in both fresh olives intended for oil production and their subproducts. Giannoutsou et al. (2004) suggested that "alpeorujo" is a good substrate for yeast growth which could be used as a feed additive, as a fertilizer in crops or as a substrate for the growth of edible mushrooms. Ciafardini and Zullo have been the first to have studied the microbial populations in olive oil. In 2002 they conducted a research for identifying lactic acid bacteria, yeasts and molds in oil extracted from olives belonging to the *cultivar* "leccino ", during the process of sedimentation at low temperatures. The results reported that yeasts were consistently present in the initial phase and during storage, moulds belonging to the genus *Aspergillus* were present only occasionally, while bacteria were not found. Moreover, the yeasts present in newly produced oil can remain active during the conservation period and, according to their metabolic capability, can both improve or worsen the oil quality (Zullo et al., 2010). Indeed, ß-glucosidase and esterase capabilities can improve the taste and the antioxidant capability of the oil, while the lipase capability can worsen the oil quality by hydrolyzing triglycerides (Ciafardini and Zullo, 2002b; Ciafardini et al., 2006a-b). On the basis of these findings, and considering the lack of information on the yeast occurrence in the different steps of the extraction process of extra virgin olive oils, a study was carried out to investigate on the presence of these microorganisms in washed and not washed olives, pastes, oil after centrifugation in a two-phase decanter, pomaces and oil after filtration in three consecutive crop seasons in the same oil mill located in Tuscany (Italy).

3.1.2 Materials and methods

Sampling throughout olive oil extraction processes

During three crop seasons (2011, 2012 and 2013), 35 batches of approx. 200 kg olives from *Frantoio, Moraiolo* and mixed cultivars were processed in an oil mill (Azienda Agricola Buonamici, Fiesole, Florence, Italy).
Plant for oil extraction (TEM, Florence, Italy) consisted in a cleaning and water washing system, an olive grinding cutter crusher (mod. FR350), a controlled-temperature vertical axis malaxation equipment (500 kg capacity) (mod. V500), a "decanter" (two-step mod. D1500) with 1500 kg/h maximum capacity and a cardboard filter press (15 µm cut-off). Plastic residue or "alperujo" from decanter was subjected to separation by centrifugation of stone fragments to obtain destoned pomace (Fig. 1).
Olives were crushed at 2,500 rpm (crusher holes 6.5 mm in diameter); malaxation was carried out at half capacity under vacuum (residual pressure of 20 kPa) at 22 ± 1°C for a mean time of 15 min to work under low oxidative stress impact conditions; decanter worked with a screw conveyor rotating at a slower speed than that of the bowl. Samples were collected in several steps of the extraction process for sensory, chemical and microbial analyses, as shown in Figure 1.
The 35 extractive processes were sampled in different days of the three consecutive years, as reported in the table 1. The sampling dates were chosen based on the maturity of the olives: sugar content, oil content, water content and phenolic compounds (Chemical analysis made by Metropoli - Laboratorio Chimico Merceologico Section, Special Agency of the Florence Chamber of Commerce). During the first year, only olives, crushed pastes, oil from decanter, filtered oil and pomaces were analyzed, while during the second and third year also the kneaded pastes have been detected. Olives were processed within 12 h from harvest, each sample was collected twice at distance of 3 minutes and transported to the laboratory in refrigeration under aseptic conditions.

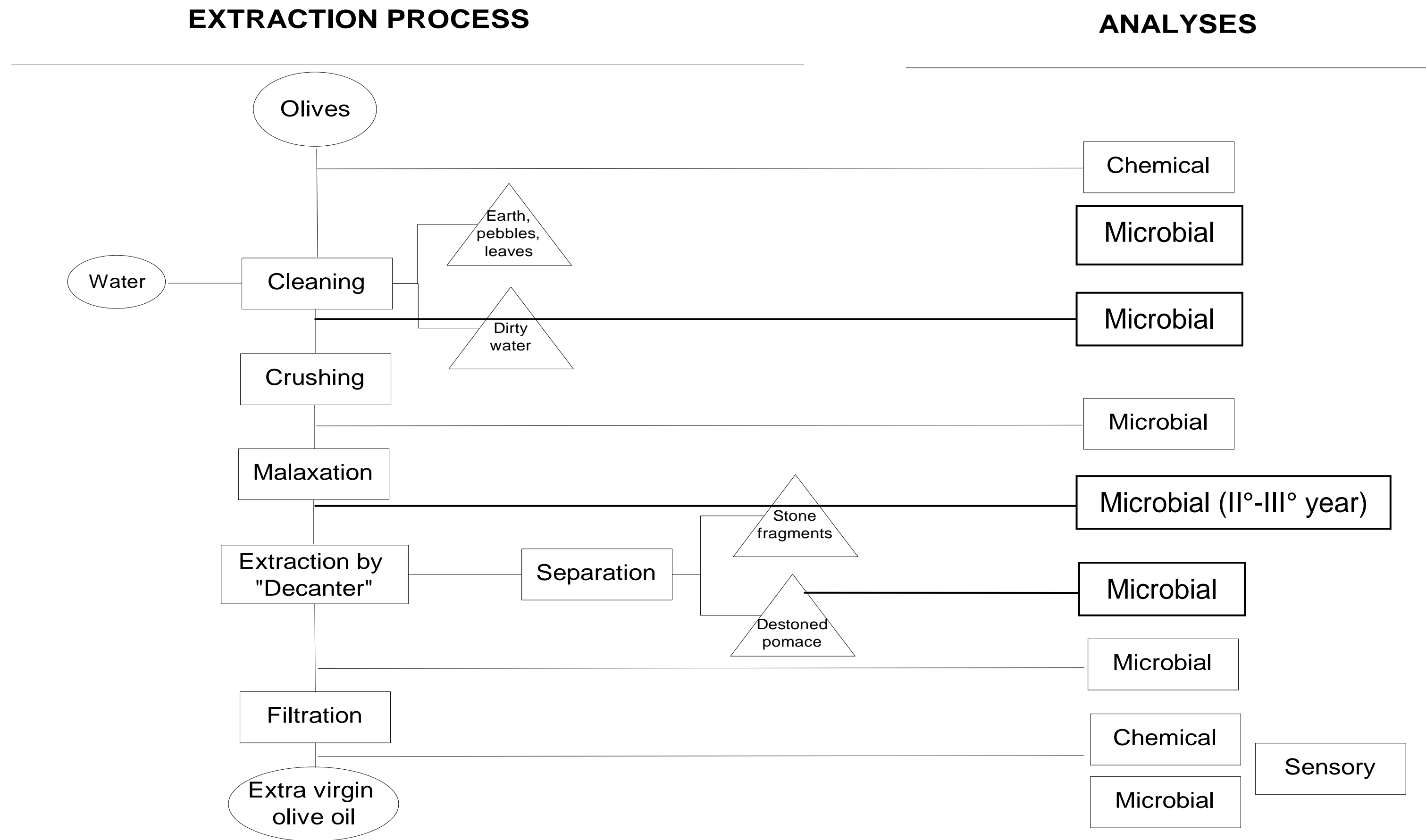


Figure 1 Plant for oil extraction used during the three years of sperimentation and steps of extraction process for sensory, chemical and microbial analyses.

Table 1 Extractive processes sampled in different harvest date of three consecutive years of study.

2011		2012		2013	
Harvest date	Extraction processes	Harvest date	Extraction processes	Harvest date	Extraction processes
		October 30	6	November 4	2
November 16	8	November 5	2	November 13	2
November 23	8	November 7	3	November 27	1
		November 23	3		

Enumeration and isolation of microorganisms

Yeasts were quantified on MYPG agar (malt extract 5g/L, yeast extract 3g/L; beef extract 5g/L, D-glucose 10g/L, agar 20g/L) containing sodium propionate (2 g/L); moulds were quantified on Sabouraud agar (glucose 40g/L, peptone 10g/L, agar 20g/L); bacteria on PCA agar (Oxoid™). The samples of olives, pastes, pomaces and oil from decanter were plated directly (unfiltered oil samples) and/or after decimal dilutions in physiological solution (NaCl, 0.86 g/L). The filtered oils were analyzed filtering 100 mL of sample on cellulose membranes (0.45 μm; Pall Corporation). Yeasts were counted after incubation for 48-72 h at 30°C under aerobic conditions. A significant number of yeast isolates from each sample was purified and stored in liquid cultures containing 50% (v/v) glycerol at - 80°C until use.

Data processing

Microbiological determinations were processed according to one way or two way ANOVA followed by Tukey's test (significance level: p = 0.05).

3.1.3 Results

3.1.3.1 Microbiota of the olive oil extractive processes carried out in 2011

During the first year of study, olives (washed or not) of two different cultivars (*Frantoio* and *Moraiolo*), crushed pastes, oil from decanter, filtered oil and pomaces were microbiologically analyzed (Figure 2 to 6).

Yeasts and/or moulds were always the dominant populations, independently of the sampling point. Concentration of bacteria only accounted for 1% of the total microbial counts on PCA plates.

The microbial concentrations in the samples analyzed ranged between values below 10 and above 10^4 CFU/g or mL.

Microbial counts of each olive, pastes or oil batch were often affected by high standard deviation values, as it typically occurs in manufacturing processes of raw materials (such as olives) at industrial scale. A rough general pattern for microbial evolution during olive processing could nonetheless be drawn.

The microbial cell counts of olives ranged between 10^3 and 10^4 CFU/g. Significant differences were found between washed and not washed olives in yeast cell counts in the second harvesting date and in microbial count of the second harvesting date of the *Moraiolo* cultivar, in both cases the not washed olives resulted more contaminated than the washed olives (Fig. 2).

The microbial cell counts of crushed pastes ranged between 10^2 and 10^4 CFU/g for yeasts, and below 100 and above 10^4 CFU/g for moulds (Fig. 3).

Oil from decanter cell counts ranged between 10^1 and above10^4 CFU/mL for yeasts, and below 100 and 10^2 CFU/mL for moulds. The second harvesting date harbored yeast and mould concentrations which were, in most cases, of about one or two orders of magnitude higher than the first harvesting date, suggesting a progressive colonization of the malaxation equipment and/or “decanter” (Fig. 4).

Pomaces cell counts ranged between 10^3 and above10^4 CFU/g for yeasts, and above 10^2 and 10^4 CFU/g for moulds. No difference between different harvesting date and different cultivars was detected (Fig. 5).
Filtered oils cell counts ranged between less than 10^2 and 10^2 CFU/100 mL (Fig. 6)
In order to generalize, mean and standard error of the yeast and mould concentrations detected in the various extractive phases sampled in 2011 are reported in figure 7. The moulds counts in crushed pastes and pomaces were significantly higher than those in oil from decanter, while yeast counts showed a different behavior. Indeed, no significant difference was found between crushed pastes and oil from decanter, but significant difference was found between the first two sampling points and pomaces (Fig. 7).
Correlation studies demonstrated that mould counts in crushed pastes (CPM) and in oil from decanter (OfDM) were positively related to each other, suggesting that mould contamination of unfiltered oil could be affected by the hygienic level of olives (Table 2). On the contrary, yeast cell densities in olive paste (CPY) and in oil from decanter (OfDY) were statistically unrelated, suggesting that yeast growth could be encouraged by malaxation and/or “decanting” steps. Finally, no correlation was found between yeast and mould concentrations in both olive paste (CPY and CPM, respectively) and filtered oil (OY and OM, respectively).

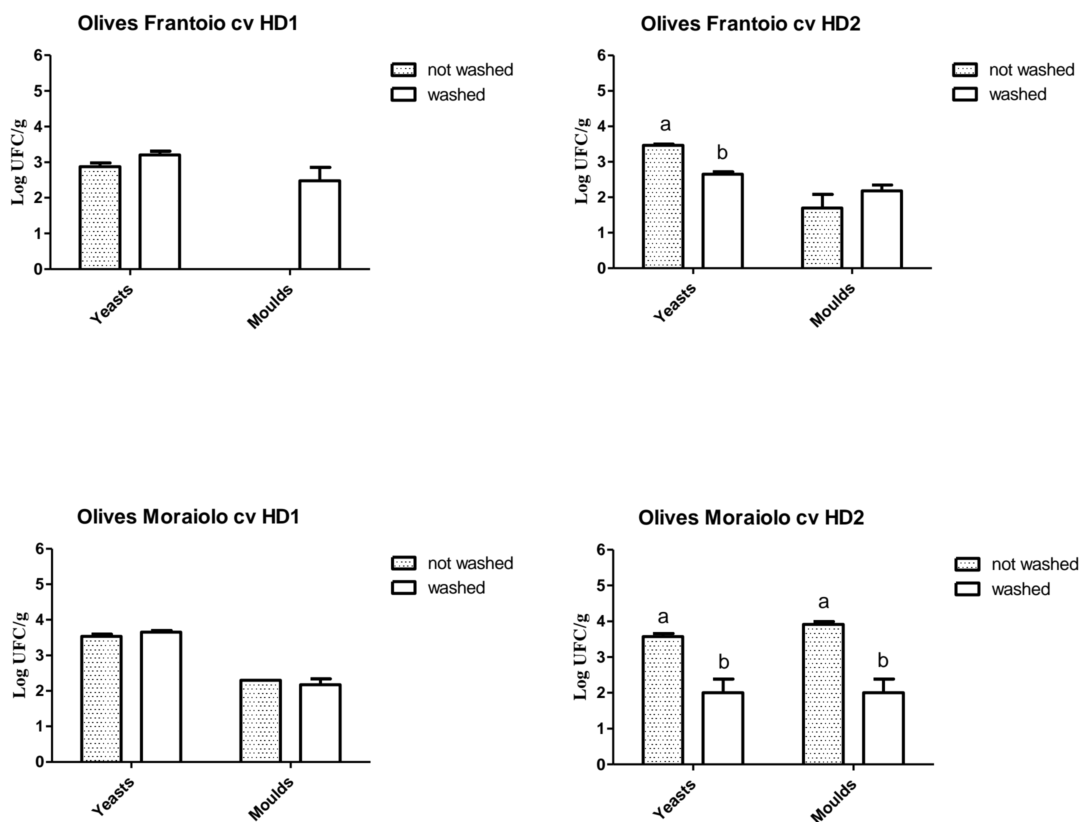


Figure 2: Microbial cell counts of *Frantoio* and *Moraiolo* olives sampled during two different harvesting dates (HD), before and after washing. Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

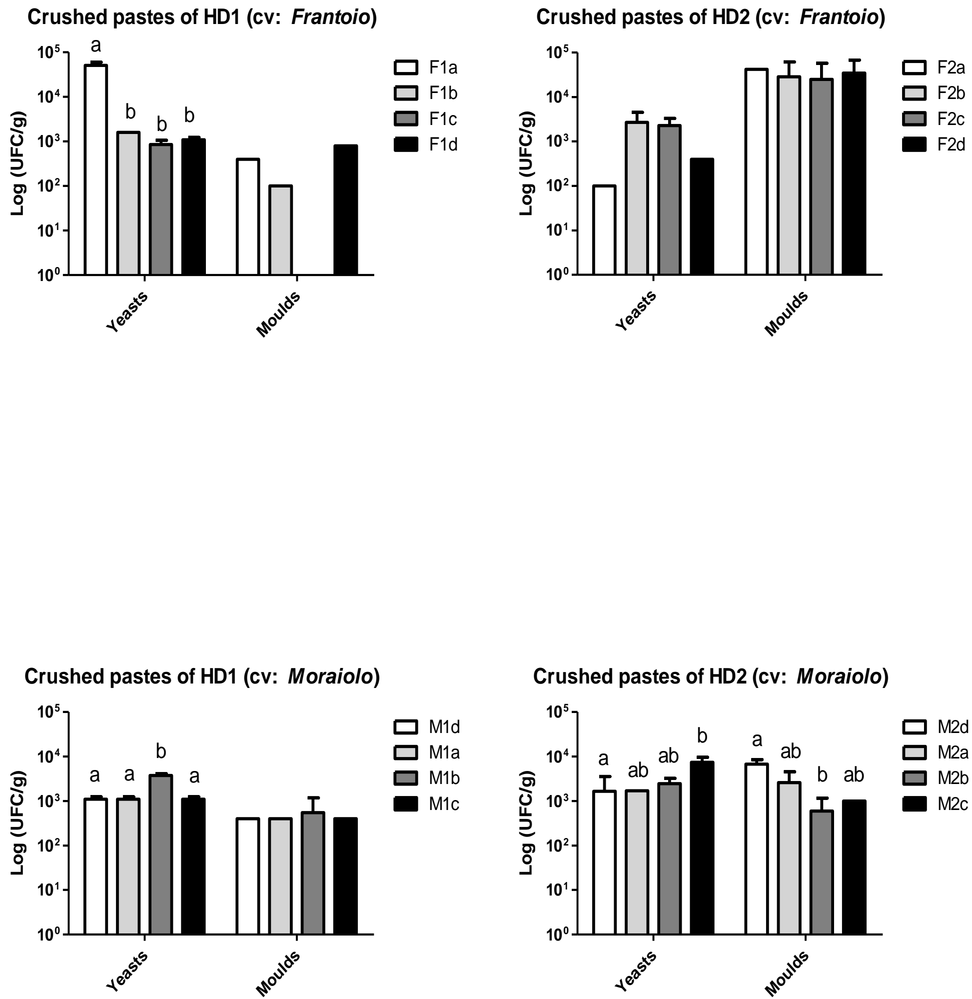


Figure 3: Microbial cell counts of crushed pastes from *Frantoio* and *Moraiolo* olives sampled during two different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

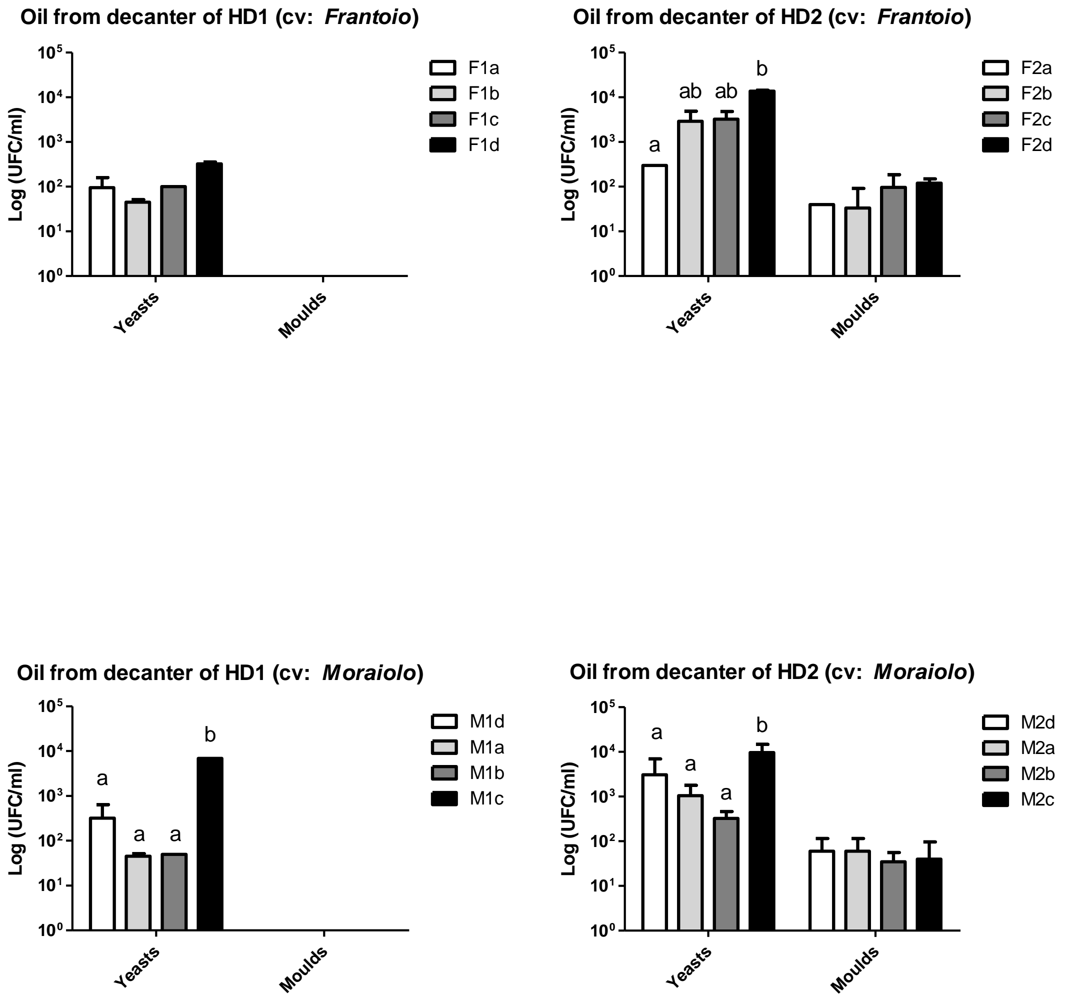


Figure 4: Microbial cell counts of oil from decanter from *Frantoio* and *Moraiolo* olives sampled during two different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

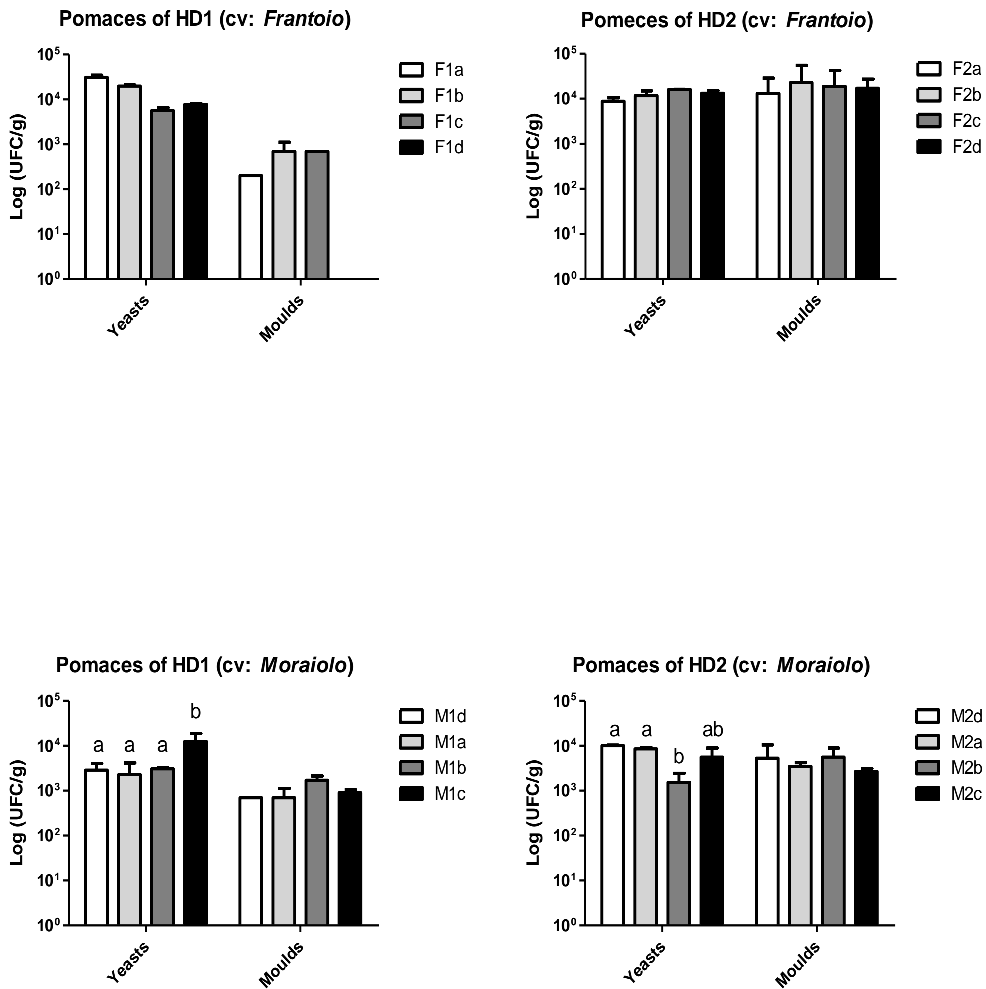


Figure 5*:* Microbial cell counts of pomaces from *Frantoio* and *Moraiolo* olives sampled during two different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

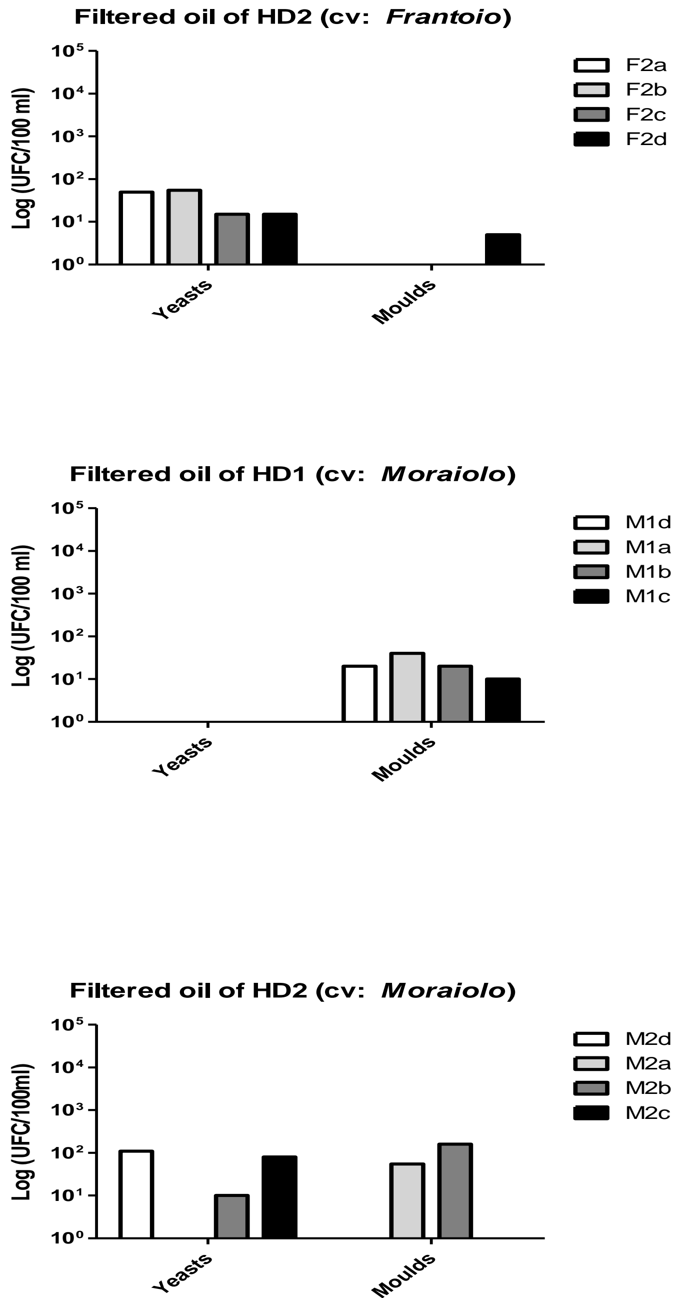


Figure 6: Microbial cell counts of filtered oil from *Frantoio* and *Moraiolo* olives sampled during two different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

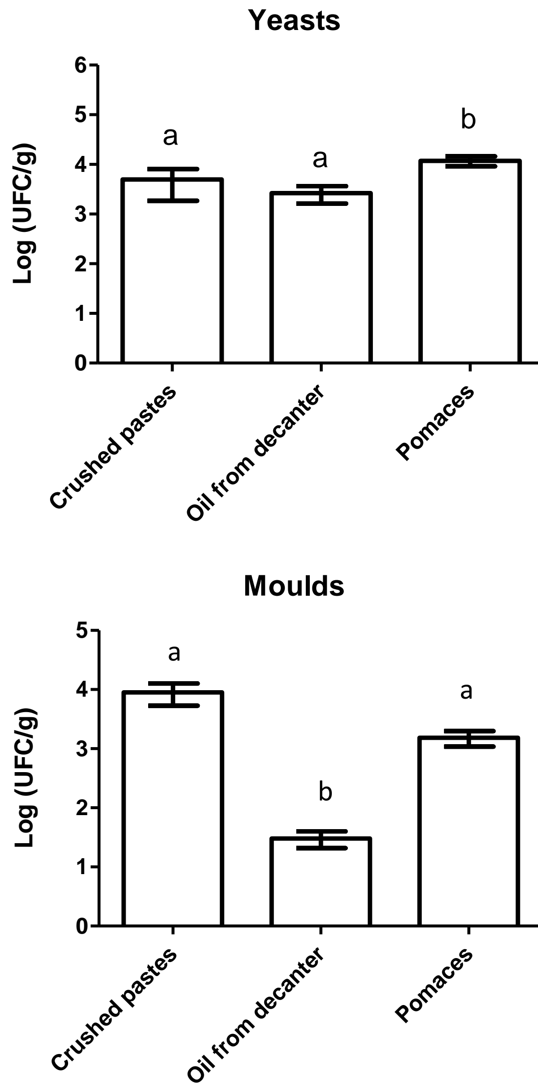


Figure 7: Concentrations of yeasts and moulds in the different extractive phases sampled in 2011 . Different letters indicate significant differences between different phases (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

Table 2: Correlation coefficients calculated between microbial contaminations (Y = yeasts; M = moulds) of crushing pastes (CP) and microbial contaminations of oil from decanter (OfD) and filtered olive oil (O). Statistically significant correlations (ANOVA; p<0.05) are underlined.

	OfDY		OfDM		OY		OM	
	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r
CPM			<u>0.8304</u>	<u>0.7347</u>			-0.1575	-0.2485
CPY	0.08641	0.05563			0.2841	0.1241		

3.1.3.2 Microbiota of the olive oil extractive processes carried out in 2012

During the second year of study, olives (washed or not, cv of *Frantoio*, *Moraiolo* or mixed), crushed pastes, kneaded pastes, oil from decanter, filtered oil and pomaces were microbiologically analyzed and the results are shown in Figures 8- 12.

Unlike the oil campaign in 2011, the study conducted during the oil campaign in 2012 showed a contamination, not only by yeasts and moulds, but also by bacteria (mainly Gram-positive rod-shaped, often spore-forming).

The microbial concentrations in the samples analyzed ranged between values below 10 and above 10^5 CFU/g or mL.

As observed in 2011, also in 2012 the microbial counts of olive, paste or unfiltered oil samples were often affected by high standard deviation values.

Olives microbial counts ranged between 10^2 and 10^5 CFU/g. In the first harvesting date bacteria cell counts of the washed olives resulted significantly higher than the not washed ones. In the second harvesting date, as regards the yeast and bacteria cell counts, and in the fourth harvesting date, as regards bacteria cell counts, the not washed olives resulted significantly higher than the washed ones (Fig. 8).

The yeast cell counts of crushed pastes, from the first to the third harvesting date, ranged between < 100 and 10^2 CFU/g; while in the fourth harvesting date yeast counts increased of about one or two orders of magnitude than the previous dates (Fig. 9).In all harvesting dates mould and bacteria cell counts of crushed pastes ranged between 10^2 and 10^3 CFU/g (Fig. 9).

The yeast cell counts of kneaded pastes were between 10^2 and 10^4 CFU/g, while mould and bacteria cell counts ranged between 10^2 and 10^3 CFU/g in all harvesting dates (Fig. 10).

Yeast cell counts in oil from decanter were characterized by a high variability between the different harvesting dates, indeed, cell counts ranged between < 100 CFU/mL and 10^1 in the first date, above 10^4 CFU/mL in the second and between 10^2 and 10^4 CFU/mL in the third and fourth date (Fig. 11). Mould and bacteria cell counts ranged between

10^1 and 10^2 CFU/mL in all dates. Only in the first extraction process of the fourth harvesting date bacteria cell counts showed values of above 10^4 CFU/mL (Fig. 11).

Yeast cell counts in pomaces harbored values of 10^2 CFU/g during the first harvesting date, up to 10^5 CFU/g in the second date and about 10^4 the other dates. Mould and bacteria cell counts ranged values of 10^2 and 10^3CFU/g respectively during all harvesting dates (Fig. 12).

The values found in the whole extraction process were higher than that obtained from filtered olive oil, which was < 10^2 CFU/100 mL (data not shown).

Finally, in order to generalize, mean and standard error of the yeast, mould and bacteria concentrations of the various extractive phases sampled in 2012 are reported in figure 13. The mould and bacteria counts in crushed and kneaded pastes were always significantly higher than those in oil from decanter (Fig 13 B and C); on the contrary, yeast counts of kneaded pastes were about one or two order of magnitude higher than those of crushed pastes, suggesting a progressive yeast colonization of the malaxation equipment, while not significant differences occurred between yeast counts of crushed pastes and oil from decanter (Fig. 13 A).

The second year of the study confirmed the findings of the first year. Indeed, in both years, yeast concentration in the pomaces resulted statistically higher than in pastes and oil from decanter (Fig. 7 and 13) suggesting a possible accumulation of yeasts during the subsequent centrifugations of the kneaded pastes in the two-phase decanter. This yeast population could be a source of oil contamination.

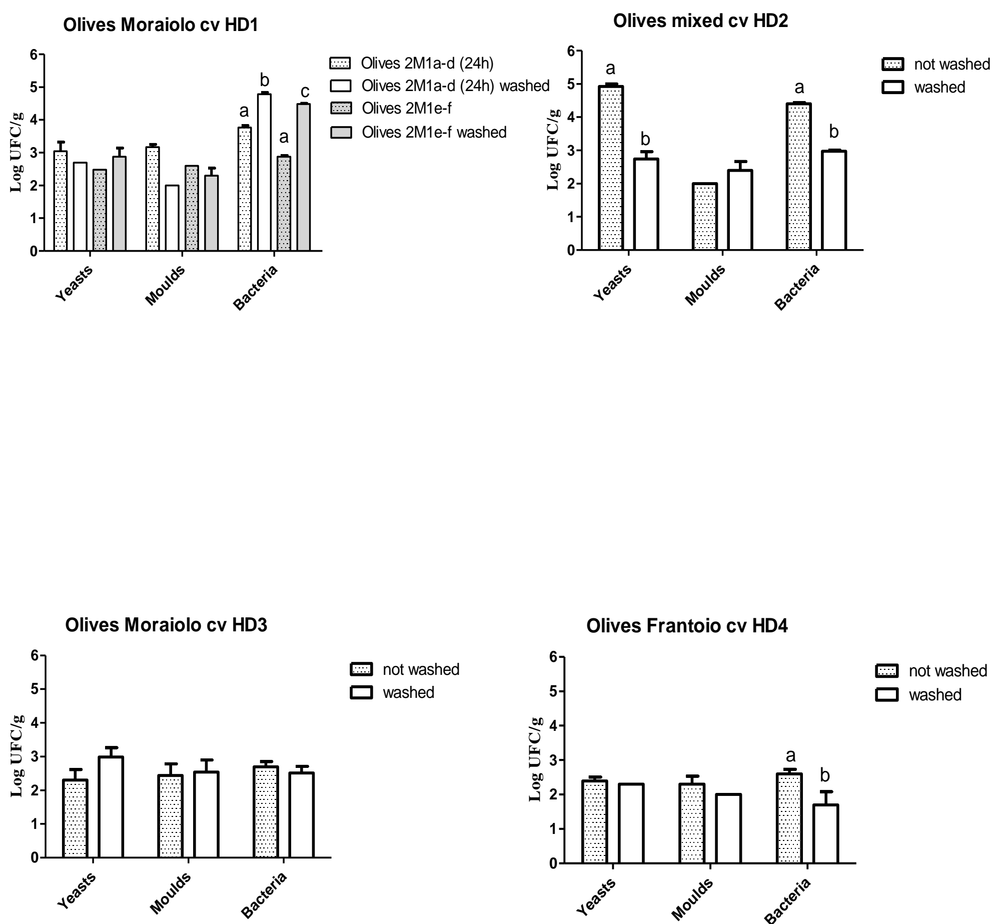


Figure 8: Microbial cell counts of *Frantoio, Moraiolo* and mixed cultivars olives sampled during four different harvesting dates (HD), before and after washing.. Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

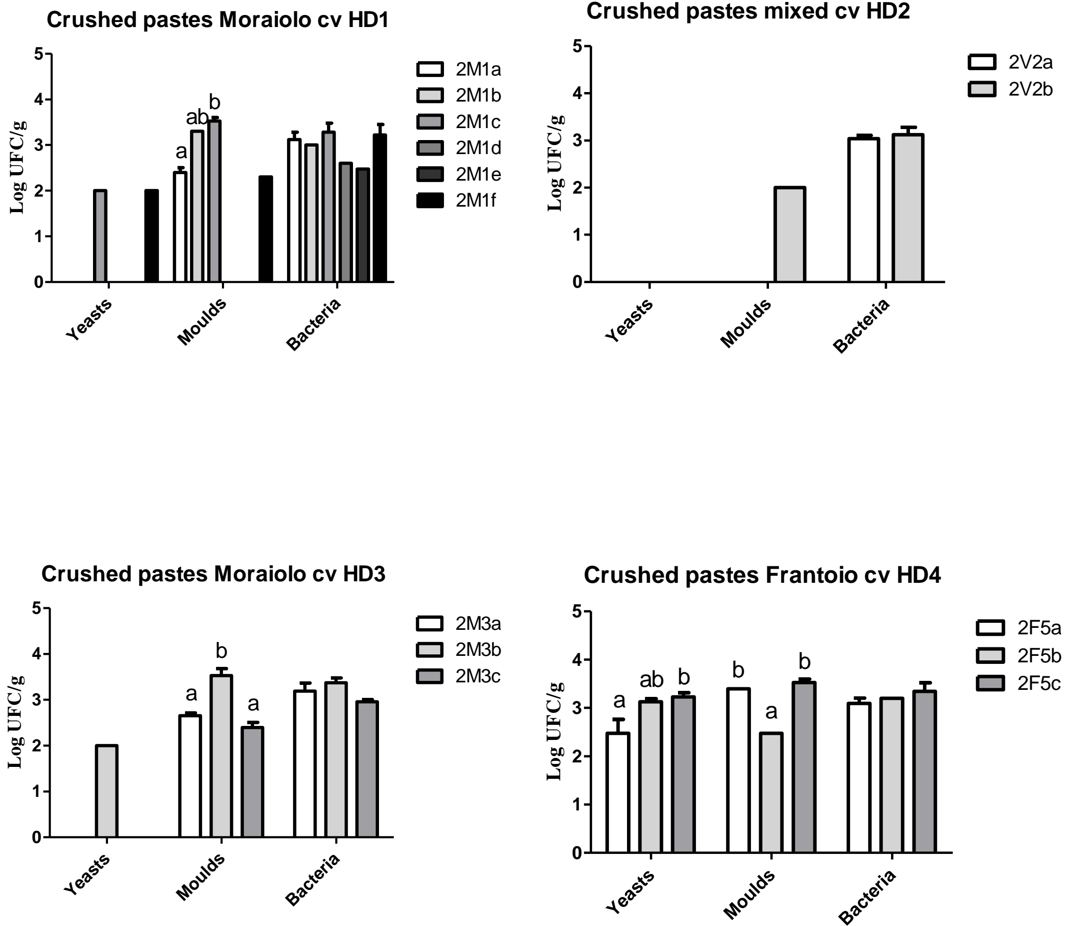


Figure 9: Microbial cell counts of crushed pastes from *Frantoio, Moraiolo* and mixed cultivars olives sampled during four different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

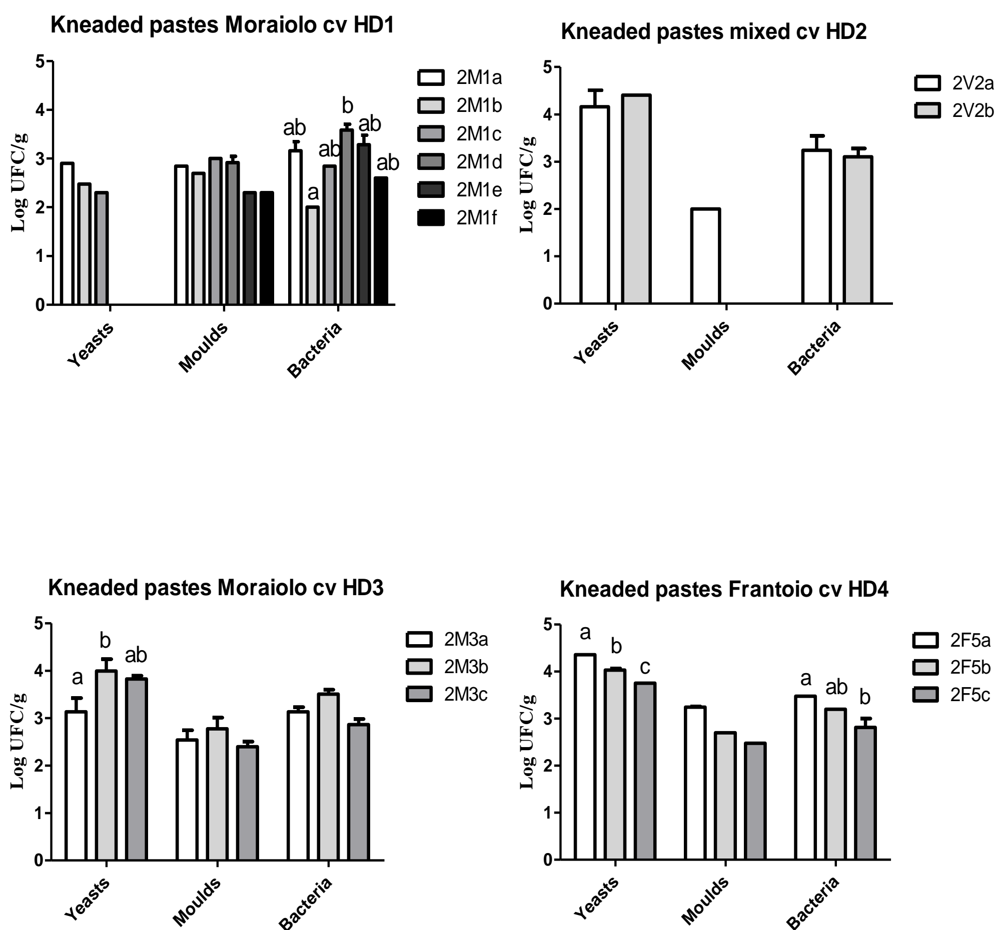


Figure 10: Microbial cell counts of kneaded pastes from *Frantoio, Moraiolo* and mixed cultivars olives sampled during four different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

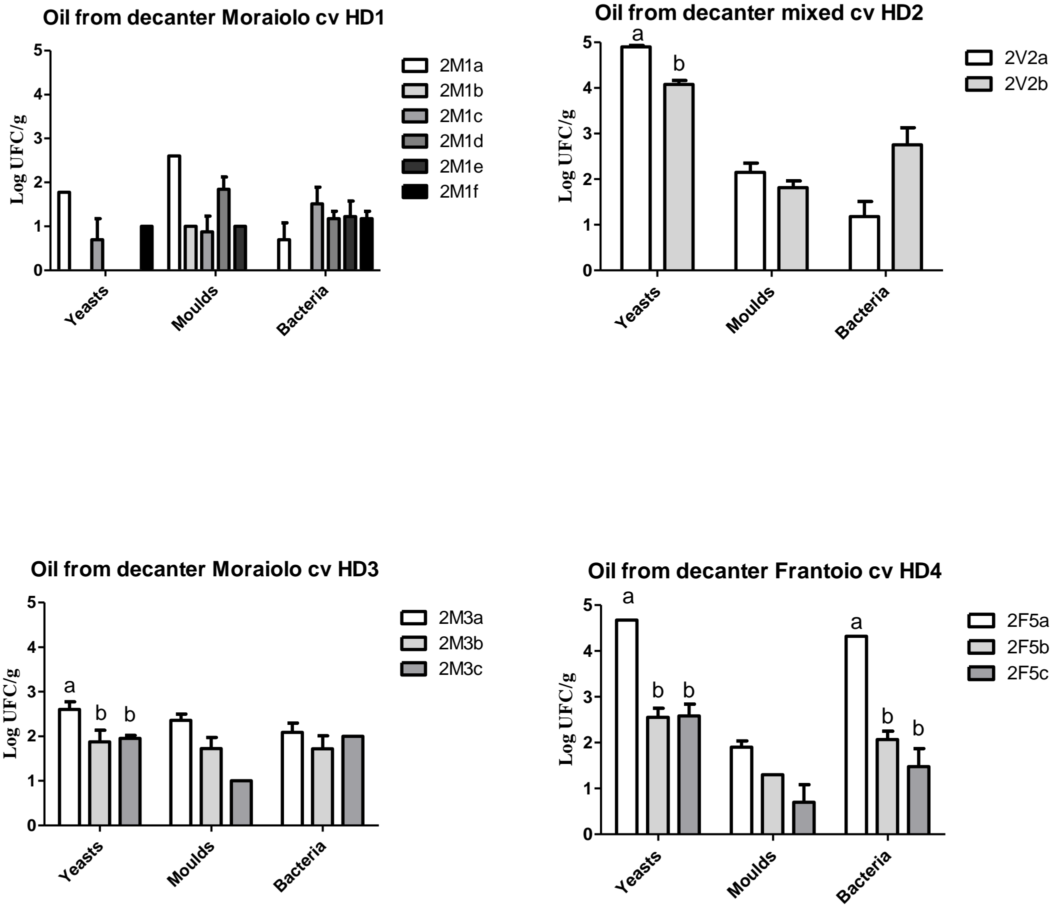


Figure 11: Microbial cell counts of oil from decanter from *Frantoio, Moraiolo* and mixed cultivars olives sampled during four different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

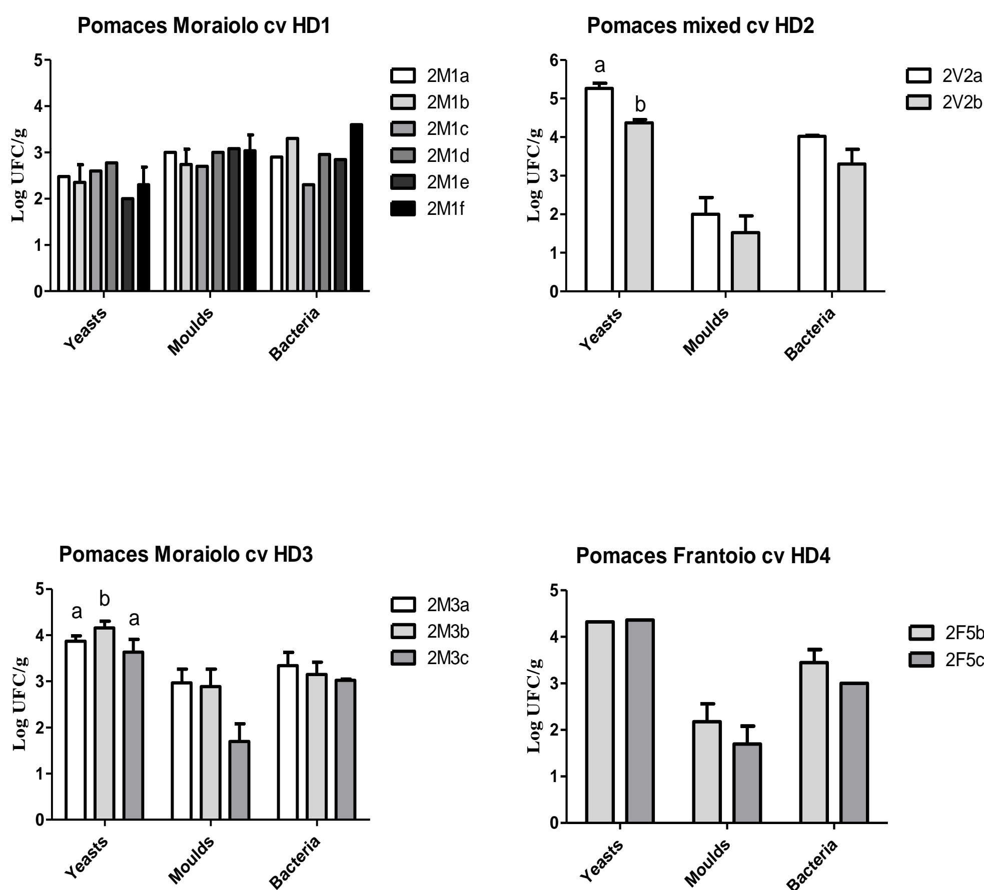


Figure 12: Microbial cell counts of pomaces from *Frantoio, Moraiolo* and mixed cultivars olives sampled during four different harvesting dates (HD). Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

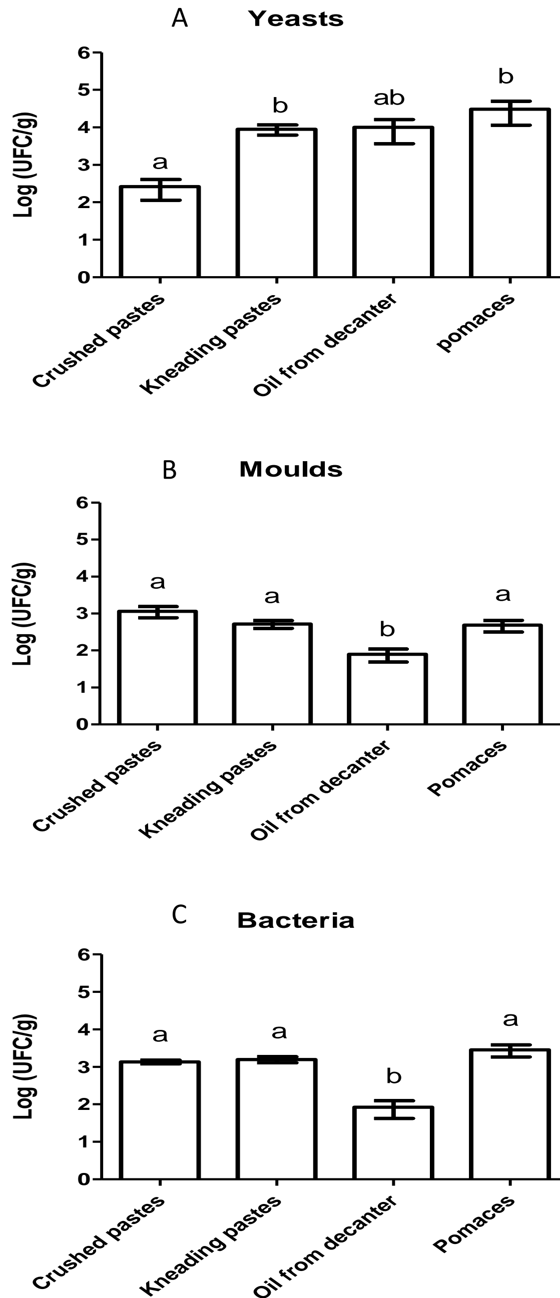


Figure 13: Concentrations of yeasts, moulds and bacteria in the different extractive phases sampled in 2012 . Different letters indicate significant differences between different phases (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

3.1.3.3 Yeast concentrations of the olive oil extractive processes carried out in 2013

During the third year of study, yeast cell concentrations of washed olives, crushed pastes, kneaded pastes, oil from decanter, filtered oil and pomaces were assayed with the aim to confirm the findings of 2011 and 2012. In particular, the aim was to confirm the accumulation of yeasts during the centrifugations of the kneaded pastes in the two-phase decanter.

Furthermore, given the absence of significant differences between yeast concentrations of different cultivar of olives and between washed and not washed olives, batches of mixed cultivar were processed and only washed olives were analyzed.

Yeast cell concentrations in the samples collected during the different phases of each oil extraction process from mixed cultivar olives are shown in Figure 14.

The microbial concentrations in the samples analyzed ranged between values below 10 and above 10^5 CFU/g or mL.

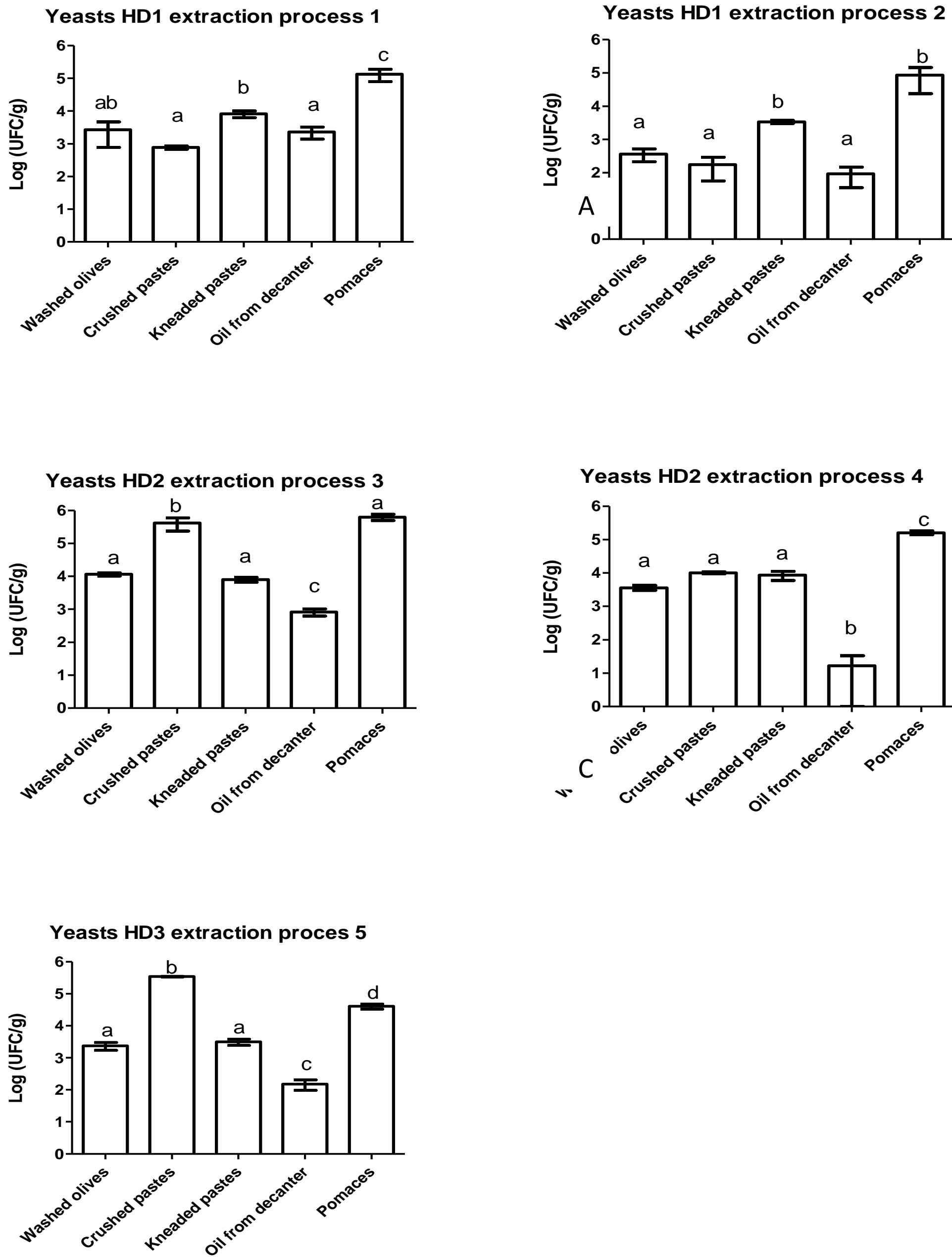
As reported in 2011 and 2012, also in 2013 the microbial counts of olive, paste and unfiltered oil samples were often affected by high standard deviation values.

The numerical enrichment in yeasts along the process of extracting extra virgin olive oil was confirmed for the first two extractive processes, where yeast counts of kneaded pastes significantly increased by about one or two orders of magnitude to crushed pastes (Fig. 14 A-B). In the other extracted process this trend was not confirmed, indeed yeast counts in crushed pastes were often significantly higher than those in kneaded pastes (Fig. 14 C-E). These different results could be due to a contamination of the faucet from which crushed pastes were sampled. Indeed in 2013 the faucet generated a stationing of olives pastes. This phenomenon could be probably responsible for the contamination. In any case, the results related to the concentration of yeasts in pomaces confirm a numerical enrichment of yeasts along the phases of the extraction process (Figure 14 A-E).

As reported in the previous years, the filtered olive oil showed yeast cell counts $< 10^2$ CFU/100 mL (data not shown).

Finally, in order to generalize, mean and standard error of the yeast concentrations of the various extractive phases sampled in 2013 is reported in figure 15. It is underlined that the yeast concentration in the pomaces resulted statistically higher than in pastes and oil from decanter (Fig. 15) suggesting a possible accumulation of yeasts during the subsequent centrifugations of the kneaded pastes in the two-phase decanter.

The third year of the study had confirmed the findings of the previous years. Indeed, also in the 2013, the yeast contamination during the centrifugations of the kneaded pastes in the two-phase decanter has occurred. This yeast population could be a source of oil contamination.



E

Figure 14: Concentrations of yeasts in the five extraction process (EP) A-B-C-D-E. Different letters indicate significant differences between different phase of the processes (ANOVA; $p < 0.05$).

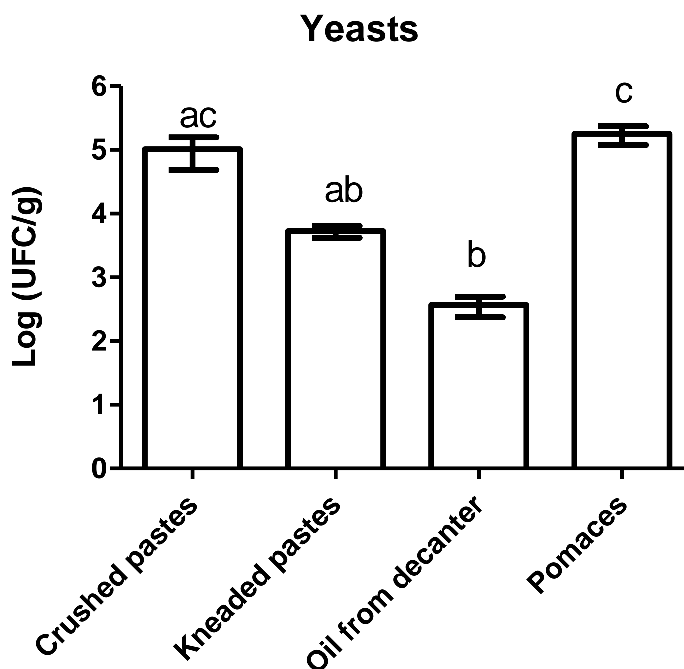


Figure 15: Concentrations of yeasts in the different extractive phases sampled in 2013 . Different letters indicate significant differences between different phases (ANOVA; $p < 0.05$).

3.1.3.4 Conclusion

Newly produced olive oils might harbor viable microbial cells which could affect, according to their metabolic capability, the oil quality (Zullo et al., 2010). 35 extraction process, carried out in the same manufacture located in Tuscany during different days of the harvest time in three consecutive crop seasons (2011, 2012 and 2013), were considered. The results showed that the microbiota occurring in the extra virgin olive oil extraction process was composed mainly by yeasts, but also by moulds and bacteria. The microbial concentrations in the samples analyzed in the three years, ranged between values below 10 and above 10^5 CFU/g and the filtered olive oil showed microbial cell counts lower than 10^2 CFU/100 mL. Correlation studies demonstrated that mould counts in crushed pastes and in oil from decanter were

positively related to each other, suggesting that mould contamination of unfiltered oil could be affected by the hygienic level of olives (Table 2). On the contrary, yeast cell densities in olive paste and in oil from decanter were not statistically related, suggesting that yeast growth could be encouraged by malaxation and/or “decanting” steps (Table 2). Indeed, in the three consecutive years considered, the yeast concentration in the pomaces resulted statistically higher than in pastes and oil from decanter (Fig. 7, 13 and 15) suggesting a possible accumulation of yeasts during the subsequent centrifugations of the kneaded pastes in the two-phase decanter.

REFERENCES

Arroyo-Lopez, F.N., Duran-Quintana, M.C., Ruiz-Barba, J.L., Querol, A., Garrido-Fernandez, A. 2006. Use of molecular methods for the identification of yeast associated with table olives. Food Microbiology, 23, 791-769.

Ciafardini, G., Zullo, B.A., 2002a. Survival of microorganisms in extra virgin olive oil during storage. Food Microbiology, 19, 105-109.

Ciafardini G., Zullo B.A. 2002b. Microbiological activity in stored olive oil. International Journal of Food Microbiology, 75, 111–118.

Ciafardini G., Zullo B.A., Iride A. 2006a. Lipase production by yeasts from extra virgin olive oil. Food Microbiology, 23, 60–67.

Ciafardini G., Zullo B.A., Cioccia G., Iride A. 2006b. Lipolytic activity of Williopsis californica and Saccharomyces cerevisiae in extra virgin olive oil. International .Journal of Food Microbiology, 107, 27–32.

Coton, E., Coton, M., Levert, D., Casaregola, S., Sohier, D. 2006. Yeast ecology in French cider and black olive natural fermentations. International Journal of Food Microbiology, 108, 130-135.

Garrido Fernández, A., Fernández Díaz, M.J., Adams, R.M. 1997. Table Olives. Production and Processing. Chapman & Hall, London, UK.

Giannoutsou, E.P., Meintanis, C., Karagouni, A.D. 2004. Identification of yeast strains isolated from a two-phase decanter system olive oil waste and investigation of their ability for its fermentation. Bioresource Technology, 93, 301-306.

Hurtado, A., Reguant, C., Esteve-Zarzoso, B., 2008. Microbial population dynamics during the processing of Arbequina table olives. Food Research International, 41, 738-744.

Rodríguez de la Borbolla J.M., (1958). Conservación de aceitunas de molino. Madrid, Spain: Sindicato Nacional del Olivo.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2010. Distribution of dimorphic yeast species in commercial extra virgin olive oil. Food Microbiology. 27, 1035-1042.

3.2 YEAST SPECIES BIODIVERSITY OF OLIVE OIL EXTRACTION PROCESS

Abstract

The yeast populations occurring in olive oil extraction process are numerically significant. Samples of crushed pastes, kneading pastes, oil from decanter and pomaces, collected during different olive oil extraction processes carried out in three consecutive years, showed yeast concentrations ranging between 10^3 and 10^5 CFU/g. Eighteen dominant yeast species were identified sequencing rRNA genes and/or their flanking ITS regions and the isolation frequencies of each species were calculated in the various collected samples. The occurrence of the various yeast species in olive oil extraction process depended not only on the yeasts contaminating the olives but also on the yeasts colonizing the plant for oil extraction. In fact, the dominant yeast species detected on the washed olives were 12, but only 3 of them were also found in oil samples at significant isolation frequency. On the contrary, 4 yeast species, showing significant isolation frequency in oil samples, were below the detection limit in washed olives. These observations suggest a phenomenon of contamination of the plant for oil extraction that selects some yeast species at the expense of others. Finally, a reproducible and rapid molecular method for differentiating the yeast species of the oleic ecosystem was also provided.

3.2.1 Introduction

Newly produced olive oils usually show an opalescent appearance due to the presence of solid particles and micro-drops of vegetation water containing microorganisms, mainly represented by yeasts (Ciafardini and Zullo, 2002a). Some yeast species do not survive a long time whereas others persist and become the typical microbiota of each oil. The yeasts occurring in newly produced oil can remain active during the conservation period and, according to their metabolic capabilities, can either improve or worsen the oil quality (Zullo et al., 2010). In fact, β-glucosidase and esterase activities can improve the taste and the antioxidant capability of the oil, while the lipase activity can deteriorate the oil quality by hydrolyzing triglycerides (Ciafardini and

Zullo, 2002a; Ciafardini et al., 2006a,b; Ciafardini and Zullo, 2015). Recently, Zullo et al. (2013) demonstrated that the presence of some yeast species might be responsible for olive oil sensory decay during storage. Despite these evidence regarding the impact of the yeasts on olive oil quality, only a few studies have investigated the yeast biodiversity in the olive oil environment. Some authors found, in commercial extra virgin olive oil, yeasts belonging to *Candida diddensiae*, *Candida boidinii*, *Candida wickerhamii*, *Williopsis californica*, *Geotrichum candidum*, and *Saccharomyces cerevisiae* species (Ciafardini and Zullo 2002a; Giannoutsou et al., 2004; Ciafardini et al. 2006a,b; Zullo and Ciafardini, 2008; Zullo et al. 2010). Zullo et al. (2010) also demonstrated within olive oil, the presence of some human opportunistic pathogen yeast species identified as *Candida parapsilosis* and *Candida guilliermondii*. Finally, Čadež et al. (2012) recently described two new yeast species (*Candida adriatica* and *Candida molendinolei*) isolated from olive oil and its by-products, while Ciafardini et al. (2013) found a new yeast species (*Yamadazyma terventina*) in Italian olive oils. Some of these yeast species were found only in oil and not in its by-products, suggesting the existence of a typical yeast microbiota for the olive oil extractive process. Actually, studies on the yeast species occurring in the different phases of this process are lacking. Only a recent study of Romo-Sanchez et al. (2010) showed the biodiversity of yeasts isolated from fresh olives, paste and pomace of two olive varieties (*Arbequina* and *Cornicabra*) by identifying fourteen yeast species that belonged to seven different genera (*Zygosaccharomyces*, *Pichia*, *Lachancea*, *Kluyveromyces*, *Saccharomyce*s, *Candida*, *Torulaspora*). Therefore, the aim of this study was to assess the yeast species occurring in washed olives, pastes (after crushing and after kneading), oil (after centrifugation in a two-phase decanter and after filtration) and pomaces sampled during 35 extra virgin olive oil extraction processes. These processes were carried out in the same oil mill located in Tuscany during different days of the harvest time in three consecutive years. Considering the great biodiversity of yeast populations associated with the oleic ecosystem, the sequencing of rRNA genes and/or their flanking ITS regions are recommended in order to reach a correct identification.

Although these techniques are reasonably precise, they are time-consuming and, thus, not suitable for a rapid screening of a large number of isolates. Therefore, the aim of this study was also to provide a rapid and reproducible molecular method for differentiating yeast species from olive oil environment.

3.2.2 Materials and methods

Randomly Amplified Polymorphic DNA analysis (RAPD).

Yeast cells picked from 24-h-old colonies were suspended in 50 µL of sterile water and then two µL were directly used for all PCR reactions. Randomly Amplified Polymorphic DNA (RAPD) analysis was performed using three different random primers: the primer M13 (5'-GAGGGTGGCGGTTCT-3') (Huey and Hall, 1989), the primer MV1 (5'-GGACGCTTCTG-3') (Venturi et al., 2012) and the primer P4 (5'-CCGCAGCGTT -3') (De Angelis et al., 2001). The PCR protocol was according to Reguant & Bordons (2003). All reactions included both negative (DNA-free) and positive controls and the PCR was processed in an Applied Biosystems® 2720 Thermal Cycler (Life Technologies, Monza, Italy). Amplicons were analyzed on 2% (w/v) agarose gel (Lonza Group Ltd, Basel, Switzerland) stained with ethidium bromide (Sigma-Aldrich, St Louis, Missouri,USA) in TEB buffer for 2,5 h at 100 V and observed by UV transillumination. Band patterns, captured as TIFF format files with a CCD camera (UVItec Gel Documentation System, Cambridge, UK), were subjected to pairwise comparison with the Dice coefficient (SD) (Sneat and Sokal 1973) and cluster analysis with the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) (Vauterin and Vauterin, 1992). All analysis steps were performed by GelCompar 4.0 software (Applied Math, Kortrijk, Belgium). Reproducibility of RAPD-PCR patterns was assessed by comparing the PCR products obtained with DNA prepared from two separate cultures of the same strains. In the RAPD-PCR analysis were also included the following type strains: *Aureobasidium pullulans var. pullulans* CBS 100524*, Candida adriatica* CBS 12504*, Candida diddensiae* CBS 2214*, Candida ishiwadae* CBS 6022, *Candida molendinolei* CBS 12508, *Candida norvegica* CBS 4239*, Candida peltata* CBS 5576, *Candida railenensis* CBS 8164*, Candida*

tenuis CBS 615, *Candida wickerhamii* CBS 2928*, Lachancea fermentati* CBS 707, *Lachancea cidri* CBS 4575, *Metschnikowia fructicola* CBS 8853*, Nakazawaea holstii* CBS 4140, *Pichia kluyveri* CBS 188*, Pichia manshurica* CBS 209, *Pichia membranifaciens* CBS 107, *Rhodotorula graminis* CBS 2826*, Rhodotorula mucilaginosa* CBS 316*, Rhodotorula slooffiae* CBS 5706*, Saccharomyces cerevisiae* CBS 1171*, Yamadazyma terventina* CBS 12510*, Zygotorulaspora mrakii* CBS 4218*.*

Restriction analysis of Internal Transcribed Spacer rDNA.

At least two yeast isolates were chosen as representative of different RAPD patterns and were assayed by PCR-RFLP analysis of the rDNA-ITS (Internal Transcribed Spacer) region as described by Granchi et al. (1999), using *Cfo*I, *Hae*III and *Hinf*I (Fermentas Inc, Burlington, Ontario, Canada) as restriction endonucleases. The restriction fragments were separated (at 100 volt for 2.5 h) on 2 % (w/v) agarose gel (Lonza Group Ltd, Basel, Switzerland), containing ethidium bromide (Sigma-Aldrich, St Louis, Missouri, USA) and TEB buffer (1 M Tris, 10 mM EDTA, 0.9 M boric acid, pH 8.3). The profiles were compared with data reported in the literature (Granchi et al., 1999; Pulvirenti et al., 2004; Arroyo-Lopez et al., 2006; Bautista-Gallego J., 2011; Esteve-Zarzoso et al., 1999; Fernandez-Espinar et al., 2000; Guillamon et al., 1998; Nisiotou et al., 2010; Pham T., 2011; de Llanos Frutos R. et al., 2004; Villa-Carvajal M., 2004). The ITS profiles of the isolates were also compared with those of the type strains listed above.

rDNA gene sequencing and sequence analysis.

To confirm the identification obtained by RFLP analysis of the 5.8S-ITS rDNA region, the D1/D2 domain of 26S rDNA gene of a significant number of isolates from each RAPD profile, was amplified using the primers NL_1 (5’-GCATATCAATAAGCGGAGGAAAAG-3’) and NL_4 (5’-GGTCCGTGTTTCAAGACGG-3’) (Kurtzman and Robnett, 1998). PCRs were performed in 50µL containing 2µL of the DNA obtained, 5µL of 10x reaction buffer (Polymed, Italy), 2.5µL dNTPs mix (10 mM

each), 2µL Bovine Serum Albumine (10mg/mL), 2.5µL $MgCl_2$ (25mM), 2µL each primers (10 µM), 0.2µL Taq DNA polymerase (Polymed, Italy) and 31.8µL of de-ionized H_2O. The following program was used: 95°C for 5 min at the start followed by 30 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 2 min and a final extension of 72°C for 7 min. In addition, the primers V9G (de Hoog & Gerrits van den Ende 1998) and LR5 (Vilgalys & Hester 1990) were used to amplify the partial nrRNA gene that includes, the 3'end of the small-subunit rDNA, the D1/D2 domain of the large subunit rDNA, as well as the ITS (internal transcribed spacer) domain (ITS1, ITS2 and the intervening 5.8S rRNA gene) as described by Knutsen et al., (2007).

The specificity of the PCR products was checked on an agarose gel 1.4% (w/v) before purification. The PCR products were purified using Nucleo Spin Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions before sending to BMR Genomics (Padua, Italy) for sequencing. The forward primer NL1 was used for sequencing the D1/D2 domain of 26S rDNA gene, while forward and reverse primers (V9G and LR5) were used to sequence both strands of the large subunit nrDNA. The sequences obtained in FASTA format were compared to sequences available in GenBank database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search tools (Altschul et al., 1990).

Phenotypic characterization.

At least two yeast isolates were chosen as representative of different RAPD patterns and were assayed to study the pattern of carbon compound assimilation, which in many cases is species-specific, the yeast identification system ID 32 C system (Biomerieux, Marcy-l'Etoile, France) was used according to the manufacturer's instructions. The capability to grow at 37°C was assayed on MYPG agar.

3.2.3 Results

3.2.3.1 Development and validation of a RAPD-PCR method for a rapid yeast identification

In order to develop a RAPD–PCR method for a rapid identification of yeasts, preliminary assays were performed by using three different primers reactions with DNA of 8 type strains of yeast species usually found in olives, in oil or in its by-products: *Candida adriatica* CBS 12504, *Candida diddensiae* CBS 2214, *Candida molendinolei* CBS 12508, *Candida railenensis* CBS 8164, *Candida tenuis* CBS 615, *Candida wickerhamii* CBS 2928, *Lachancea fermentati* CBS 707, *Saccharomyces cerevisiae* CBS 1171, (Ciafardini and Zullo, 2002a,b; Giannoutsou et al., 2004; Zullo and Ciafardini, 2008; Zullo et al., 2010¸ Čadež et al., 2012). The suitability of each primer was evaluated and one of them was selected on the basis of its differentiating capability between isolates belonging to different yeast species, number and intensity of the bands and reproducibility (Table 3). Indeed, as shown in Table 3, only M13 RAPD reaction was able to differentiate the largest number of the strains. Nevertheless, RAPD patterns generated with primers MV1 and P4 did not show an adequate reproducibility level and therefore these primers were discarded. On the contrary, reproducibility level of 98% was achieved with the primer M13.

Table 3: Differentiating value (expressed as percentage of number of different profiles/number of total isolates), number of DNA fragments and reproducibility level of each primer.

PRIMER	Differentiating value (%)	Number of DNA fragments (170-5000 pb)	Reproducibility level
M13	100	8-15	98 %
P4	82	2-13	95%
MV1	64	2-11	90%

3.2.3.2 Molecular yeast identification

Yeast isolates from the different samples, as reported in table 4, were identified by combining various molecular methods such as RAPD-PCR with primer M13, RFLP analysis of the rDNA-ITS region, and sequencing of specific rDNA regions. Type strains of yeast species usually found in olives, in oil or in its by-products (Ciafardini and Zullo, 2002a,b; Giannoutsou et al., 2004; Zullo and Ciafardini, 2008; Zullo et al., 2010¸ Čadež et al., 2012, Ciafardini et al., 2013) were also assayed. Cluster analysis of the 27 RAPD-PCR patterns generated the dendrogram shown in Figure 16. Seventeen clusters, those designated from A to Q, were detected at about a 50% similarity level (Fig. 16). This similarity level was chosen on the basis of the reproducibility between different RAPD–PCR patterns for the same isolate (≥ 95%) and of the similarity levels between type strains of different yeast species (≤ 48%). Among the seventeen RAPD-PCR clusters, twelve included the type strain of a yeast species while the remaining five clusters did not include any tested type strain (Table 5). Therefore, according to these results, yeast isolates belonging to the following clusters A, E, F, H, I, J, K, L, N, O, P and Q, were respectively assigned to twelve putative yeast species: *Pichia manshurica*, *Rhodotorula sloffiae, Candida diddensiae*, *Candida norvegica*, *Candida adriatica*, *Metschnikowia fructicola*, *Candida molendinolei, Candida wickerhamii*, *Saccharomyces cerevisiae*, *Pichia kluyveri*, *Rhodotorula mucilaginosa* and *Zygotorulaspora mrakii*. In order to confirm the identification of these yeast species and to accomplish the identification of yeast isolates belonging to the clusters not including a type strain, two isolates from each RAPD-PCR cluster along with the relative type strains were assayed by PCR-RFLP analysis of the rDNA ITS region and sequence analysis of D1/D2 region. The isolates and the type strains belonging to the same RAPD-PCR cluster showed very similar ITS restriction patterns by using the endonucleases *Cfo*I, *Hae*III, and *Hinf*I (Tab. 5) and these were in accordance with data obtained by other Authors (Esteve-Zarzoso et al., 1999; Fernandez-Espinar et al., 2000; de Llanos Frutos et al., 2004; Villa-Carvajal et al., 2004; Pham et al., 2011; Nisiotou et al., 2012). In addition, sequence comparison of the D1/D2 region of the yeasts, included in the same RAPD-cluster, yielded

similarity values, with the closest relative species, between 99.8 and 100%, thus confirming the identifications obtained by ITS polymorphism analysis (Tab. 5). These findings demonstrated that all the putative above-mentioned twelve species were properly identified by the RAPD-PCR with primer M13. In particular, it was able to discriminate *Candida molendinolei* and *Candida wickerhamii*, which were indistinguishable with ITS-RFLP as they showed the same ITS restriction fragments with *Cfo*I, *Hae*III, and *Hinf*I (Tab. 5).

Regarding yeast isolates grouped into the five RAPD-clusters (B, C, D, G and M) not containing a type strain, RFLP analysis of the rDNA ITS region and sequence analysis of the D1/D2 region suggested that they belonged, respectively, to *Candida oleophila*, *Candida railenensis*, *Candida tenuis*/*Yamadazyma terventina*, *Lachancea fermentati/Lachancea cidri* and *Rhodotorula glutinis* (Tab. 5) (Groenewald et al., 2011; de Llanos Frutos et al., 2004; Čadež et al., 2010; Esteve-Zarzoso et al., 1999). It is underlined that type strains of the species *Candida oleophila* and *Rhodotorula glutinis* were not tested in this study. However, since BLAST analysis of D1/D2 sequences of the isolates ascribed to these species yielded similarity values of 100 and 99.7%, respectively, the two yeast species were considered correctly designated (Kurtzman and Robnett 1998) by RAPD-PCR with the primer M13. On the contrary, the type strains of the other five putative species were here analysed and displayed RAPD-patterns different from those of the isolates comprised in clusters C, D and G, as shown in the dendrogram reported in figure 16. Therefore, to assess the identity of these isolates and considering that previous molecular tests gave in two cases ambiguous identifications, sequence analysis of their 5.8S-ITS region and of 26S rRNA gene were, additionally, carried out. According to the highest similarity value obtained by sequence comparisons to GenBank database by using BLAST (tab. 6), isolates belonging to clusters C, D and G were respectively assigned to the species *Candida railenensis*, *Yamadazyma terventina* and *Lachancea fermentati*. The fact that the type strains of these yeast species exhibited different RAPD-PCR patterns with primer M13

could be due to the intraspecific genetic variation. Indeed, also restriction profiles of 5.8S-ITS region obtained with *Cfo*I for the type strain of *Yamadazyma terventina* species, showed some differences in the size of the restriction fragments (data not shown), although the enzyme *Hae*III and *Hinf*I generated restriction patterns in agreement with Ciafardini et al. (2013).

In conclusion, RAPD-PCR with primer M13 allowed to distinguish seventeen different yeast species, three of them recently isolated from olive oil and/or its by-products and recognised as new species: *Yamadazyma terventina* (Ciafardini et al., 2013), *Candida molendinolei* and *Candida adriatica* (Čadež et al., 2012).

On the contrary, this rapid method did not give reproducible RAPD-PCR patterns for some assayed isolates, which showed black colonies on agar plates. Based on their microscopic morphology and on the results of RFLP analysis of rITS region and on the sequencing of the D1/D2 26S rDNA region, all these isolates were identified as belonging to *Aureobasidium pullulans* species, a ubiquitous yeast-like fungus also associated with olive fermentation (Nisiotou et al., 2010). In particular, all these isolates along with the type strain *Aureobasidium pullulans* CBS 100524 showed a PCR product of ca. 600 bp in the ITS region and restriction profiles of 190, 180, 110 and 100 bp fragments with *Cfo*I, of 440 and 150 bp with *Hae*III and of 290, 180 and 140 with *Hinf*I, according to Nisiotou et al. (2010). Furthermore, these isolates and the type strain of *Aureobasidium pullulans* showed 99.8% similarity of the nucleotide sequence of the D1/D2 region, confirming their identification.

Table 4: Origin of the yeast isolates from 35 olive oil extractive processes carried out during the harvest time in three consecutive years.

Olive cultivar	Origin	Number of yeast
	I year	
Moraiolo	Olives	24
	Crushed pastes	46
	Oil from decanter	44
	Pomaces	20
Frantoio	Olives	22
	Crushed pastes	56
	Oil from decanter	48
	Pomaces	26
	II year	
Moraiolo	Olives	42
	Crushed pastes	10
	Kneaded pastes	90
	Oil from decanter	72
	Pomaces	62
Frantoio	Olives	14
	Crushed pastes	34
	Kneaded pastes	20
	Oil from decanter	28
	Pomaces	20
	III year	
Mixed	Olives	38
	Crushed pastes	90
	Kneaded pastes	56
	Oil from decanter	84
	Pomaces	60

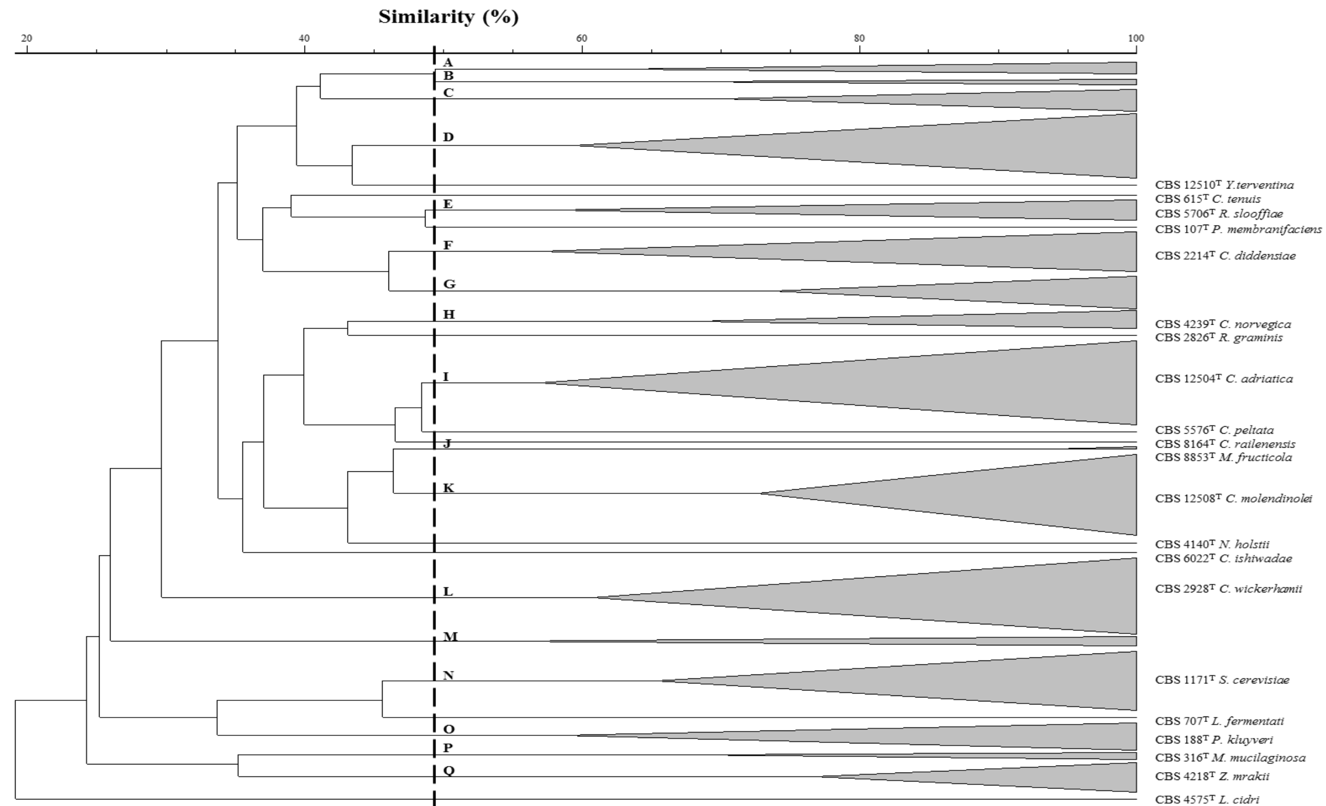


Figure 16: UPGMA dendrogram derived from comparison of the RAPD–PCR patterns obtained with primer M13 for the yeast isolates tested. The vertical dotted line indicates the 49% similarity level.

Table 5: Restriction analysis of the 5.8-ITS rDNA region and sequence information for the D1/D2 region of 26 rDNA gene of yeasts isolates grouped in RAPD clusters and of some relative yeast type strains (*Identification of yeast isolates according to ITS-5.8S RFLPs and 26S rRNA gene D1/D2 region sequences*)

RAPD cluster	Type strain included in RAPD cluster	CBS code	ITS Restriction fragments (pb) of type strains			ITS (pb)	ITS Restriction fragments (pb) of isolates			Sequence of D1/D2 region of isolates and type strains		Closest relative species
			*Cfo*I	*Hae*III	*Hinf*I		*Cfo*I	*Hae*III	*Hinf*I	Matching nucleotides (Identity %)*		
A	*Pichia manshurica*	209	230-110-80	310-90	280-220	470	240-110-50	320-100-50	210-190-90	526/526	(100)	*Pichia manshurica*
B	-	-	-	-	-	620	300-300	400-150	310-310	558/558	(100)	*Candida oleophila*
C	-	-	-	-	-	610	280-280-50	410-140	310-310	540/541	(99.8)	*Candida railenensis*
D	-	-	-	-	-	650	290-220-50	410-130-90	310-310	508/510	(99.6)	*Candida tenuis/Yamadazyma terventina*
E	*Rhodotorula sloffiae*	5706	600	600	330-270	600	600	600	330-280	574/575	(99.8)	*Rhodotorula sloffiae*
F	*Candida diddensiae*	2214	280-170-130-	410-130-80	310-310	650	290-180-130	410-130-90	310-310	511/512	(99.8)	*Candida diddensiae*
G	-	-	-	-	-	680	310-280-80	300-210-90	320-320	552/554	(99.6)	*Lachancea fermentati/L. cidri*
H	*Candida norvegica*	4239	500	380-190	290-270	590	510	370-190	290-260	559/560	(99.8)	*Candida norvegica*
I	*Candida adriatica*	12504	210-130-100-80	400-90	310-310	610	210-140-100-80	400-90	300-300	550/550	(100)	*Candida adriatica*
J	*Metschnikowia fructicola*	8853	210-120-100	290-110	210-190	400	210-120-100	290-110	200-180	416/420	(99)	*Metschnikowia fructicola*
K	*Candida molendinolei*	12508	600	580-80	320-320	660	590	580-90	320-320	506/506	(100)	*Candida molendinolei*
L	*Candida wickerhamii*	2928	590	590-80	320-320	660	590	580-90	320-320	552/552	(100)	*Candida wickerhamii*
M	-	-	-	-	-	650	300-220-110	650	220-130-100	429/430	(99.7)	*Rhodotorula glutinis*
N	*Saccharomyces cerevisiae*	1171	375-325-150	320-230-170-125	375-365-110	850	370-330-140	310-240-180-120	370-360-120	558/558	(100)	*Saccharomyces cerevisiae*
O	*Pichia kluyveri*	188	180-100	380-80	250-200	450	170-110-50	370-80	260-210	556/556	(100)	*Pichia kluyveri*
P	*Rhodotorula mucilaginosa*	316	300-220-120	400-210	340-210-50	650	300-230-120	400-220	360-230-50	547/547	(100)	*Rhodotorula mucilaginosa*
Q	*Zygotorulaspora mrakii*	4218	300-290	390-120-70-50	310-200-130	650	300-280	400-120-70-50	310-200-130	565/565	(100)	*Zygotorulaspora mrakii*

* Identical nucleotide percentages in the sequence obtained from the D1/D2 region of the 26S rRNA gene and the sequence found in Genbank

Table 6: Homology (%) for the 5.8S–ITS region and LSU rRNA gene among isolates belonging to different RAPD-PCR cluster and their putative yeast species based on RFLP-ITS and D1/D2 sequence analysis (N.D. = not detected)

Sequence comparison	5.8-ITS	26S rRNA
Isolates in the cluster C *vs Candida railenensis*	98.9	98.6
Isolates in the cluster D *vs Candida tenuis*	92.0	94.0
Isolates in the cluster D *vs Yamadazyma terventina*	99.6	99.8
Isolates in the cluster G *vs Lachancea fermentati*	96.6	N.D.
Isolates in the cluster G *vs Lachancea cidri*	94.0	N.D.

3.2.3.3 Phenotypic yeast characterization

After molecular identification, the same isolates were also assayed for their capability to metabolize different 32 carbon sources and to grow at 37°C with the aim to further assess the identification at species level obtained with molecular methods. In the table 7 are shown the results compared with those reported in literature (Kurtzman et al., 2011; Čadež et al., 2012; Ciafardini et al., 2013). In dark grey and white are indicated the results respectively in contrast or in agreement with literature, while in light grey are shown the not confirmable results do to a lacking in literature. The comparison between the results obtained by each isolate and the respective type strain was evaluated calculating the "percentage of identity". This percentage was obtained from the following formula:

$$\frac{\text{number of assays common among those obtained and those reported in literature}}{\text{the total number of assays}} \times 100$$

The percentages of identity calculated for each of the species, are shown in Table 8. Isolates belonging to *P. manshurica*, *C. diddensiae*, *L. fermentati, C. norvegica*, *C. wickerhamii*, and *R. mucilaginosa* species were in total agreement with literature (100% identity), while the isolates belonging to other species showed some differences (from 97% to 75% identity) evidently due to intraspecific diversity (Table

8). This diversity was particularly marked for the isolates belonging to *R. glutinis* and *Y. terventina* species. Concerning the latter yeast species, Ciafardini et al. (2013) mentioned the ability to assimilate lactic acid (verified on three strains) as a useful tool to distinguish *Y. terventina* species from the others included in the *Yamadazyma* clade, but this ability was absent in all the isolates assayed in this study (Table 7). Also this difference, added to the fact that the type strains of these yeast species exhibited different RAPD-PCR patterns with primer M13 and the differences in the size of the *CfoI* restriction fragments of 5.8S-ITS region, could be due to the intraspecific genetic variation.

Table 7: Carbohydrate assimilation and growth at 37°C of two yeast isolates representative of each RAPD cluster / species; dark grey: results in contrast with literature, white: results in agreement with literature, light grey: results unreported in literature +: positive, -: negative; v: variable; w: weak.

RAPD cluster	Yeast species	D-Galactose	Actidione	D-saccharose	N-Acetiyl -glucosamine	Lactic acid	L-Arabinose	D-cellobiose	D-raffinose	D-maltose	D-trehalose	potassium.-2-ketogluconate	Methyl-D-glucopyranoside	D-mannitol	D-lactose	Inositol	D-sorbitol	D-xylose
A	***P. manshurica***	-	-	-	+	w	-	-	-	-	-	-	-	-	-	-	-	-
B	***C. oleophila***	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	+
C	***C. railenensis***	+	+	+	+	+	-	-	-	w	w	+	-	+	-	+	+	+
D	***Y. terventina***	+	-	+	+	-	+	+	-	+	+	w	w	+	w	-	+	+
E	***R. sloffiae***	-	-	+	-	-	+	-	-	-	+	+	-	+	-	-	w	+
F	***C. diddensiae***	+	-	+	+	-	+	+	-	+	+	+	+	+	-	-	+	+
G	***L. fermentati***	+	+	+	w	+	w	w	+	w	w	w	-	+	-	-	+	+
H	***C. norvegica***	-	-	-	-	+	-	w	-	-	-	w	-	+	-	-	+	+
I	***C. adriatica***	-	-	+	-	-	-	+	-	+	-	+	+	+	-	-	+	+
J	***M. fructicola***	+	-	+	+	-	-	+	-	+	+	+	+	+	w	-	+	+
K	***C. molendinolei***	-	+	+	+	+	+	+	-	-	+	+	w	+	-	w	+	+
L	***C. wickerhamii***	v	+	-	+	v	v	+	-	-	-	+	-	+	-	-	+	+
M	***R. glutinis***	+	-	+	-	-	-	-	+	-	+	+	-	+	-	-	+	+
N	***S. cerevisiae***	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
O	***P. kluyveri***	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	w
P	***R. mucilaginosa***	+	-	+	v	v	v	-	+	+	+	+	-	+	-	-	v	+
Q	***Z. mrakii***	v	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-

RAPD cluster	Yeast species	D-ribose	Glycerol	L-rhamnose	Palatinose	Erythritol	D-melibiose	Sodium glucoronate	D-melizitose	Potassium gluconate	Levulinic acid	D-glucose	L-sorbose	Glucosamine	Asculine ferric citrate	Growth at 37°
A	***P. manshurica***	-	w	-	-	-	-	-	-	-	-	+	-	+	-	+
B	***C. oleophila***	+	+	-	+	-	-	-	+	-	-	+	+	+	+	-
C	***C. railenensis***	+	+	-	+	-	-	+	+	+	-	+	+	-	w	-
D	***Y. terventina***	-	-	-	+	+	-	-	-	+	w	+	-	-	+	-
E	***R. sloffiae***	w	+	-	-	-	-	+	+	+	-	+	-	-	-	-
F	***C. diddensiae***	+	+	w	+	+	-	-	+	+	w	+	-	+	+	+
G	***L. fermentati***	w	w	w	w	w	+	-	-	-	-	+	-	-	-	+
H	***C. norvegica***	w	+	+	-	-	-	-	-	-	w	+	w	-	-	-
I	***C. adriatica***	w	+	-	+	-	-	-	-	+	-	+	-	-	+	-
J	***M. fructicola***	+	+	+	+	-	-	-	+	+	w	+	+	+	+	-
K	***C. molendinolei***	+	+	+	-	-	-	-	-	w	-	+	-	-	+	+/w
L	***C. wickerhamii***	v	+	v	-	-	-	-	-	+	-	+	-	-	+	-
M	***R. glutinis***	+	+	v	-	-	-	-	-	-	-	+	+	-	-	-
N	***S. cerevisiae***	-	-	-	-	-	+	w	-	-	-	+	-	-	-	v
O	***P. kluyveri***	-	+	-	-	-	-	-	-	-	w	+	+	+	-	+
P	***R. mucilaginosa***	+	+	-	-	-	v	-	+	v	-	+	v	-	-	-
Q	***Z. mrakii***	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-

Table 8: Percentage of identity between the phenotypic characterizations of the various isolates and the respective type strains.

RAPD cluster	Yeast species	% of identity
A	***P. manshurica***	**100**
B	***C. oleophila***	**97**
C	***C. railenensis***	**84**
D	***Y. terventina***	**78**
E	***R. sloffiae***	**97**
F	***C. diddensiae***	**100**
G	***L. fermentati***	**100**
H	***C. norvegica***	**100**
I	***C. adriatica***	**94**
J	***M. fructicola***	**90**
K	***C. molendinolei***	**94**
L	***C. wickerhamii***	**100**
M	***R. glutinis***	**75**
N	***S. cerevisiae***	**87**
O	***P. kluyveri***	**97**
P	***R. mucilaginosa***	**100**
Q	***Z. mrakii***	**94**

3.2.3.4 Distribution of yeast species in the different extractive phases

To evaluate the yeast biodiversity occurring in olive oil extraction process, the frequencies of each yeast species was calculated grouping the data according to: (1) the type of sample (pastes, oil, or pomaces), (2) the olive cultivars (*Frantoio* or *Moraiolo*) used in the various extractive processes, and finally (3) the years in which the extraction processes were carried out.

Table 9 indicates the distribution of the various yeast species (expressed as isolation frequencies) in the different samples (olives, pastes, oil, and pomaces) collected during the 35 olive oil extraction processes taken into consideration. Eleven dominant yeast species, besides the yeast-like fungus *A. pullulans,* were isolated from washed olives. *A. pullulans* was also found in crushed pastes, even if at lower percentage than in the washed olives, and below 1% in the other samples. All the yeast species characterizing the olives, except *Z. mrakii*, were also isolated from the crushed pastes

and the *C. molendinolei* species exhibited the highest isolation frequency (almost 50%). Kneaded pastes showed nine different yeast species, six being in common with those isolated from crushed pastes. *Z. mrakii* was the dominant species of the kneaded pastes showing an isolation frequency of about 40%, although this yeast was isolated from washed olives and crushed pastes with a very low frequency (≤1%). The samples of oil from decanter were characterized by eight dominant yeast species, six shared with those from kneaded pastes. *C. molendinolei* and *Y. terventina* were both the dominant yeast species in these samples with the same isolation frequencies. Nevertheless, the distribution of these two yeast species in the olive oil extraction processes were very different. Indeed, *C. molendinolei* was found in all the types of samples (pastes, oil, and pomaces) with isolation frequency ranging from 10 to 52%, while *Y. terventina* was detected at significant frequency (> 1%) only from oil and pomace samples (38 and 2%, respectively). Finally, pomaces substantially showed the same yeast species detected in oil even if with different isolation frequencies.

Table 10 shows the isolation frequencies of yeast species in the different types of samples according to the olive cultivars (*Frantoio* or *Moraiolo*) used in the extractive processes. The results did not demonstrate the existence of relationships between the olive cultivar used in the extractive process and the yeast species isolated from the various samples: no yeast species was uniquely associated with one of the two olive cultivars assayed.

Finally, the distribution of yeast species in the different samples is reported in relation to the year of collection (Tab. 11). The results demonstrated differences in the dominant yeast species isolated from washed olives according to the different year of collection. Indeed, washed olives in 2011 were characterized by a significant presence of *C. adriatica*, *C. molendinolei*, and *C. wickerhamii*; in 2012 by *C. norvegica*; in 2013 by *C. railenensis* and *C. oleophila*. *A. pullulans* was found in all olive samples even if with very different isolation frequencies (the highest being in 2012 and the lowest in 2011). Differences based on the years were also observed in the pastes. Indeed, the dominant species of pastes in 2011 were *C. molendinolei*, in 2012 *C. norvegica*, *A.*

pullulans and *Z.mrakii*, while in 2013 *S. cerevisiae*. On the contrary, the dominant yeast species, isolated from oil and pomaces during the three assayed years, were almost the same. In particular, the oil was mainly characterized by the presence of *Y. terventina* and *C. molendinolei*, while the pomaces by *C. adriatica* and *C. molendinolei*.

As reported in this study, the yeast populations occurring in olive oil extraction processes were originate not only from the yeasts contaminating the olives but also from the yeasts colonizing the oil extractive plants. Indeed, the dominant yeast species detected on the washed olives were eleven but only three of them were also found in oil samples at significant isolation frequencies: *C. adriatica*, *C. molendinolei*, and *C. wickerhamii*. On the contrary, same yeast species showed significant isolation frequencies only in oil samples, such as *Y. terventina*, or in kneaded pastes and pomaces, such as *Z. mrakii*. These observations suggest a possible contamination of the plant for oil extraction (malaxation equipment and decanter in particular) that might select some yeast species at the expense of others.

Table 9: Distribution (%) of yeast species and of the yeast-like fungus *A. pullulans* in different samples collected during 35 olive oil extraction processes carried out in three years; (the symbol “-“ indicates isolation frequency <1%).

	Washed olives	Crushed pastes	Kneaded pastes	Oil from decanter	Pomaces
A. pullulans	22	12	-	-	-
C. adriatica	7	6	-	10	20
C. diddensiae	-	3	2	3	1
C. kluyveri	-	-	1	-	-
C. molendinolei	10	47	28	38	52
C. norvegica	13	6	-	-	-
C. oleophila	10	-	1	-	-
C. railenensis	9	3	1	-	-
C. wickerhamii	13	5	12	7	7
L. fermentati	-	5	-	-	4
M. fructicola	1	1	-	-	-
R. glutinis	6	2	-	-	-
R. mucilaginosa	4	1	-	-	-
R. sloffiae	3	2	1	1	-
S. cerevisiae	-	4	19	2	2
Y. terventina	-	-	-	38	2
Z. mrakii	1	-	38	1	11
Others	1	3	-	-	1

Table 10: Distribution of yeast species (%) in the different samples collected during 30 olive oil extraction processes of two different years and subdivided according to olive cultivar (*Frantoio* or *Moraiolo*) ; (the symbol "-" indicates isolation frequency <1%).

	Washed olives		**Crushed pastes**		**Kneaded pastes**		**Oil from decanter**		**Pomaces**	
	Frantoio	*Moraiolo*	*Frantoio*	*Moraiolo*	*Frantoio*	*Moraiolo*	*Frantoio*	*Moraiolo*	*Frantoio*	*Moraiolo*
A. pullulans	19	25	23	-	-	-	-	-	-	-
C. adriatica	12	8	7	2	-	-	17	19	10	13
C. diddensiae	-	-	4	3	-	-	8	-	3	-
C. kluyveri	-	-	-	-	-	2	-	-	-	-
C. molendinolei	10	18	31	21	13	6	21	28	34	28
C. norvegica	33	5	7	20	-	-	-	-	-	-
C. oleophila	-	-	-	-	-	-	-	-	-	-
C. railenensis	-	-	5	2	-	-	-	-	-	-
C. wickerhamii	17	19	9	3	26	3	4	22	10	18
L. fermentati	-	-	2	11	-	-	-	8	7	8
M. fructicola	2	-	2	-	-	-	-	2	-	-
R. glutinis	3	8	4	-	-	-	-	-	-	-
R. mucilaginosa	1	11	-	10	-	-	-	-	-	-
R. sloffiae	3	6	-	20	-	-	-	-	-	-
S. cerevisiae	-	-	2	8	8	3	2	-	3	2
Y. terventina	-	-	-	-	-	-	46	21	8	4
Z. mrakii	-	-	-	-	53	85	2	-	25	26
Others	-	-	4	-	-	1	-	-	-	1

Table 11: Distribution of yeast species (%) in the different samples collected during 35 olive oil extraction processes and subdivided according to the different years (2011, 2012, and 2013) ; (the symbol "-" indicates isolation frequency <1%).

	Washed olives			Crushed pastes			Kneaded pastes		Oil from decanter			Pomaces		
	2011	**2012**	**2013**	**2011**	**2012**	**2013**	**2012**	**2013**	**2011**	**2012**	**2013**	**2011**	**2012**	**2013**
A. pullulans	2	42	23	-	36	-	-	-	-	-	-	-	-	-
C. adriatica	20	-	-	8	-	1	-	33	4	27	-	13	37	9
C. diddensiae	-	-	-	8	-	-	-	-	9	-	-	3	-	-
C. kluyveri	-	-	-	-	-	-	-	1	-	-	-	-	-	-
C. molendinolei	28	-	3	48	4	1	7	10	29	9	75	55	12	88
C. norvegica	-	38	-	-	19	-	-	-	-	-	-	-	-	-
C. oleophila	-	-	30	-	-	-	-	2	-	-	-	-	-	-
C. railenensis	-	-	26	2	7	-	-	-	-	-	-	-	-	-
C. wickerhamii	36	-	4	10	4	-	13	4	7	11	3	10	10	-
L. fermentati	-	-	-	14	-	10	-	-	-	-	-	13	-	-
M. fructicola	2	-	-	-	4	-	-	-	-	-	-	-	-	-
R. glutinis	3	8	8	-	7	-	-	-	-	-	-	-	-	-
R. mucilaginosa	-	12	-	-	4	-	-	1	-	-	-	-	-	-
R. slooffiae	9	-	-	-	7	-	-	-	-	-	4	-	-	-
S. cerevisiae	-	-	-	10	-	88	4	48	2	-	4	3	1	3
Y. terventina	-	-	-	-	-	-	-	1	49	50	14	-	6	-
Z. mrakii	-	-	3	-	-	-	76	-	-	3	-	-	33	-
Others	-	-	3	-	8	-	-	-	-	-	-	3	1	-

3.2.3.5 Evolution of the yeasts contamination during the activity of the olive oil extractive plant: focus on 2012

In order to investigate on the evolution of the contamination of the yeasts during the activity of the oil extractive plants, a focus on the distribution of the various yeast species (expressed as isolation frequencies) in the different samples (crushed and kneaded pastes, oil, and pomaces) collected during four different days of the same harvesting year (2012) was evaluated (Figure 17). Samples were collected at the beginning of the harvesting year (first harvesting date), at the middle (second and third harvesting date), and at the end (fourth harvesting date). The isolation frequencies of the dominant yeast species detected the first harvesting day in the different samples, often decreased over the other harvesting days, while other species increased their relative abundance (Fig. 17 A-D). In the samples of oil from decanter, for example, the first harvesting day was characterized by a significant presence of *C. wickerhamii, M. fructicola* and *C. molendinolei*; during the other harvesting days the relative abundance *of M. fructicola* decreased below the detection limit (< 10 UFC/ml) while *C.adriatica* and *Y.terventina* increased (Fig.17 C).

These observations confirm a possible contamination of the plant for oil extraction during the process. Probably, the environmental conditions promote a selective enrichment of specific yeast species that could affect olive oil quality.

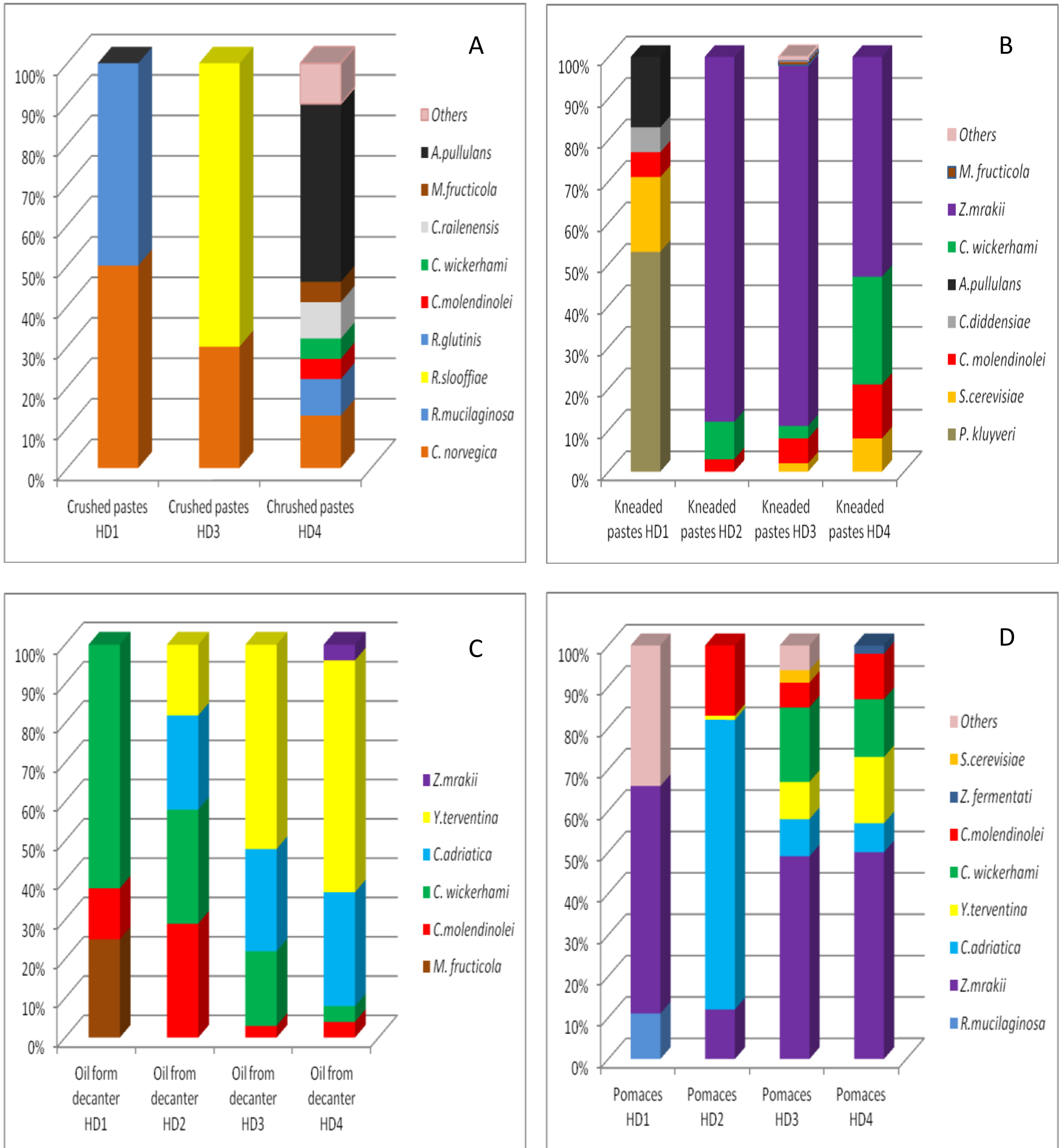


Figure 17: Distribution of the yeast species (expressed as isolation frequencies) in the different extractive phases sampled during four different harvesting dates (HD) in 2012. A: distribution of the yeast species in crushed pastes; the data of the second harvesting date is not available because the yeast concentration was below the detection limit (< 100 UFC/g). B: distribution of the yeast species in kneaded pastes. C: distribution of the yeast species in oil from decanter. D: distribution of the yeast species in pomaces.

3.2.4 Conclusion

The yeast populations occurring in olive oil extraction processes are numerically significant and originate not only from the yeasts contaminating the olives but also from the yeasts colonizing the oil extractive plants. Indeed, as reported in this study, the dominant yeast species detected on the washed olives were eleven (*A. pullulans, C. norvegica*, *C. adriatica*, *C. railenensis*, *C. molendinolei, C. wickerhamii*, *C. oleophila*, *M. fructicola, R. glutinis, R. mucilaginosa R. sloffiae* and *Z. mrakii*), but only three of them, were also found in oil samples at significant isolation frequencies (*C. adriatica*, *C. molendinolei*, and *C. wickerhamii)*. On the contrary, same yeast species showed significant isolation frequencies only in oil samples, such as *Y. terventina*, or in kneaded pastes and pomaces, such as *Z. mrakii*. These observations suggest a possible contamination of the plant for oil extraction (malaxation equipment and decanter in particular) that might select some yeast species at the expense of others. Probably, during the extractive process, the environmental conditions promote a selective enrichment of specific yeast species that could affect olive oil quality. Only few studies were carried out on the metabolic capabilities of these yeast species in modifying the chemical and organoleptic properties of olive oil. In particular, *C. wickerhamii* species was frequently isolated from commercial oils and various strains belonging to this species demonstrated to possess lipase and β-glucosidase activities (Zullo and Ciafardini, 2008; Ciafardini and Zullo, 2002b). The strain *C. wickerhamii* DAPES 1885 showed also esterase activity and, once inoculated in olive oil, it was responsible of “Muddy-sediment” and “rancid” (Zullo et al., 2013). On the contrary, little information are available on the metabolic capabilities of *C. adriatica, C. molendinolei,* and *Y. terventina*, because of their recent classification as new species (Čadež et al., 2012; Ciafardini et al., 2013). Indeed, only two studies have been currently carried out on this topic. The first demonstrated that the strain *C. adriatica* DAPES 1933 possess esterase activity with similar effects to those caused by *C. wickerhamii* DAPES 1885 when inoculated in oil (Zullo et al., 2013). The latter proved that the lipolytic activity of lipase-producer yeast strains belonging to *C. adriatica* and *Y. terventina* species can

influence the acidity of the olive oil (Ciafardini and Zullo 2015). *Z. mrakii* was often isolated from table olives during spontaneous or industrial fermentation (Bleve et al., 2014; Bautista-Gallego et al., 2011), but no information is currently available regarding the impact of this species on the organoleptic quality of olive oil. Finally, the application of RAPD method with primer M13 proved to be an effective, low cost and efficient tool to identify at level species the yeasts isolated from olives, olive oil and its by-products.

It must be highlighted that the species *Candida wickerhamii, Candida molendinolei*, *Candida peltata* and *Candida ishiwadae* are transferred in the genus *Nakazawaea* (Cletus et al.,2014) but in the present study the previous classification was maintained.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. Journal of Molecular Biology, 215, 403-410.

Arroyo-Lopez, F.N., Duran-Quintana, M.C., Ruiz-Barba, J.L., Querol, A., Garrido-Fernandez, A. 2006. Use of molecular methods for the identification of yeast associated with table olives. Food Microbiology, 23, 791-769.

Bautista-Gallego, J., Rodríguez-Gómez, F., Barrio, E., Querol, A., Garrido-Fernández, A., Arroyo-López, F.N. 2011. Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. International Journal of Food Microbiology, 147, 89-96.

Bleve, G., Tufariello, M., Durante, M., Perbellini, E., Ramires, F.A., Grieco, F., Cappello, M.S., De Domenico, S., Mita G., Tasioula-Margari, M., Logrieco A.F. 2014. Physico-chemical and microbiological characterization of spontaneous fermentation of Cellina di Nardò and Leccino table olives. Frontiers in Microbiology, 5, 1-18.

Čadež, N., Raspor, P., Turchetti, B., Cardinali, G., Ciafardini, G., Veneziani, G., Peter, G. 2012. *Candida adriatica* sp. nov. and *Candida molendinolei* sp. nov., two novel yeast species isolated from olive oil and its by-products. International Journal of *Systematic and Evolutionary* Microbiology, 62, 2296-2302.

Ciafardini, G., Zullo, B.A. 2002a. Survival of microorganisms in extra virgin olive oil during storage. Food Microbiology, 19, 105-109.

Ciafardini G., Zullo B.A. 2002b. Microbiological activity in stored olive oil. International Journal of Food Microbiology, 75, 111–118.

Ciafardini G., Zullo B.A. 2015 Effect of lipolytic activity of *Candida adriatica, Candida diddensiae* and *Yamadazyma terventina* on the acidity of extra-virgin olive oil with a different polyphenol and water content. Food Microbiology, 47, 12-20.

Ciafardini G., Zullo B.A., Iride A. 2006a. Lipase production by yeasts from extra virgin olive oil. Food Microbiology, 23, 60–67.

Ciafardini G., Zullo B.A., Cioccia G., Iride A. 2006b. Lipolytic activity of Williopsis californica and Saccharomyces cerevisiae in extra virgin olive oil. International .Journal of Food Microbiology, 107, 27–32.

Ciafardini G., Zullo B.A., Antonielli L., Corte L., Roscini L., Cardinali G., 2013. *Yamadazyma terventina* sp.nov., a yeast species of the *Yamadazyma* clade from Italian olive oils. International Journal of Systematic and Evolutionary *Microbiology*, 63, 372-376.

Cletus P., Kurtzman C.P., Robnett C.J. 2014. Description of *Kuraishia piskuri* f.a., sp. nov., a new methanol assimilating yeast and transfer of phylogenetically related *Candida* species to the

genera *Kuraishia* and *Nakazawaea* as new combinations. FEMS Yeast Research, 14, 1028-1035.

de Hoog, G.S., Gerrits van den Ende, A.H.G. 1998. Molecular diagnostics of clinical strains of filamentous basidiomycetes. Mycoses, 41, 183 –189.

de Llanos Frutos, R., Fernandez-Espinar M., T., Querol, A. 2004. Identification of species of the genus *Candida* by analysis of 5.8S rRNA gene and the two ribosomal internal transcribed spacer. Antonie van Leeuwenhoek. 85, 175-185.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. International Journal of Systematic Bacteriology, 49, 329–337.

Fernandez-Espinar, M.T., Esteve-Zarzoso, B., Querol, A. and Barrio, E. 2000. RFLP analysis of the ribosomal internal transcribed spacer and the5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of flor yeasts. Antonie van Leeuwenhoek, 78, 87–97.

Giannoutsou, E.P., Meintanis, C., Karagouni, A.D. 2004. Identification of yeast strains isolated from a two-phase decanter system olive oil waste and investigation of their ability for its fermentation. Bioresource Technology, 93, 301-306.

Granchi, L., Bosco, M., Messini, A., Vincenzini, M., 1999. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. Journal of Applied Microbiology, 87, 949-956.

Groenewald M., Robert V., Smith M.Th. 2011. The value of the D1/D2 and internal transcribed spacers (ITS) domains for the identification of yeast species belonging to the genus *Yamadazyma*. Persoonia, 26, 40-46.

Guillamon, J.M., Sabate, J., Barrio, E., Cano, J., Querol, A. 1998. Rapid identification of wine yeast species based on the RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Archives *of* Microbiology, 169, 387–392.

Huey, B. and Hall, J., 1989. Hypervariable DNA fingerprinting in *Escherichia coli*. Minisatellite probe from bacteriophage M13. Journal of Bacteriology, 171, 2528–2532.

Knutsen, A.K., Robert, V., Poot, G.A., Epping, W., Figge, M., Holst-Jensen, A., Skaar, I., Smith, M.Th. 2007. Polyphasic re-examination of Yarrowia lipolytica strains and the description of three novel Candida species: *Candida osloensis* sp. nov., *Candida alimentaria* sp. nov. and *Candida hollandica* sp. nov. International Journal of Systematic and Evolutionary Microbiology 57, 2426–2435.

Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), 2011. The Yeasts: A Taxonomic Study. Fifth edition, Elsevier, San Diego USA.

Kurtzman, C.P., Robnett, C.J. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequence. Antonie van Leeuwenhoekm, 73, 331-371.

Nisiotou, A.A., Chorianopoulos, N., Nychas, G.J.E., Panagou, E.Z. 2010. Yeast heterogeneity during spontaneous fermentation of black Conservolea olives in different brine solutions. Journal of Applied Microbiology, 108, 396-405.

Pham, T., Wimalasena T., Box W.G., Koivuranta K., Storgards E., Smart K.A., Gibson B.R., 2011. Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery "wild" yeast contaminants. Journal of The Institute of Brewing, 117, 556-568.

Pulvirenti, A., Solieri, L., Gullo, M., De Vero, L., Giudici, P., 2004. Occurrence and dominance of yeast species in sourdough. Letters *i*n Applied Microbiology, 38, 113-117.

Romo-Sánchez, S., Alves-Baffi, M., Arévalo-Villenaa, M., Úbeda-Iranzoa, J., Briones-Pérez, A., 2010. Yeast biodiversity from oleic ecosystems: Study of their biotechnological properties. Food Microbiology 27, 487-492

Sneat, P.H.A. and Sokal, R.R. (Eds.), 1973. Numerical taxonomy. The Principles and Practise of Numerical Classification. San Francisco: W.H. Freeman.

Vauterin, L. and Vauterin, P., 1992. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. European Microbiol. 1, 37–41.

Venturi M., Guerrini S., Granchi L., Vincenzini M. 2012. Typing of *Lactobacillus sanfranciscensis* isolates from traditional sourdoughs by combining conventional and multiplex RAPD–PCR profiles. International Journal of Food Microbiology, 156, 122-126.

Vilgalys, R., Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology, 172, 4238–4246.

Villa-Carvajal, M., R. Coque J.J., Alvarez-Rodrıguez M.L., Uruburua F., Belloch C., 2004. Polyphasic identification of yeasts isolated from bark of cork oak during the manufacturing process of cork stoppers. FEMS Yeast Research. 4, 745–750.

Zullo B.A. and Ciafardini G. 2008. Lypolitic yeast distribution in commercial extra virgin olive oil. Food Microbiology, 25, 970-977.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2010. Distribution of dimorphic yeast species in commercial extra virgin olive oil. Food Microbiology. 27, 1035-1042.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2013. Effects of some oil-born yeasts on the sensory characteristics of Italian virgin olive oil during its storage. Food Microbiol. 36, 70-78

1 **Enumeration and rapid identification of yeasts during extraction processes of extra virgin olive oil in Tuscany**

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Abstract

In this work, with the aim to evaluate the occurrence of yeast populations in extra virgin olive oil extraction process, samples of crushed pastes, kneaded pastes, oil from decanter and pomaces were collected during different olive oil extraction processes carried out in three consecutive years in Tuscany (Italy). The results showed yeast concentrations ranging between 10^3 and 10^5 CFU/g. Seventeen dominant yeast species were identified by RAPD-PCR with primer M13 and their identification was confirmed by RFLP-rITS and sequencing rRNA genes. The isolation frequencies of each species in the collected samples pointed out that the occurrence of the various yeast species in olive oil extraction process was dependent not only on the yeasts contaminating the olives but also on the yeasts colonizing the plant for oil extraction. In fact, eleven dominant yeast species were detected from the washed olives, but only three of them were also found in oil samples at significant isolation frequency. On the contrary, four yeast species, showing significant isolation frequency in oil samples, were below the detection limit in washed olive samples. These findings suggest a phenomenon of contamination of the plant for oil extraction that selects some yeast species that could affect the quality of olive oil.

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Keywords

Olive oil extraction, yeasts, extra virgin olive oil quality, RAPD-PCR (Random Amplified Polymorphic DNA).

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1. Introduction

Newly produced olive oils usually show an opalescent appearance due to the presence of solid particles and micro-drops of vegetation water containing microorganisms, mainly represented by yeasts (Ciafardini and Zullo, 2002b). The yeasts found in the oil come from the olives' carposphere, since during the crushing of olives the yeasts pass on, along with the solid particles of the fruit and the micro-drops of the vegetation water, into the oil. Some yeast species do not survive a long time whereas others persist and become the typical microbiota of each oil. The yeasts occurring in newly produced oil can remain active during the conservation period and, according to their metabolic capabilities, can either improve or worsen the oil quality (Zullo et al., 2010). In fact, β-glucosidase and esterase activities can improve the taste and the antioxidant capability of the oil, while the lipase activity can deteriorate the oil quality by hydrolyzing triglycerides (Ciafardini and Zullo, 2002a; Ciafardini and Zullo, 2015; Ciafardini et al., 2006a,b). Recently, Zullo et al. (2013) demonstrated that the presence of some yeast species might be responsible for olive oil sensory decay during storage. Despite these evidences regarding the impact of the yeasts on olive oil quality, only a few studies have investigated the yeast communities in the olive oil environment. Some Authors found, in commercial extra virgin olive oil, yeasts belonging to *Candida diddensiae*, *Candida boidinii*, *Candida wickerhamii* (now *Nakazawaea wickerhamii* according to

58 Kurtzman and Robnett, 2014) *Williopsis californica*, and *Saccharomyces cerevisiae* species (Ciafardini and Zullo
59 2002b; Ciafardini et al. 2006a,b; Zullo and Ciafardini, 2008; Zullo et al. 2010). Zullo et al. (2010) also demonstrated
60 within olive oil, the presence of some human opportunistic pathogen yeast species identified as *Candida parapsilosis*
61 and *Candida guilliermondii*. Finally, Čadež et al. (2012, 2013) recently described some new yeast species (*Candida*
62 *adriatica*, *Candida molendini-olei*, *Ogataea kolombanensis, Ogataea histrianica*) isolated from olive oil and its by-
63 products, while Ciafardini et al. (2013) found a new yeast species (*Yamadazyma terventina*) in Italian olive oils. Some
64 of these yeast species were found only in oil and not in its by-products, suggesting the existence of a typical yeast
65 microbiota for the olive oil extraction process. Actually, studies on the yeast species occurring in the different phases of
66 this process are lacking. Only a recent study of Romo-Sanchez et al. (2010) showed the biodiversity of yeasts isolated
67 from fresh olives, paste and pomace of two olive varieties (*Arbequina* and *Cornicabra*) by identifying fourteen yeast
68 species that belonged to seven different genera (*Zygosaccharomyces*, *Pichia*, *Lachancea*, *Kluyveromyces*,
69 *Saccharomyces*, *Candida*, *Torulaspora*). Therefore, the aim of this study was to assess the yeast species occurring in
70 different stages of various extra virgin olive oil extraction processes carried out in the same oil mill located in Tuscany
71 during different days of the harvest time in three consecutive years. For this purpose, in each process different samples
72 were analyzed: washed olives, pastes (after crushing and after kneaded), oil (after centrifugation in a two-phase
73 decanter and after filtration) and pomaces. Moreover, in order to perform a rapid detection of different yeast species
74 from the olive oil extraction processes, at first, efforts were made to develop a reproducible molecular method that was
75 suitable for the screening of a large number of isolates.
76
77 **2. Materials and methods**
78 *2.1 Sampling throughout olive oil extraction processes*
79 During the harvest time in three consecutive years, 35 batches of approximately 200 kg olives from Frantoio and/or
80 Moraiolo *cultivars* were processed in an oil mill (Azienda Agricola Buonamici, Fiesole, Florence, Italy).
81 Plant for oil extraction (TEM, Florence, Italy) consisted of a cleaning and water washing system, an olive grinding
82 cutter crusher (mod. FR350), a controlled-temperature vertical axis malaxation equipment (500 kg capacity) (mod.
83 V500), a "decanter" (two-step mod. D1500) with 1500 kg/h maximum capacity and a cardboard filter press (15-µm cut-
84 off). Plastic residue or "alperujo" from decanter was subjected to separation by centrifugation of stone fragments to
85 obtain destoned pomace. Olives were processed within 12 h from harvest. Olives were crushed at 2,500 rpm (crusher
86 holes 6.5 mm in diameter); malaxation was carried out at half capacity under vacuum (residual pressure of 20 kPa) at 22
87 ± 1°C for a mean time of 15 min to work under low oxidative stress impact conditions; decanter worked with a screw
88 conveyor rotating at a slower speed than that of the bowl.

89 The 35 extractive processes were sampled in different days of three consecutive years, as reported in the table 1. During
90 the first year, only crushed pastes, oil from decanter, filtered oil and pomaces were analyzed, while during the second
91 and third year also the kneaded pastes. Indeed, during the second and third years a faucet to collect kneaded pastes was
92 added between the malaxian equipment and the decanter. Each sample was collected twice at distance of 3 minutes and
93 transported to the laboratory in refrigeration under aseptic conditions. Each sample was analyzed in duplicate.
94
95 *2.2 Enumeration and isolation of yeasts*
96 Yeasts were quantified on MYPG agar (malt extract 5g/L, yeast extract 3g/L; beef extract 5g/L, D-glucose 10g/L)
97 containing sodium propionate (2 g/L) and chloramphenicol (30 mg/mL) in order to inhibit growth of moulds and
98 bacteria, respectively. The samples of olives, pastes, pomaces and oil from decanter were plated directly and/or after
99 decimal dilutions in physiological solution (NaCl, 0.86 g/L). The samples of filtered oil were analyzed by filtration of
100 100 mL and subsequent washings with physiological solution through 0.45-µm cellulose membranes (Pall Corporation).
101 Yeast colonies were counted after incubation for 48-72 h at 30°C under aerobic conditions. A significant number of
102 yeast isolates from each sample was purified and stored in liquid cultures containing 50% (v/v) glycerol at - 80°C until
103 use.
104
105 *2.3 Statistical analysis*
106 Microbiological determinations, performed in duplicate, were elaborated according nonparametric ANOVA followed
107 by Bonferroni Test (Statistica 7.0 software package). Differences were reported at a significance level of $p < 0.05$.
108
109 *2.4 Identification of the yeasts by molecular methods*
110 *2.4.1 Randomly Amplified Polymorphic DNA analysis*
111 Yeast cells picked from 24-h-old colonies were suspended in 50 µL-sterile water and then two µL were directly used for
112 all PCR reactions. Randomly Amplified Polymorphic DNA (RAPD) analysis was performed using the primer M13 (5'-
113 GAGGGTGGCGGTTCT-3') (Huey and Hall, 1989) and the PCR protocol according to Reguant and Bordons (2003).
114 All reactions included both negative (DNA-free) and positive controls and the PCR was processed in an Applied
115 Biosystems® 2720 Thermal Cycler (Life Technologies, Monza, Italy). Amplicons were analyzed on 2% (w/v) agarose
116 gel (Lonza Group Ltd, Basel, Switzerland) stained with ethidium bromide (Sigma-Aldrich, St Louis, Missouri,USA) in
117 TEB buffer for 2.5 h at 100 V and observed by UV transillumination. Band patterns, captured as TIFF format files with
118 a CCD camera (UVItec Gel Documentation System, Cambridge, UK), were subjected to pairwise comparison with the
119 Dice coefficient (SD) (Sneat and Sokal 1973) and cluster analysis with the Unweighted Pair Group Method using

120 Arithmetic Averages (UPGMA) (Vauterin and Vauterin, 1992). All analysis steps were performed by GelCompar 4.0
121 software (Applied Math, Kortrijk, Belgium). Reproducibility of RAPD-PCR patterns was assessed by comparing the
122 PCR products obtained with DNA prepared from two separate cultures of the same strains. In the RAPD-PCR analysis
123 were also included the following type strains: *Aureobasidium pullulans var. pullulans* CBS 100524, *Candida adriatica*
124 CBS 12504, *Candida diddensiae* CBS 2214, *Candida norvegica* CBS 4239, *Candida railenensis* CBS 8164, *Candida*
125 *tenuis* CBS 615, *Lachancea fermentati* CBS 707, *Lachancea cidri* CBS 4575, *Metschnikowia fructicola* CBS 8853,
126 *Nakazawaea holstii* CBS 4140, *Nakazawaea ishiwadae* CBS 6022, *Nakazawaea molendini-olei* CBS 12508,
127 *Nakazawaea peltata* CBS 5576, *Nakazawaea wickerhamii* CBS 2928, *Pichia kluyveri* CBS 188, *Pichia manshurica*
128 CBS 209, *Pichia membranifaciens* CBS 107, *Rhodotorula graminis* CBS 2826, *Rhodotorula mucilaginosa* CBS 316,
129 *Rhodotorula slooffiae* CBS 5706, *Saccharomyces cerevisiae* CBS 1171, *Yamadazyma terventina* CBS 12510,
130 *Zygotorulaspora mrakii* CBS 4218.
131
132 *2.4.2 Restriction analysis of Internal Transcribed Spacer rDNA*
133 At least two yeast isolates were chosen as representative of different RAPD patterns and were assayed by PCR-RFLP
134 analysis of the rDNA-ITS (Internal Transcribed Spacer) region as described by Granchi et al. (1999), using *Cfo*I, *Hae*III
135 and *Hinf*I (Fermentas Inc, Burlington, Ontario, Canada) as restriction endonucleases. The restriction fragments were
136 separated (at 100 volt for 2.5 h) on 2 % (w/v) agarose gel (Lonza Group Ltd, Basel, Switzerland), containing ethidium
137 bromide (Sigma-Aldrich, St Louis, Missouri, USA) and TEB buffer (1 M Tris, 10 mM EDTA, 0.9 M boric acid, pH
138 8.3). The profiles were compared with data reported in the literature (Arroyo-Lopez et al., 2006; Bautista-Gallego J.,
139 2011; de Llanos Frutos R. et al., 2004; Esteve-Zarzoso et al., 1999; Fernandez-Espinar et al., 2000; Granchi et al., 1999;
140 Guillamon et al., 1998; Nisiotou et al., 2010; Pulvirenti et al., 2004; Pham T., 2011; Villa-Carvajal M., 2004). The ITS
141 profiles of the isolates were also compared with those of the type strains listed above.
142
143 *2.4.3 rDNA gene sequencing and sequence analysis*
144 To confirm the identification obtained by RFLP analysis of the 5.8S-ITS rDNA region, the D1/D2 domain of 26S
145 rDNA gene of a significant number of isolates from each RAPD profile, was amplified using the primers NL_1 (5'-
146 GCATATCAATAAGCGGAGGAAAAG-3') and NL_4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett,
147 1998). PCRs were performed in 50µL containing 2µL of the DNA obtained, 5µL of 10x reaction buffer (Polymed,
148 Italy), 2.5µL dNTPs mix (10 mM each), 2µL Bovine Serum Albumine (10mg/mL), 2.5µL $MgCl_2$ (25mM), 2µL each
149 primers (10 µM), 0.2µL Taq DNA polymerase (Polymed, Italy) and 31.8µL of de-ionized H_2O. The following program
150 was used: 95°C for 5 min at the start followed by 30 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 2 min and a

151 final extension of 72°C for 7 min. In addition, the primers V9G (de Hoog and Gerrits van den Ende 1998) and LR5
152 (Vilgalys and Hester 1990) were used to amplify the partial rRNA gene that includes, the 3'end of the small-subunit
153 rDNA, the D1/D2 domain of the large subunit rDNA, as well as the ITS (internal transcribed spacer) domain (ITS1,
154 ITS2 and the intervening 5.8S rRNA gene) as described by Knutsen et al., (2007).
155 The specificity of the PCR products was checked on an agarose gel 1.4% (w/v) before purification. The PCR products
156 were purified using Nucleo Spin Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the
157 manufacturer's instructions before sending to BMR Genomics (Padua, Italy) for sequencing. The forward primer NL1
158 was used for sequencing the D1/D2 domain of 26S rDNA gene, while forward and reverse primers (V9G and LR5)
159 were used to sequence both strands of the large subunit nrDNA. The sequence obtained in FASTA format were
160 compared to sequences available in GenBank database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search
161 tools (Altschul et al., 1990).
162
163 *2.5 Phenotypic characterization*
164 At least two yeast isolates were chosen as representative of different RAPD patterns and were assayed to study the
165 pattern of carbon compound assimilation, which in many cases is species-specific, the yeast identification system ID 32
166 C system (Biomerieux, Marcy-l'Etoile, France) was used according to the manufacturer's instructions. The capability to
167 grow at 37°C was assayed on MYPG agar.
168
169 **3. Results**
170
171 *3.1 Yeast populations occurring in extractive olive oil processes*
172 The yeast concentration in samples from the 35 olive oil extraction processes ranged between 10^3 CFU/g and 10^5
173 CFU/g (Figure 1), with the exception of filtered oils that showed concentrations lower than 10 CFU/100g. These
174 concentrations remained unchanged during oil conservation in dark-green glass bottles at room temperature over a
175 period of six months. It is underlined that the yeast concentration in the pomaces resulted statistically higher than in
176 pastes and oil from decanter (Fig. 1) suggesting a possible accumulation of yeasts during the subsequent centrifugations
177 of the kneaded pastes in the two-phase decanter. This yeast population could be a source of oil contamination.
178
179 *3.2 Molecular yeast identification*
180 Yeast isolates from the different samples, as reported in table 1, were identified by combining various molecular
181 methods such as RAPD-PCR, RFLP analysis of the rDNA-ITS region, and sequencing of specific rDNA regions, with

182 the aim to detect the most suitable tool for a rapid differentiation of the yeasts at species level. First, all the isolates and
183 type strains of yeast species usually found in olives, in oil or in its by-products (Čadež et al., 2012; Ciafardini and Zullo,
184 2002a,b; Giannoutsou et al., 2004; Zullo and Ciafardini, 2008; Zullo et al., 2010) were assayed using RAPD-PCR with
185 primer M13, since it was previously applied successfully to the identification of yeasts at species level (Andrighetto et
186 al., 2000). This method yielded 27 distinctive and reproducible patterns containing from five to fifteen bands ranging in
187 size from approximately 170 to 5,000 bp. Indeed, repetition of RAPD analysis on DNA from two separate cultures of
188 the same strains produced patterns with more than 90% similarity (data not shown). Cluster analysis of the 27 RAPD-
189 PCR patterns generated the dendrogram shown in Figure 2. Seventeen clusters, designated clusters A-Q, were detected
190 at about a 50% similarity level (Fig. 2). This similarity level was chosen on the basis of the reproducibility between
191 different RAPD–PCR patterns for the same isolate (≥ 95%) and of the similarity levels between type strains of different
192 yeast species (≤ 48%). Among the seventeen RAPD-PCR clusters, twelve included the type strain of a yeast species
193 while the remaining five clusters did not include any tested type strain (Table 2). Therefore, according to these results,
194 yeast isolates belonging to the following clusters A, E, F, H, I, J, K, L, N, O, P and Q, were respectively assigned to
195 twelve putative yeast species: *Pichia manshurica*, *Rhodotorula slooffiae*, *Candida diddensiae*, *Candida norvegica*,
196 *Candida adriatica*, *Metschnikowia fructicola*, *Nakazawaea molendini-olei*, *Nakazawaea wickerhamii*, *Saccharomyces*
197 *cerevisiae*, *Pichia kluyveri*, *Rhodotorula mucilaginosa* and *Zygotorulaspora mrakii*. In order to confirm the
198 identification of these yeast species and to accomplish the identification of yeast isolates belonging to the clusters not
199 including a type strain, two isolates from each RAPD-PCR cluster along with the relative type strains were assayed by
200 PCR-RFLP analysis of the rDNA ITS region and sequence analysis of D1/D2 region. The isolates and the type strain
201 belonging to the same RAPD-PCR cluster showed very similar ITS restriction patterns by using the endonucleases *Cfo*I,
202 *Hae*III, and *Hinf*I (Tab. 2) and these were in accordance with data obtained by other Authors (de Llanos Frutos et al.,
203 2004; Esteve-Zarzoso et al., 1999; Fernandez-Espinar et al., 2000; Nisiotou et al., 2012; Pham et al., 2011; Villa-
204 Carvajal et al., 2004). In addition, sequence comparison of the D1/D2 region of the yeasts, included in the same RAPD-
205 cluster, yielded similarity values, with the closest relative species, between 99.8 and 100%, thus confirming the
206 identifications obtained by ITS polymorphism analysis (Tab. 2). These findings demonstrated that all the putative
207 twelve species above-mentioned were properly identified by the RAPD-PCR with primer M13. In particular, it was able
208 to discriminate *Nakazawaea molendini-olei* and *Nakazawaea wickerhamii*, which were indistinguishable with ITS-
209 RFLP as they showed the same ITS restriction fragments with *Cfo*I, *Hae*III, and *Hinf*I (Tab. 2).
210 Regarding yeast isolates grouped into the five RAPD-clusters (B, C, D, G and M) not containing a type strain, RFLP
211 analysis of the rDNA ITS region and sequence analysis of the D1/D2 region suggested that they belonged, respectively,
212 to *Candida oleophila*, *Candida railenensis*, *Candida tenuis*/*Yamadazyma terventina*, *Lachancea fermentati*/*Lachancea*

213 *cidri* and *Rhodotorula glutinis* (Tab. 2) (Čadež et al., 2010; de Llanos Frutos et al., 2004; Esteve-Zarzoso et al., 1999;
214 Groenewald et al., 2011). It is underlined that type strains of the species *Candida oleophila* and *Rhodotorula glutinis*
215 were not tested in this study. However, since BLAST analysis of D1/D2 sequences of the isolates ascribed to these
216 species yielded similarity values of 100 and 99.7%, respectively, the two yeast species were considered correctly
217 designated (Kurtzman and Robnett 1998) by RAPD-PCR with the primer M13. On the contrary, the type strains of the
218 other five putative species were here analysed and displayed RAPD-patterns different from those of the isolates
219 comprised in clusters C, D and G, as shown in the dendrogram reported in figure 2. Therefore, to assess the identity of
220 these isolates and considering that previous molecular tests gave in two cases ambiguous identifications, sequence
221 analysis of their 5.8S-ITS region and of 26S rRNA gene were, additionally, carried out. According to the highest
222 similarity value obtained by sequence comparisons to GenBank database by using BLAST (tab. 3), isolates belonging to
223 clusters C, D and G were respectively assigned to the species *Candida railenensis*, *Yamadazyma terventina* and
224 *Lachancea fermentati*. The fact that the type strains of these yeast species exhibited different RAPD-PCR patterns with
225 primer M13 could be due to the intraspecific genetic variation. Indeed, also restriction profiles of 5.8S-ITS region
226 obtained with *Cfo*I for the type strain of *Yamadazyma terventina* species, showed some differences in the size of the
227 restriction fragments (data not shown), although the enzyme *Hae*III and *Hinf*I generated restriction patterns in
228 agreement with Ciafardini et al. (2013).
229 In conclusion, RAPD-PCR with primer M13 allowed to distinguish seventeen different yeast species, three of them
230 recently isolated from olive oil and/or its by-products and recognised as new species: *Yamadazyma terventina*
231 (Ciafardini et al., 2013), *Candida molendinolei* (classified as *Nakazawaea molendini-olei* according to Kurtzman and
232 Robnett, 2014) and *Candida adriatica* (Čadež et al., 2012).
233 On the contrary, this rapid method did not give reproducible RAPD-PCR patterns for some assayed isolates, which
234 showed black colonies on agar plates. Based on their microscopic morphology and on the results of RFLP analysis of
235 rITS region and on the sequencing of the D1/D2 26S rDNA region, all these isolates were identified as belonging to
236 *Aureobasidium pullulans* species, a ubiquitous yeast-like fungus also associated with olive fermentation (Nisiotou et al.,
237 2010). In particular, all these isolates along with the type strain *Aureobasidium pullulans* CBS 100524 showed a PCR
238 product of ca. 600 bp in the ITS region and restriction profiles of 190, 180, 110 and 100 bp fragments with *Cfo*I, of 440
239 and 150 bp with *Hae*III and of 290, 180 and 140 with *Hinf*I, according to Nisiotou et al. (2010). Furthermore, these
240 isolates and the type strain of *Aureobasidium pullulans* showed 99.8% similarity of the nucleotide sequence of the
241 D1/D2 region, confirming their identification.
242
243 *3.3 Phenotypic yeast characterization*

244 After molecular identification, the same isolates were also assayed for their capability to metabolize different 32 carbon
245 sources and to grow at 37°C with the aim to further assess the identification at species level obtained with molecular
246 methods. In the table 4 are shown the results compared with those reported in literature (Čadež et al., 2012; Ciafardini et
247 al., 2013; Kurtzman et al., 2011). In dark grey and white are indicated the results in contrast and in agreement with
248 literature, respectively, while in light grey the not confirmable results because lacking in literature. Isolates belonging to
249 *P. manshurica*, *C. diddensiae*, *L. fermentati*, *C. norvegica*, *N. wickerhamii*, and *R. mucilaginosa* species were in total
250 agreement with literature, while the isolates belonging to other species showed some differences (from 1 to 8 fonts of
251 carbons), evidently due to intraspecific diversity. This diversity was particularly marked for the isolates belonging to *R.*
252 *glutinis* and *Y. terventina* species. Concerning the latter yeast species, Ciafardini et al. (2013) mentioned the ability to
253 assimilate lactic acid (verified on three strains) as a useful tool to distinguish *Y. terventina* species from the others
254 included in the *Yamadazyma* clade, but this ability was absent in all the isolates assayed in this study (Table 4).
255
256 *3.4 Yeast species biodiversity*
257 To evaluate the yeast biodiversity occurring in olive oil extraction process, the frequencies of each yeast species was
258 calculated grouping the data according to: (1) the type of sample (pastes, oil, or pomaces), (2) the olive cultivars
259 (*Frantoio* or *Moraiolo*) used in the various extractive processes, and finally (3) the years in which the extraction
260 processes were carried out.
261 Table 5 indicates the distribution of the various yeast species (expressed as isolation frequencies) in the different
262 samples (olives, pastes, oil, and pomaces) collected during the 35 olive oil extraction processes taken into consideration.
263 Eleven dominant yeast species, besides the yeast-like fungus *A. pullulans,* were isolated from washed olives. *A.*
264 *pullulans* was also found in crushed pastes, even if at lower percentage than in the washed olives, and below 1% in the
265 other samples. All the yeast species characterizing the olives, except *Z. mrakii*, were also isolated from the crushed
266 pastes and the *N. molendini-olei* species exhibited the highest isolation frequency (almost 50%). Kneaded pastes
267 showed nine different yeast species, six being in common with those isolated from crushed pastes. *Z. mrakii* was the
268 dominant species of the kneaded pastes showing an isolation frequency of about 40%, although this yeast was isolated
269 from washed olives and crushed pastes with a very low frequency (≤1%). The samples of oil from decanter were
270 characterized by eight dominant yeast species, six shared with those from kneaded pastes. *N. molendini-olei* and *Y.*
271 *terventina* were both the dominant yeast species in these samples with the same isolation frequencies. Nevertheless, the
272 distribution of these two yeast species in the olive oil extraction processes were very different. Indeed, *N. molendini-*
273 *olei* was found in all the types of samples (pastes, oil, and pomaces) with isolation frequency ranging from 10 to 52%,
274 while *Y. terventina* was detected at significant frequency (> 1%) only from oil and pomace samples (38 and 2%,

275 respectively). Finally, pomaces substantially showed the same yeast species detected in oil even if with different
276 isolation frequencies.
277 Table 6 shows the isolation frequencies of yeast species in the different types of samples according to the olive cultivars
278 (*Frantoio* or *Moraiolo*) used in the extractive processes. The results did not demonstrate the existence of relationships
279 between the olive cultivar used in the extractive process and the yeast species isolated from the various samples: no
280 yeast species was uniquely associated with one of the two olive cultivars assayed.
281 Finally, the distribution of yeast species in the different samples is reported in relation to the year of collection (Tab. 7).
282 The results demonstrated differences in the dominant yeast species isolated from washed olives according to the
283 different year of collection. Indeed, washed olives in 2011 were characterized by a significant presence of *C. adriatica*,
284 *N. molendini-olei*, and *N. wickerhamii*; in 2012 by *C. norvegica*; in 2013 by *C. railenensis* and *C. oleophila*. *A.*
285 *pullulans* was found in all olive samples even if with very different isolation frequencies (the highest being in 2012 and
286 the lowest in 2011). Differences based on the years were also observed in the pastes. Indeed, the dominant species of
287 pastes were, in 2011 *N. molendini-olei*, in 2012 *A. pullulans* and *Z. mrakii*, while in 2013 *S. cerevisiae*. On the contrary,
288 the dominant yeast species, isolated from oil and pomaces during the three assayed years, were almost the same. In
289 particular, the oil was mainly characterized by the presence of *Y. terventina* and *N. molendini-olei*, while the pomaces
290 by *C. adriatica* and *N. molendini-olei*.
291
292 **5. Discussion**
293 The yeast populations occurring in olive oil extraction processes are numerically significant and originate not only from
294 the yeasts contaminating the olives but also from the yeasts colonizing the oil extractive plants. Indeed, as reported in
295 this study, the dominant yeast species detected on the washed olives were eleven, but only three of them, were also
296 found in oil samples at significant isolation frequencies: *C. adriatica*, *N. molendini-olei*, and *N. wickerhamii*. On the
297 contrary, same yeast species showed significant isolation frequencies only in oil samples, such as *Y. terventina*, or in
298 kneaded pastes and pomaces, such as *Z. mrakii*. These observations suggest a possible contamination of the plant for oil
299 extraction (malaxation equipment and decanter in particular) that might select some yeast species at the expense of
300 others. Probably, during the extractive process, the environmental conditions promote a selective enrichment of specific
301 yeast species that could affect olive oil quality. Only few studies were carried out on the metabolic capabilities of these
302 yeast species in modifying the chemical and organoleptic properties of olive oil. *C. wickerhamii* (now *Nakazawaea*
303 *wickerhamii* according to Kurtzman and Robnett, 2014) as species was frequently isolated from commercial oils and
304 various strains belonging to this species demonstrated to possess lipase and β-glucosidase activities (Ciafardini and
305 Zullo, 2002a; Zullo and Ciafardini, 2008). The strain *C. wickerhamii* DAPES 1885 showed also esterase activity and,

306 once inoculated in olive oil, it was responsible of "Muddy-sediment" and "rancid" (Zullo et al., 2013). On the contrary,
307 little information are available on the metabolic capabilities of *C. adriatica, N. molendini-olei,* and *Y. terventina*,
308 because of their recent classification as new species (Čadež et al., 2012; Ciafardini et al., 2013, Kurtzman and Robnett,
309 2014)). Indeed, only two studies have been currently carried out on this topic: the first demonstrated that the strain *C.*
310 *adriatica* DAPES 1933 possess esterase activity with similar effects to those caused by *C. wickerhamii* DAPES 1885
311 when inoculated in oil (Zullo et al., 2013); while the latter proved that the lipolytic activity of lipase-producer yeast
312 strains belonging to *C. adriatica* and *Y. terventina* species can influence the acidity of the olive oil (Ciafardini and Zullo
313 2015). Laboratory tests highlighted a substantial increase in free fatty acid in the inoculated olive oil characterized by
314 high water content and low polyphenol concentration. *Z. mrakii* was often isolated from table olives during spontaneous
315 or industrial fermentation (Bleve et al., 2014; Bautista-Gallego et al., 2011), but no information is currently available
316 regarding the impact of this species on the organoleptic quality of olive oil.

317 Certainly, the organoleptic characteristics of the olive oil are influenced by the hygienic quality of the olives (Morales et
318 al., 2005). Indeed, as reported by Vichi et al. (2011), the metabolic activities of olive microbiota during the oil
319 extraction process could be a critical point for virgin olive oil, and their influences could be greater than those exerted
320 by malaxation time and temperature. Based on the results presented in this paper, however, also the yeast species
321 contaminating the oil extraction plant could be a critical point in determining the organoleptic quality of olive oil.
322 Therefore, further studies are needed to evaluate the role of yeasts during the extraction process as well as during the
323 storage of oil after filtration (if performed) in order to assess its efficiency in removing yeasts, completely.

324 Actually, to our knowledge only one recent study reported the microbial concentrations of samples collected in several
325 steps (pastes, extracted oil, and filtered oil) of various extraction processes with chemical and sensory determination of
326 the filtered oil (Guerrini et al., 2015). In this paper, the Authors showed that the olive oil samples with sensory defects
327 were significantly correlated with specific volatile compounds (i.e., 2-butanone, butyric acid, 2-heptanol, octanoic acid,
328 1-octen-3-ol) and the same volatile compounds were correlated to both yeast and mould counts quantified in extracted
329 and filtered oil.

330 Finally, the application of RAPD method with primer M13 proved to be an effective, low cost and efficient tool to
331 identify at level species the yeasts isolated from olives, olive oil and its by-products.

332 In conclusion, yeast occurrence in olive oil extraction process is numerically significant and characterized by a great
333 biodiversity. These observations suggest a possible role of yeasts in determining of the quality of olive oil and the
334 possibility that each plant for oil extraction is characterized by a typical microbiota, possibly with different metabolic
335 capabilities. Both observations deserve further studies, which could be useful to improve the quality of extra virgin
336 olive oils.

337

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341

342 **References**

343 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-
344 410.

345 Andrighetto C, Psomas E, Tzanetakis N, Suzzi G, Lombardi A (2000) Randomly amplified polymorphic DNA (RAPD)
346 PCR for the identification of yeasts isolated from dairy products. Lett Appl Microbiol 30: 5-9.

347 Arroyo-Lopez FN, Duran-Quintana MC, Ruiz-Barba JL, Querol A, Garrido-Fernandez A (2006) Use of molecular
348 methods for the identification of yeast associated with table olives. Food Microbiol 23: 791-769.

349 Bautista-Gallego J, Rodríguez-Gómez F, Barrio E, Querol A, Garrido-Fernández A, Arroyo-López FN (2011)
350 Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. Int J Food
351 Microbiol 147: 89-96.

352 Bleve G, Tufariello M, Durante M, Perbellini E, Ramires FA, Grieco F, Cappello MS, De Domenico S, Mita G,
353 Tasioula-Margari M, Logrieco AF (2014) Physico-chemical and microbiological characterization of spontaneous
354 fermentation of Cellina di Nardò and Leccino table olives. Front Microbiol 5: 1-18.

355 Čadež N, Raspor P, Zupan J (2010) The effect of fungicides on yeast communities associated with grape berries. FEMS
356 Yeast Res 10: 619–630.

357 Čadež N, Raspor P, Turchetti B, Cardinali G, Ciafardini G, Veneziani G, Peter G (2012) *Candida adriatica* sp. nov. and
358 *Candida molendinolei* sp. nov., two novel yeast species isolated from olive oil and its by-products. Int J Syst Evol
359 Microbiol 62: 2296-2302.

360 Čadež N, Dlauchy D, Raspor P, Péter G (2013) *Ogataea kolombanensis* sp. nov., *Ogataea histrianica* sp. nov. and
361 *Ogataea deakii* sp. nov., three novel yeast species from plant sources. Int J Syst Evol Microbiol 63: 3115–3123.

362 Ciafardini G, Zullo BA (2002a) Microbiological activity in stored olive oil. Int J Food Microbiol 75: 111-118.

363 Ciafardini G, Zullo BA (2002b) Survival of microorganisms in extra virgin olive oil during storage. Food Microbiol 19:
364 105-109.

365 Ciafardini G, Zullo BA (2015) Effect of lipolytic activity of *Candida adriatica*, *Candida diddensiae* and *Yamadazyma*
366 *terventina* on the acidity of extra-virgin olive oil with a different polyphenol and water content. Food Microbiol 47: 15-
367 20.

368 Ciafardini G, Zullo BA, Antonielli L, Corte L, Roscini L, Cardinali G (2013) *Yamadazyma terventina* sp.nov., a yeast
369 species of the *Yamadazyma* clade from Italian olive oils. Int J Syst Evol Microbiol 63: 372-376.
370 Ciafardini G, Zullo BA, Cioccia G, Iride A (2006a) Lipolytic activity of *Williopsis californica* and *Saccharomyces*
371 *cerevisiae* in extra virgin olive oil. Int J Food Microbiol 107: 27-32.
372 Ciafardini G, Zullo BA, Iride A (2006b). Lipase production by yeasts from extra virgin olive oil. Food Microbiol 23:
373 60-67.
374 de Llanos Frutos R, Fernandez-Espinar MT, Querol A (2004) Identification of species of the genus *Candida* by analysis
375 of 5.8S rRNA gene and the two ribosomal internal transcribed spacer. Antonie van Leeuwenhoek 85: 175-185.
376 Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA
377 gene and the two ribosomal internal transcribed spacers. Int J Syst Bacteriol 49: 329–337.
378 Fernandez-Espinar MT, Esteve-Zarzoso B, Querol A, Barrio E (2000) RFLP analysis of the ribosomal internal
379 transcribed spacer and the5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification
380 and the differentiation of flor yeasts. Antonie van Leeuwenhoek 78: 87–97.
381 Giannoutsou EP, Meintanis C, Karagouni AD (2004) Identification of yeast strains isolated from a two-phase decanter
382 system olive oil waste and investigation of their ability for its fermentation. Bioresour Technol 93: 301-306.
383 Granchi L, Bosco M, Messini A, Vincenzini M (1999) Rapid detection and quantification of yeast species during
384 spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. J Appl Microbiol 87: 949-956.
385 Groenewald M, Robert V, Smith MTh (2011) The value of the D1/D2 and internal transcribed spacers (ITS) domains
386 for the identification of yeast species belonging to the genus *Yamadazyma*. Persoonia 26: 40-46.
387 Guerrini S, Mari E, Migliorini M, Cherubini C, Trapani S, Zanoni B, Vincenzini M (2015). Investigation on
388 microbiology of olive oil extraction process. Ital J Food Sci 27: 237-247.
389 Guillamon JM, Sabate J, Barrio E, Cano J, Querol A (1998) Rapid identification of wine yeast species based on the
390 RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch Microbiol 169: 387–392.
391 Hoog GS de, Gerrits van den Ende AHG (1998) Molecular diagnostics of clinical strains of filamentous
392 basidiomycetes. Mycoses 41: 183 –189.
393 Huey B, Hall J (1989) Hypervariable DNA fingerprinting in *Escherichia coli*. Minisatellite probe from bacteriophage
394 M13. J Bacteriol 171: 2528–2532.
395 Knutsen AK, Robert V, Poot GA, Epping W, Figge M, Holst-Jensen A, Skaar I, Smith MTh (2007) Polyphasic re-
396 examination of *Yarrowia lipolytica* strains and the description of three novel *Candida* species: *Candida osloensis* sp.
397 nov., *Candida alimentaria* sp. nov. and *Candida hollandica* sp. nov. Int J Syst Evol Microbiol 57: 2426–2435.

398 Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large
399 subunit (26S) ribosomal DNA partial sequence. Antonie van Leeuwenhoek 73: 331-371.
400 Kurtzman CP, Fell JW, Boekhout T (eds) (2011) The Yeasts: A Taxonomic Study. Fifth edn. Elsevier, San Diego USA.
401 Kurtzman CP, Robnett CJ (2014) Description of *Kuraishia piskuri* f.a., sp. nov., a new methanol assimilating yeast and
402 transfer of phylogenetically related *Candida* species to the genera *Kuraishia* and *Nakazawaea* as new combinations.
403 FEMS Yeast Res 14: 1028–1036.
404 Morales MT, Luna G, Aparicio R (2005) Comparative study of virgin olive oil sensory defects. Food Chem 91: 293–
405 301.
406 Nisiotou AA, Chorianopoulos N, Nychas GJE, Panagou EZ (2010) Yeast heterogeneity during spontaneous
407 fermentation of black Conservolea olives in different brine solutions. J Appl Microbiol 108: 396-405.
408 Pham T, Wimalasena T, Box WG, Koivuranta K, Storgards E, Smart KA, Gibson BR (2011) Evaluation of ITS PCR
409 and RFLP for differentiation and identification of brewing yeast and brewery "wild" yeast contaminants. J Inst Brew
410 117: 556-568.
411 Pulvirenti A, Solieri L, Gullo M, De Vero L, Giudici P (2004) Occurrence and dominance of yeast species in
412 sourdough. Lett Appl Microbiol 38: 113-117.
413 Reguant C, Bordons A (2003) Typification of *Oenococcus oeni* strains by multiplex RAPD-PCR and study of
414 population dynamics during malolactic fermentation. J Appl Microbiol 95: 344-353.
415 Romo-Sánchez S, Alves-Baffi M, Arévalo-Villenaa M, Úbeda-Iranzoa J, Briones-Pérez A. (2010) Yeast biodiversity
416 from oleic ecosystems: Study of their biotechnological properties. Food Microbiol 27: 487-492.
417 Sneat PHA, Sokal RR (eds) (1973) Numerical taxonomy. The Principles and Practise of Numerical Classification. W.H.
418 Freeman, San Francisco USA.
419 Vauterin L, Vauterin P (1992) Computer-aided objective comparison of electrophoresis patterns for grouping and
420 identification of microorganisms. European Microbiol 1: 37–41.
421 Vichi S, Romero A, Tous J, Caixach J (2011) The Activity of Healthy Olive Microbiota during Virgin Olive Oil
422 Extraction Influences Oil Chemical Composition. J Agr Food Chem 59: 4705-4714.
423 Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from
424 several *Cryptococcus* species. J Bacteriol 172: 4238–4246.
425 Villa-Carvajal M, Coque RJJ, Alvarez-Rodriguez ML, Uruburua F, Belloch C (2004) Polyphasic identification of yeasts
426 isolated from bark of cork oak during the manufacturing process of cork stoppers. FEMS Yeast Research 4: 745–750.
427 Zullo BA, Ciafardini G (2008) Lipolytic yeast distribution in commercial extra virgin olive oil. Food Microbiol 25: 970-
428 977.

429 Zullo BA, Cioccia G, Ciafardini G (2010) Distribution of dimorphic yeast species in commercial extra virgin olive oil.
430 Food Microbiol 27: 1035-1042.
431 Zullo BA, Cioccia G, Ciafardini G (2013) Effects of some oil-born yeasts on the sensory characteristics of Italian virgin
432 olive oil during its storage. Food Microbiol 36: 70-78.
433
434 **Figure Captions**
435 **Fig. 1** Yeast concentrations at different steps of the 35 oil extraction processes here studied. Different letters indicate
436 significant different concentrations ($p < 0.05$)
437 **Fig. 2** UPGMA dendrogram derived from comparison of the RAPD–PCR patterns obtained with primer M13 for the
438 yeast isolates tested. The vertical dotted line indicates the 49% similarity level

1 **Table 1.** Origin of the yeast isolates from 35 olive oil extractive processes carried out during the
2 harvest time in three consecutive years.
3

Olive *cultivar*	Origin	Number of yeast isolates
	I year	
Moraiolo	Olives	24
	Crushed pastes	46
	Oil from decanter	44
	Pomaces	20
Frantoio	Olives	22
	Crushed pastes	56
	Oil from decanter	48
	Pomaces	26
	II year	
Moraiolo	Olives	42
	Crushed pastes	10
	Kneaded pastes	90
	Oil from decanter	72
	Pomaces	62
Frantoio	Olives	14
	Crushed pastes	34
	Kneaded pastes	20
	Oil from decanter	28
	Pomaces	20
	III year	
Frantoio+ Moraiolo+others	Olives	38
	Crushed pastes	90
	Kneaded pastes	56
	Oil from decanter	84
	Pomaces	60

Table 2. Restriction analysis of the 5.8-ITS rDNA region and sequence information for the D1/D2 region of 26 rDNA gene of yeasts isolates grouped in different RAPD clusters.

RAPD cluster	Type strain included in RAPD cluster	CBS code	ITS Restriction fragments (pb) of type strains			ITS (pb)	ITS Restriction fragments (pb) of isolates			Sequence of D1/D2 region of isolates and type strains Matching nucleotides (Identity %)*		Closest relative species
			*Cfo*I	*Hae*III	*Hinf*I		*Cfo*I	*Hae*III	*Hinf*I			
A	*Pichia manshurica*	209	230-110-80	310-90	280-220	470	240-110-50	320-100-50	210-190-90	526/526	(100)	*Pichia manshurica*
B	-	-	-	-	-	620	300-300	400-150	310-310	558/558	(100)	*Candida oleophila*
C	-	-	-	-	-	610	280-280-50	410-140	310-310	540/541	(99.8)	*Candida railenensis*
D	-	-	-	-	-	650	290-220-50	410-130-90	310-310	508/510	(99.6)	*Candida tenuis/Yamadazyma terventina*
E	*Rhodotorula sloffiae*	5706	600	600	330-270	600	600	600	330-280	574/575	(99.8)	*Rhodotorula sloffiae*
F	*Candida diddensiae*	2214	280-170-130-	410-130-80	310-310	650	290-180-130	410-130-90	310-310	511/512	(99.8)	*Candida diddensiae*
G	-	-	-	-	-	680	310-280-80	300-210-90	320-320	552/554	(99.6)	*Lachancea fermentati/L. cidri*
H	*Candida norvegica*	4239	500	380-190	290-270	590	510	370-190	290-260	559/560	(99.8)	*Candida norvegica*
I	*Candida adriatica*	12504	210-130-100-80	400-90	310-310	610	210-140-100-80	400-90	300-300	550/550	(100)	*Candida adriatica*
J	*Metschnikowia fructicola*	8853	210-120-100	290-110	210-190	400	210-120-100	290-110	200-180	416/420	(99)	*Metschnikowia fructicola*
K	*Nakazawaea molendinolei*	12508	600	580-80	320-320	660	590	580-90	320-320	506/506	(100)	*Candida molendinolei*
L	*Nakazawaea wickerhamii*	2928	590	590-80	320-320	660	590	580-90	320-320	552/552	(100)	*Candida wickerhamii*
M	-	-	-	-	-	650	300-220-110	650	220-130-100	429/430	(99.7)	*Rhodotorula glutinis*
N	*Saccharomyces cerevisiae*	1171	375-325-150	320-230-170-125	375-365-110	850	370-330-140	310-240-180-120	370-360-120	558/558	(100)	*Saccharomyces cerevisiae*
O	*Pichia kluyveri*	188	180-100	380-80	250-200	450	170-110-50	370-80	260-210	556/556	(100)	*Pichia kluyveri*
P	*Rhodotorula mucilaginosa*	316	300-220-120	400-210	340-210-50	650	300-230-120	400-220	360-230-50	547/547	(100)	*Rhodotorula mucilaginosa*
Q	*Zygotorulaspora mrakii*	4218	300-290	390-120-70-50	310-200-130	650	300-280	400-120-70-50	310-200-130	565/565	(100)	*Zygotorulaspora mrakii*

* Identical nucleotide percentages in the sequence obtained from the D1/D2 region of the 26S rRNA gene and the sequence found in Genbank

Table 3. Homology (%) for the 5.8S–ITS region and LSU rRNA gene among isolates belonging to different RAPD-PCR cluster and their putative yeast species based on RFLP-ITS and D1/D2 sequence analysis (N.D. = not detected)

Sequence comparison	5.8-ITS	26S rRNA
Isolates in the cluster C *vs Candida railenensis*	98.9	98.6
Isolates in the cluster D *vs Candida tenuis*	92.0	94.0
Isolates in the cluster D *vs Yamadazyma terventina*	99.6	99.8
Isolates in the cluster G *vs Lachancea fermentati*	96.6	N.D.
Isolates in the cluster G *vs Lachancea cidri*	94.0	N.D.

12 **Table 4:** Carbohydrate assimilation and growth at 37°C of two yeast isolates representative of each RAPD cluster / species; dark grey: results in contrast
13 with literature, white: results in agreement with literature, light grey: results unreported in literature +: positive, -: negative; v: variable; w: weak
14

RAPD cluster	Yeast species	D-Galactose	Actidione	D-saccharose	N-Acetyl -glucosamine	Lactic acid	L-Arabinose	D-cellobiose	D-raffinose	D-maltose	D-trehalose	potassium-2-ketogluconate	Methyl-D-glucopyranoside	D-mannitol	D-lactose	Inositol	D-sorbitol	D-xylose	D-ribose	Glycerol	L-rhamnose	Palatinose	Erythritol	D-melibiose	Sodium glucuronate	D-melizitose	Potassium gluconate	Levulinic acid	D-glucose	L-sorbose	Glucosamine	Asculine ferric citrate	Growth at 37°
A	*P. manshurica*	-	-	-	+	w	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	-	-	+	-	+	-	+
B	*C. oleophila*	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	+	+	+	-	+	-	-	-	+	-	-	+	+	+	+	-
C	*C. railenensis*	+	+	+	+	+	-	-	-	w	w	+	-	+	-	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+	-	w	-
D	*Y. terventina*	+	-	+	+	-	+	+	-	+	+	w	w	+	w	-	+	+	-	-	-	+	+	-	-	-	+	w	+	-	-	+	-
E	*R. sloffiae*	-	-	+	-	-	+	-	-	-	+	+	-	+	-	-	w	+	w	+	-	-	-	-	+	+	+	-	+	-	-	-	-
F	*C. diddensiae*	+	-	+	+	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	w	+	+	-	-	+	+	w	+	-	+	+	+
G	*L. fermentati*	+	+	+	w	+	w	w	+	w	w	w	-	+	-	-	+	+	w	w	w	w	w	+	-	-	-	-	+	-	-	-	+
H	*C. norvegica*	-	-	-	-	+	-	w	-	-	-	w	-	+	-	-	+	+	w	+	+	-	-	-	-	-	-	w	+	w	-	-	-
I	*C. adriatica*	-	-	+	-	-	-	+	-	+	-	+	+	+	-	-	+	+	w	+	-	+	-	-	-	-	+	-	+	-	-	+	-
J	*M. fructicola*	+	-	+	+	-	-	+	-	+	+	+	+	+	w	-	+	+	+	+	+	+	-	-	-	+	+	w	+	+	+	+	-
K	*N. molendini-olei*	-	+	+	+	+	+	+	-	-	+	+	w	+	-	w	+	+	+	+	+	-	-	-	-	-	w	-	+	-	-	+	+/w
L	*N. wickerhamii*	v	+	-	+	v	v	+	-	-	-	+	-	+	-	-	+	+	v	+	v	-	-	-	-	-	+	-	+	-	-	+	-
M	*R. glutinis*	+	-	+	-	-	-	-	+	-	+	+	-	+	-	-	+	+	+	+	v	-	-	-	-	-	-	-	+	+	-	-	-
N	*S. cerevisiae*	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	w	-	-	-	+	-	-	-	v
O	*P. kluyveri*	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	w	-	+	-	-	-	-	-	-	-	w	+	+	+	-	+
P	*R. mucilaginosa*	+	-	+	v	v	v	-	+	+	+	+	-	+	-	-	v	+	+	+	-	-	-	v	-	+	v	-	+	v	-	-	-
Q	*Z. mrakii*	v	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-

15 **Table 5**: Distribution (%) of yeast species and of the yeast-like fungus *A. pullulans* in different samples collected during 35 olive oil extraction processes carried
16 out in three years; (the symbol "-" indicates isolation frequency <1%).
17

	Washed olives	Crushed pastes	Kneaded pastes	Oil from decanter	Pomaces
A. pullulans	22	12	-	-	-
C. adriatica	7	6	-	10	20
C. diddensiae	-	3	2	3	1
C. kluyveri	-	-	1	-	-
N. molendini-olei	10	47	28	38	52
C. norvegica	13	6	-	-	-
C. oleophila	10	-	1	-	-
C. railenensis	9	3	1	-	-
N. wickerhamii	13	5	12	7	7
L. fermentati	-	5	-	-	4
M. fructicola	1	1	-	-	-
R. glutinis	6	2	-	-	-
R. mucilaginosa	4	1	-	-	-
R. sloffiae	3	2	1	1	-
S. cerevisiae	-	4	19	2	2
Y. terventina	-	-	-	38	2
Z. mrakii	1	-	38	1	11
Others	1	3	-	-	1

18 **Table 6**: Distribution of yeast species (%) in the different samples collected during 30 olive oil extraction processes of two different years and subdivided
19 according to olive cultivar (*Frantoio* or *Moraiolo*) ; (the symbol "-" indicates isolation frequency <1%).
20

	Washed olives		Crushed pastes		Kneaded pastes		Oil from decanter		Pomaces	
	Frantoio	*Moraiolo*	*Frantoio*	*Moraiolo*	*Frantoio*	*Moraiolo*	*Frantoio*	*Moraiolo*	*Frantoio*	*Moraiolo*
A. pullulans	19	25	23	-	-	-	-	-	-	-
C. adriatica	12	8	7	2	-	-	17	19	10	13
C. diddensiae	-	-	4	3	-	-	8	-	3	-
C. kluyveri	-	-	-	-	-	2	-	-	-	-
N. molendini-olei	10	18	31	21	13	6	21	28	34	28
C. norvegica	33	5	7	20	-	-	-	-	-	-
C. oleophila	-	-	-	-	-	-	-	-	-	-
C. railenensis	-	-	5	2	-	-	-	-	-	-
N. wickerhamii	17	19	9	3	26	3	4	22	10	18
L. fermentati	-	-	2	11	-	-	-	8	7	8
M. fructicola	2	-	2	-	-	-	-	2	-	-
R. glutinis	3	8	4	-	-	-	-	-	-	-
R. mucilaginosa	1	11	-	10	-	-	-	-	-	-
R. sloffiae	3	6	-	20	-	-	-	-	-	-
S. cerevisiae	-	-	2	8	8	3	2	-	3	2
Y. terventina	-	-	-	-	-	-	46	21	8	4
Z. mrakii	-	-	-	-	53	85	2	-	25	26
Others	-	-	4	-	-	1	-	-	-	1

Table 7: Distribution of yeast species (%) in the different samples collected during 35 olive oil extraction processes and subdivided according to the different years (2011, 2012, and 2013) ; (the symbol "-" indicates isolation frequency <1%).

	Washed olives			Crushed pastes			Kneaded pastes		Oil from decanter			Pomaces		
	2011	2012	2013	2011	2012	2013	2012	2013	2011	2012	2013	2011	2012	2013
A. pullulans	2	42	23	-	36	-	-	-	-	-	-	-	-	-
C. adriatica	20	-	-	8	-	1	-	33	4	27	-	13	37	9
C. diddensiae	-	-	-	8	-	-	-	-	9	-	-	3	-	-
C. kluyveri	-	-	-	-	-	-	-	1	-	-	-	-	-	-
N. molendini-olei	28	-	3	48	4	1	7	10	29	9	75	55	12	88
C. norvegica	-	38	-	-	19	-	-	-	-	-	-	-	-	-
C. oleophila	-	-	30	-	-	-	-	2	-	-	-	-	-	-
C. railenensis	-	-	26	2	7	-	-	-	-	-	-	-	-	-
N. wickerhamii	36	-	4	10	4	-	13	4	7	11	3	10	10	-
L. fermentati	-	-	-	14	-	10	-	-	-	-	-	13	-	-
M. fructicola	2	-	-	-	4	-	-	-	-	-	-	-	-	-
R. glutinis	3	8	8	-	7	-	-	-	-	-	-	-	-	-
R. mucilaginosa	-	12	-	-	4	-	-	1	-	-	-	-	-	-
R. slooffiae	9	-	-	-	7	-	-	-	-	-	4	-	-	-
S. cerevisiae	-	-	-	10	-	88	4	48	2	-	4	3	1	3
Y. terventina	-	-	-	-	-	-	-	1	49	50	14	-	6	-
Z. mrakii	-	-	3	-	-	-	76	-	-	3	-	-	33	-
Others	-	-	3	-	8	-	-	-	-	-	-	3	1	-

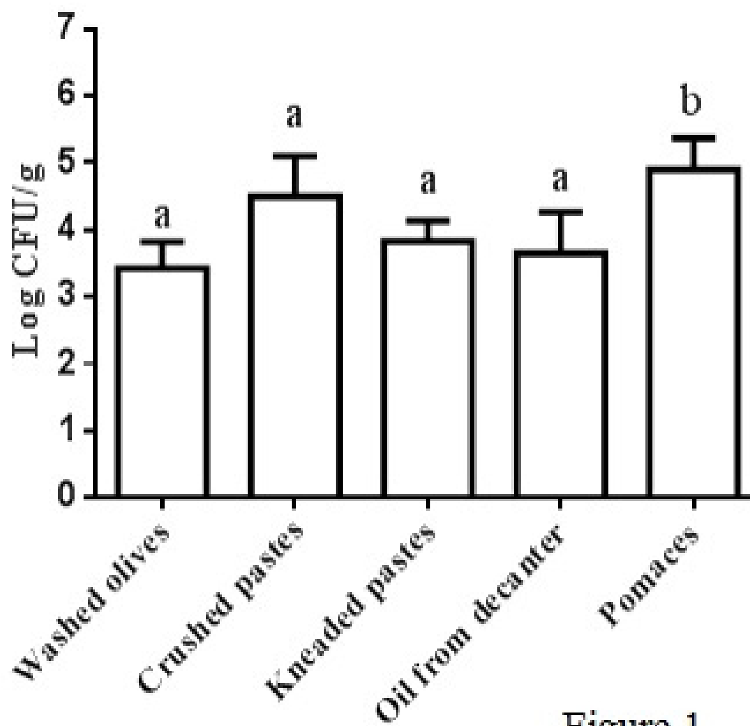
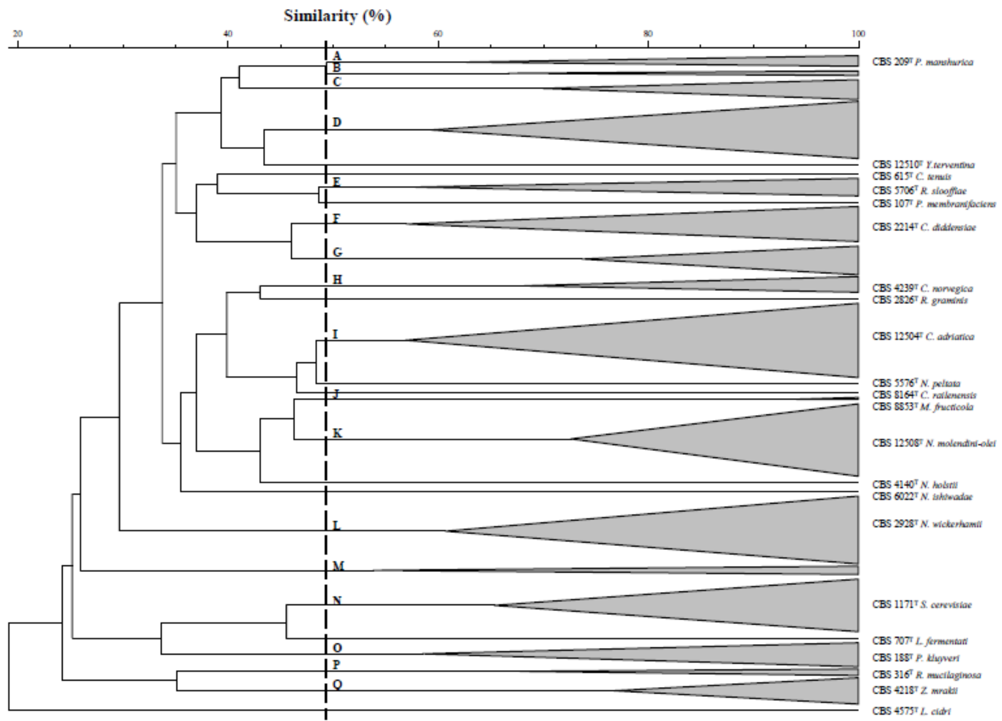


Figure 1


Similarity (%)
20
40
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A
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Q
CBS 209T P. manshurica
CBS 12510T Y.terventina
CBS 615T C. tenuis
CBS 5706T R. slooffiae
CBS 107T P. membranifaciens
CBS 2214T C. diddensiae
CBS 4239T C. norvegica
CBS 2826T R. graminis
CBS 12504T C. adriatica
CBS 5576T N. peltata
CBS 8164T C. railenensis
CBS 8853T M. fructicola
CBS 12508T N. molendini-olei
CBS 4140T N. holstii
CBS 6022T N. ishiwadae
CBS 2928T N. wickerhamii
CBS 1171T S. cerevisiae
CBS 707T L. fermentati
CBS 188T P. kluyveri
CBS 316T R. mucilaginosa
CBS 4218T Z. mrakii
CBS 4575T L. cidri

AL39

IDENTIFICATION AND ENUMERATION OF THE YEAST POPULATIONS OCCURRING IN THE EXTRACTION PROCESS OF EXTRA VIRGIN OLIVE OIL PRODUCED IN TUSCANY

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Newly produced olive oils usually show an opalescent appearance due to the presence of solid particles and micro-drops of vegetation water containing microorganisms, mainly represented by yeasts (Ciafardini and Zullo, 2002). The yeasts found in the oil derive from the olive carposphere which, during the crushing of the olives, migrate into the oil together with the solid particles of the fruit and the micro-drops of vegetation water. Some yeast species do not survive a long time whereas others persist and become the typical microflora of each oil. The yeasts present in newly produced oil can remain active during the conservation period and, according to their metabolic capability, can both improve or worsen the oil quality (Zullo *et al.*, 2010). Indeed, beta-glucosidase and esterase capabilities can improve the taste and the antioxidant capability of the oil, while the lipase capability can worsen the oil quality by hydrolyzing triglycerides (Ciafardini and Zullo, 2002; Ciafardini *et al.*, 2006). On the basis of these findings, and considering the lack of information on the yeast occurrence in the different steps of the extraction process of extra virgin olive oils, a study was carried out to investigate on the presence of these microorganisms in the pastes after crushing, in the oil after centrifugation in a two phase decanter, in the oil after filtration, and in the oil after three months of conservation in dark-green bottles. A total of 16 extraction processes, carried out in the same manufacture located in Tuscany, were considered. The yeast densities in the pastes and in the oil before the filtration step ranged between values of about 10^2 and 10^4 CFU/g, while in the filtrated oil the yeast density was below 10^2 CFU/100 mL. After three months of conservation, the yeast densities were below 1 CFU/100 mL in all the oil samples. Correlation studies showed that yeast densities in the pastes and in the oils before and after the filtration were unrelated, suggesting a role of the environment in the oil contaminations. The yeast isolates from each step (108 in total) were purified and identified with a polyphasic approach. At first, the yeast isolates were analyzed by PCR-RFLP of the rDNA internal transcribed spacer (ITS) region. Then, representative isolates of each ITS pattern group were randomly chosen for sequence analysis of D1/D2 domain of the large subunit ribosomal RNA gene. Finally, the capabilities of these isolates to assimilate different carbon sources were assayed using the ID 32 yeast identification system (Biomérieux). According to the genotypic and phenotypic results, most of the yeast isolates belonged to *Candida* genus, *Candida molendinolei* being the species most frequently isolated from the pastes. *C. molendinolei* is a novel species recently described by Čadež *et al.* (2012) and isolated from unfiltred extra virgin olive oil. This work may be considered a preliminary study to understand the microbial ecology of the extra virgin olive oil and its effective impact on the organoleptic quality of this product.

Keywords: extra virgin olive oil microrganisms, olive-oil yeast identification, Candida molendinolei

References:

Čadež N., Raspor P., Turchetti B., Cardinali G., Ciafardini G., Veneziani G., Gàbor P. (2012). International Journal of Systematic and Evolutionary Microbiologi (in press).
Ciafardini G., Zullo B. A. (2002). International Journal of Food Microbiology 75: 111-118.

Identification and enumeration of the yeast populations occurring in the extraction process of extra virgin olive oil produced in Tuscany.

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Introduction

- Newly produced olive oils usually show the presence of solid particles and micro-drops of vegetation water containing microorganisms, mainly represented by yeasts (Ciafardini and Zullo, 2002).
- The yeasts found in the oil derive from the olive carposphere which, during the crushing of the olives, migrate into the oil.
- The yeasts present in newly produced oil can remain active during the conservation period and, according to their metabolic capability, can both improve or worsen the oil quality (Zullo et al., 2010).
- Information on the yeast occurrence in the different steps of the extraction process of extra virgin olive oils are lacking.

AIM

to investigate on the presence of yeasts:

- in the pastes after crushing,
- in the oil after centrifugation in a two phase decanter,
- in the oil after filtration,
- in the oil after three months of conservation in dark-green bottles.

Materials and methods

- Viable counts of the yeasts were carried out on MYPG agar.
- The yeast isolates were identified by PCR-RFLP of the rDNA internal transcribed spacer (ITS) region and by sequencing of D1/D2 domain of the large subunit ribosomal RNA gene.
- The capabilities of yeasts to assimilate different carbon sources were assayed using the ID 32 C yeast identification system (bioMérieux).

Results

- A total of 16 extraction processes, carried out in the same manufacture located in Tuscany, were considered.
- The yeast densities in the pastes and in the oil before the filtration step ranged between values of about 10^2 and 10^4 CFU/g, while in the filtrated oil the yeast density was below 10^2 CFU/100 mL (fig. 1).
- After three months of conservation, the yeast densities were below 1 CFU/100 mL in all the oil samples.
- Correlation studies showed that yeast densities in the pastes and in the oils before and after the filtration were unrelated, suggesting a role of the oil-mill environment in the oil contaminations.

FIG. 1

Pastes | Oil before the filtration | Filtered oil

TAB. 1

Number of isolates	Sampling 1	Sampling 2
Paste	27	26
Oil before filtration	21	24
Filtered oil	-	10
Total (108)	48	60

- The yeast isolates (Tab. 1) from each step were purified and identified with molecular methods:
  1. the yeast isolates were analyzed by PCR-RFLP of the ITS rDNA (Tab. 2).
  2. representative isolates of each ITS pattern group were randomly chosen for sequence analysis of D1/D2 domain of the large subunit ribosomal RNA gene (Tab. 2).

TAB. 2

Profile	PCR Product (bp)	ITS Restriction fragments (bp)			Sequence analysis of D1/D2 domain of the large subunit ribosomal RNA gene	
		CfoI	HaeIII	HinfI	Closest relative species	Matching nucleotides (identity %)
A	640	310-290-90	300-210-90	320-320	Zygosaccharomyces "fermentati"	552/554 (99.6%)
B	610	210-140-100-[illegible]	400-90	300-300	*Candida adriatica*	550/550 (100%)
C	850	370-330-140	310-240-180-130	370-360-130	*Saccharomyces cerevisiae**	-*
D	640	590	590-90	320-320	*Candida molendinolei*	552/552 (100%)
E	680	290-180-130-	450-130-90	310-310	*Candida diddensiae*	515/515 (100%)
F	640	590	590-90	320-320	[illegible]	552/552 (100%)
H	470	240-110-90	380-100-90	210-190-90	[illegible]	537/538 (99.8%) 537/538 (99.8%)

*Identification with multiplex PCR ([illegible] et al., 2008)

TAB. 3

S. cerevisiae / *C. adriatica* / *C. molendinolei* / *C. diddensiae* / *C. parapsilosis*

- The capabilities of the yeast isolates to assimilate different carbon sources and to growth at 37°C on MYPG agar medium were assayed (Tab. 3) and the results were compared with the literature (Kurtzman, 2011).

- According to the polyphasic approach → most yeast isolates belonged to *Candida* genus (Fig. 2).
- *Candida molendinolei*, a novel species recently described by Čadež et al. (2012) and isolated from unfiltred extra virgin olive oil, was the species most frequently isolated from the pastes.

FIG. 2

Pastes: Sampling 1, Sampling 2 | Oil before the filtration: Sampling 1, Sampling 2 | Filtered oil: Sampling 2

Conclusions

This work may be considered a preliminary study to understand the microbial ecology of the extra virgin olive oil and its effective impact on the quality of this product.

References
Čadež N., Raspor P., Turchetti B., Cardinali G., Ciafardini G., Veneziani G., Péter G. (2012) International Journal of Systematic and Evolutionary Microbiology (in press).
Ciafardini G., Zullo B.A. (2002). International Journal of Food Microbiology 75: 111-118.
Kurtzman C.P. (2011) ... Amsterdam: Elsevier
Vaudano E., Garcia-Moruno E. (2008) Food Microbiology 25: 56-64.
Zullo B.A., Cioccia G., Ciafardini G. (2010) Food Microbiology 27: 1035-1042.

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PS7-02

A SURVEY ON YEAST SPECIES OCCURRING IN THE OLIVE OIL EXTRACTION PROCESS

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During the olive oil extraction process, the yeasts contaminating the olives' carposphere pass on into the oil along with the solid particles of the fruit and the micro-drops of the vegetation water. The yeast species, able to persist in freshly produced oil, can remain active during the conservation period and, according to their metabolic capabilities, can either improve or worsen the oil quality. Studies on the yeast ecology during the olive oil extraction process are lacking. Therefore, the aim of this study was to quantify and identify the yeast populations occurring in different samples (olives, crushed and kneaded pastes, centrifuged oil and pomaces) collected during 14 olive oil extraction processes were carried out in the same oil mill at the beginning, in the middle, and at the end of the same harvest year. The results showed that the yeast concentrations associated with the olives exhibited quite similar values independently of the sampling day, while the yeast concentrations occurring in the pastes, unfiltered oil, and pomaces in the first day-sampling were significantly lower than those found in the last day (ANOVA, $p<0.05$). Similarly, the yeast species identified (by RFLP-rITS and sequencing rRNA genes) in pastes, unfiltered oil, and pomaces samples were more numerous than in olive samples (10 and 3 respectively) and their isolation frequencies changed over the sampling time. Therefore, the occurrence of various yeast species in olive oil extraction process was dependent not only on yeasts contaminating the olives but also on yeasts colonizing the plant for oil extraction. These findings suggest a progressive contamination of the oil mill plant that selects some yeast species. In particular, the two-phase decanter during pastes centrifugation determined the oil contamination with *Yamadazima terventina* and *Candida adriatica*, two yeasts able to influence the oil quality.

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A survey on yeast species occurring in the olive oil extraction process

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INTRODUCTION

- During the olive oil extraction process, the yeasts contaminating the olives' carposphere pass on into the oil along with the solid particles of the fruit and the micro-drops of the vegetation water[1].
- The yeast species, able to persist in freshly produced oil, can remain active during the conservation period and, according to their metabolic capabilities, can either improve or worsen the oil quality[2].
- Studies on the yeast ecology during the olive oil extraction process are lacking[3].

AIM

To quantify and identify the yeast populations occurring in different samples: olives (A, B) crushed (C) and kneaded pastes (D), unfiltered oil (E) and pomaces (F) collected during 14 olive oil extraction processes carried out in the same oil mill at the beginning, the middle, and the end of the same harvest year.

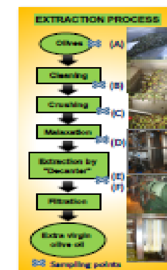


Fig. 1

MATERIALS AND METHODS

- Yeast concentrations were quantified on MYPG agar after three days of incubation at 30°C.
- Yeast isolates coming from olives (washed or not), from pastes (crushed pastes, kneaded pastes), unfiltered oil and pomaces (Fig. 1), were randomly collected during 14 olive oil extraction processes (5 at the beginning, 4 at the middle, 5 at the end).
- The yeast isolates (56 from olives, 154 from pastes, 100 from unfiltered oil, 82 from pomaces) were purified and identified by using PCR-RFLP of rITS and sequencing rDNA genes (data not shown) and the results used to calculate the isolation frequencies expressed as "% of isolates".

RESULTS

- The yeast concentrations associated with the olives exhibited quite similar values (t-test), independently of the sampling day (Fig. 2).
- The yeast concentrations occurring in the pastes, unfiltered oil, and pomaces in the first day-sampling (beginning) were significantly (ANOVA, $p = 0,05$) lower than those found in the last day (end) (Fig. 3).

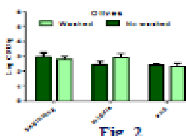


Fig. 2

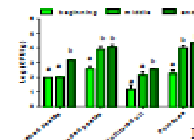


Fig. 3

Species (%)	Washed olives		
	Beginning	Middle	End
Yeast-like fungus			
Aureobasidium pullulans	-	25	100
Yeasts			
Candida norvegica	64	50	-
Rhodotorula glutinis	-	25	-
Rhodotorula mucilaginosa	36	-	-

- The yeast species identified in pastes, unfiltered oil, and pomace samples were more numerous than in olive samples (10 and 3, respectively) and their isolation frequencies changed over the sampling time (Fig. 4 and table 1). Therefore, the occurrence of various yeast species in olive oil extraction process was dependent:
  - on yeasts contaminating the olives;
  - on yeasts colonizing the plant for oil extraction.

- *Yamadazyma terventina* and *Candida adriatica* were isolated:
  - only from unfiltered oil or pomaces;
  - only the second and last day-sampling (middle and end);
  - never from olives or pastes.

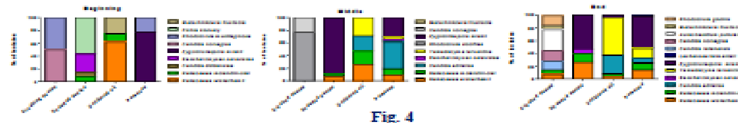


Fig. 4

CONCLUSIONS

These findings suggest a progressive contamination of the oil mill plant that selects some yeast species. In particular, the two-phase decanter during pastes centrifugation, determined the oil contamination with *Yamadazyma terventina* and *Candida adriatica*, two yeasts able to influence the oil quality[2].

REFERENCES
[1] Ciafardini, G., Zullo, B.A. (2002): Microbiological activity in stored olive oil. International Journal of Food Microbiology, 75: 111-118.
[2] Zullo B.A., Cioccia G., Ciafardini G. (2013): Effects of some oil-born yeasts on the sensory characteristics of Italian virgin olive oil during its storage. Food Microbiology, 36: 70-78.
[3] Guerrini S., Mari E., Migliorini M., Cherubini C., Trapani S., Zanoni B., Vincenzini M. (2015): Investigation on microbiology of olive oil extraction process. Italian Journal of Food Science, 27: 108-119.

3.3 CHEMICAL ANALYSIS OF OILS

Abstract

Extra virgin olive oil is characterized by pleasant sensory note, moreover, the absence of sensory defects is necessary for olive oil to be marketed as ‘‘extra virgin’’ in the EU (EU Reg. 1348/2013). The aromatic and polyphenolic compounds of oils from 35 extraction processes of three consecutive crop seasons in the same oil mill were analyzed. Trans-2-hexenal, considered with other compounds to be responsible for olive oil “fruity”, “grassy” and other positive attribute (Aparicio *et al.*, 2012) was the most abundant compound present in all three years of study. As regards the aromatic compounds related to olive oil defects (Morales et al., 2005), all the oils of the 2011 showed a content of trans-2-heptenal, trans-2-decenal and 1-octen-3-ol significantly above the odour threshold, while in the 2012 only 1-octen-3-ol was detected above the odour threshold in all oils extracted. On the contrary, in the oils of the 2013, compounds associated with defect were not detected or detected below the odor threshold. Correlation studies showed that 24, 13 and 12 volatile compounds were significantly correlated with the yeast concentrations quantified in one, two and all steps (crushed pastes, oil from decanter and pomaces) of the extraction process respectively. The results regarding the polyphenolic compounds of oils confirmed that the presence of oleuropein and its derivatives was predominant compared to the other classes of phenolic compounds. 3,4-DHPEA-EDA was the most abundant phenolic compound in 2011 and 2013 oils, while in 2012 oils the most abundant phenolic compound was oleuropein. Correlation studies showed that a total of 10, 5 and 2 polyphenolic compounds were significantly correlated with the yeast concentrations quantified respectively in one, two and all steps of the extraction process. Finally, Principal Component Analysis of the various analyzed olive batches, considering as variables the yeast cell concentrations during various extraction process steps (crushed pastes, oil from decanter and pomaces); the volatile compounds and the polyphenolic compounds showed that the samples were grouped depending on the harvesting year.

3.3.1 Introduction

The absence of sensory defects is necessary for olive oil to be marketed as ‘‘extra virgin’’ in the EU. Extra virgin olive oil is characterized by pleasant sensory notes. They are mainly originated by aldehydes, esters, alcohols and ketones, which are responsible for oil sensory attributes such as “green” and “fruity” (Aparicio and Morales, 1998; Morales *et al*., 2005, Bendini *et al.*, 2012). However, several phenomena can alter the initial pleasant flavour, giving rise to unpleasant sensory notes. The current olive oil regulations (EU Reg. 1348/2013) classify the most frequent sensory defects into four groups as follows: “fusty”, “musty”, “winey–vinegary”, and “rancid”. Both biogenesis of volatile compounds and transformation phenomena of phenolic compounds can be significantly influenced by microbial contamination of olives. Effects of olive microbiota on oil characteristics are considered even greater than time-temperature conditions of malaxation (Vichi *et al*., 2011). Oil quality may be affected by microorganisms, according to their metabolic activities. During olive crushing, microorganisms might migrate into oil through both solid particles of olive fruit and micro-drops of vegetation water (Ciafardini and Zullo, 2002a). Some microorganisms do not survive a long time, but others may persist and become a typical microbiota of olive oil. For example, yeasts may remain metabolically active during olive oil storage and thus modify olive oil characteristics (Zullo *et al*., 2010). Enzymatic activities of yeasts and moulds isolated from either olives or extra virgin olive oil have been reported to include β-glucosidase, β-glucanase, polyphenoloxidases, peroxidase and, in some cases, lipase and cellulase activities (Ciafardini and Zullo, 2002b; Ciafardini *et al*., 2006a-b; Zullo and Ciafardini, 2008; Romo-Sanchez *et al*., 2010). Enzymes such as β-glucosidase are known to improve oil quality by increasing phenolic compound extractability, while others such as lipase, polyphenoloxidases and peroxidase are known to cause detrimental effects (Romo-Sanchez *et al*., 2010; Vichi *et al*., 2011; Migliorini *et al*., 2012). The aim of this study was to investigate on the aromatic and polyphenolic compounds of oils from the studied extraction processes (paragraph 3.1: 35 extraction processes of three

consecutive crop season in the same oil mill) and carry out correlations between these compounds and yeast concentrations in the different phases of the process (crushed pastes, kneaded pastes, oil from decanter and pomaces).

3.3.2 Materials and methods

Chemical analysis

Chemical analysis were performed by PromoFirenze - Laboratorio Chimico Merceologico, Special Agency of the Florence Chamber of Commerce, Florence, Italy. Extraction, identification and determination of phenolic compounds were performed in agreement with IOC Official Method (IOC, 2009) by an HPLC equipment consisting of a Hewlett Packard 1200 diode-array detector system and a Hewlett Packard model 1200 autosampler (Agilent Technologies, Santa Clara, California, USA). Secoiridoids, lignans, flavonoids and phenolic acids were quantified in $mg_{tyrosol}kg_{oil}^{-1}$. The content of the total phenolic compounds ($mg_{tyrosol}kg_{oil}^{-1}$) was determined using the sum of the peak areas of phenols recorded at 280 nm. The tocopherol content was determined according to ISO 9936:2006 (ISO, 2006) using a Hewlett Packard mod. 1050 liquid chromatograph with quaternary pump and fluorescence detector, provided with Hewlett Packard mod.1100 autosampler (Agilent Technologies, Santa Clara, California, USA). Quantitative analysis was carried out using the external standard method. Results were expressed as mg of total tocopherols per kg of oil. The volatile compound content was determined according to the literature (VICHI *et al.*, 2003), using HS-SPME-GC-MS technique (solid phase microextraction of the head space, coupled with a gas chromatograph with a mass spectrometer as a detector). Analysis was performed using the Trace CG instrument combined with a Trace DSQ Thermo Finnigan instrument (Fisher Scientific SAS, Illkirch, France). Quantitative analysis was performed using 4-methyl-2-pentanol as an internal standard. Results were expressed as mg of aromatic compound per kg of oil.

Data processing

Chemical determinations were processed according to one-way ANOVA followed by Tukey's test (significance level: $p = 0.05$).

Principal Component Analysis (PCA) was used to classify samples by Statistica 7.0 software package (Stasoft GmbH, Hamburg, Germany). Correlation studies between microbial cell density and the volatile and polyphenolic compounds content of oil samples were carried out by calculating both Pearson and Spearman rank correlation coefficients (significance level: $\alpha = 0.05$).

3.3.3 Results

3.3.3.1 Aromatic compounds analysis

The contents of volatile compounds of olive oil samples in the three years of study were subdivided into chemical classes, as reported in Tables 12-14.

Oils obtained in the 2013 harvesting year showed lower values of volatile compounds than the oils produced during the first and second crop seasons (Tab. 12-14). Probably this is related to the degree of ripeness of the olives at the time of processing, indeed, transforming olives at the time of ripeness technology, allows improving the contents of drupes.

Underlined volatile compounds are intermediate of LOX pathway and they are considered (Di Giacinto *et al*., 2010; Kotti *et al.*, 2011; Aparicio *et al*., 2012) to be responsible for olive oil "fruity", "grassy" and other positive attribute. In the 2011 crop season there were not differences on the total of the compounds of the lipoxygenase pathway in oils obtained from different cultivars, while in the 2012 campaign, oils from *Moraiolo* cultivar resulted with a higher content of cis-3-hexenal and trans-2-hexenal (first harvesting date) than oils from *Frantoio* cultivar (Tab. 12B-13B). Moreover, trans-2-hexenal was the most abundant compound present in all three years of study with concentration ranged between 5.121 and 15.65 mg/Kg oil (Tables 12-14 B).

The oils characterized by high values of trans-2-heptenal, trans-2-decenal, 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol, 1-octen-3-one and 2-octanone are related to olive oil defects. Indeed, as reported by Morales et al., 2005, (Tab. 15) these compounds have been associated with "musty", "winey–vinegary", "fusty" and "rancid" defect. All the oils of the 2011 showed a content of trans-2-heptenal, trans-2-decenal and 1-octen-3-ol well above the odour threshold (Tab. 12 B,C), while in the 2012 only 1-octen-3-ol was detected above the dour threshold in all oils extracted. On the contrary butyric acid and trans-2-decenal were detected above the odor threshold only in three oils from the first harvesting date and in the oils from the third and fourth harvesting date respectively (Tab. 13 A,B,C). In the oils of the 2013 compounds associated with defect were not detected or detected below the odor threshold (Tab. 14).

Table 12: Concentration of volatile compounds in olive oils obtained during 2011 crop season. Samples are encoded in relation to olive cultivar(*Moraiolo* or *Frantoio*), harvesting date (1 or 2), batch (a, b, c or d). *A, B, C, D* and the chemical classes of the compounds analyzed. HD: harvesting date; n.d. not determined. Underlined volatile compounds are intermediate of LOX pathway and they are considered to be responsible for olive oil positive attribute.

A. Class of esters, acids and hydrocarbons

	HD	Batch code	Methyl acetate (mg/Kg)	Ethyl acetate (mg/Kg)	Hexyl acetate (mg/Kg)	Butyl acetate (mg/Kg)	Cis-3-hexenyl acetate (mg/Kg)	Trans-2-hexenyl acetate (mg/Kg)	Butyric acid (mg/Kg)	Pentanoic acid (mg/Kg)	Hexanoic acid (mg/Kg)	Octanoic acid (mg/Kg)	Heptane (mg/Kg)	Octane (mg/Kg)	Ocimene (mg/Kg)
Frantoio	1	**F1a**	0.036	0.019	0.153	0.002	0.144	0.003	nd	0.010	0.274	0.070	0.007	0.048	0.084
		F1b	0.022	0.017	0.089	0.002	0.131	0.002	nd	0.013	0.333	0.089	0.006	0.043	0.051
		F1c	0.015	0.020	0.110	0.002	0.134	0.001	nd	0.011	0.255	0.047	0.004	0.038	0.041
	2	**F2a**	0.015	0.042	0.107	0.002	0.070	nd	0.011	0.007	0.210	0.121	0.005	0.053	0.056
		F2b	0.007	0.035	0.184	0.002	0.409	nd	0.010	0.008	0.243	0.135	0.002	0.039	0.061
		F2c	0.008	0.027	0.235	0.001	0.614	nd	0.012	0.013	0.237	0.170	0.003	0.036	0.064
		F2d	0.006	0.023	0.228	0.001	0.619	nd	0.010	0.010	0.235	0.166	0.003	0.036	0.063
Moraiolo	1	**M1a**	0.005	0.018	0.215	0.001	0.747	0.003	nd	0.004	0.236	0.095	0.003	0.032	0.041
		M1b	0.005	0.016	0.208	0.002	1.070	0.027	nd	0.007	0.259	0.083	0.005	0.031	0.044
		M1c	0.006	0.019	0.224	0.003	1.173	0.004	nd	0.008	0.277	0.065	0.003	0.033	0.043
		M1d	0.005	0.015	0.249	0.001	0.480	0.006	nd	0.004	0.214	0.065	0.005	0.028	0.041
	2	**M2a**	0.006	0.021	0.237	0.001	1.032	nd	0.013	0.012	0.277	0.185	0.003	0.035	0.049
		M2b	0.005	0.020	0.199	0.002	0.858	nd	0.013	0.011	0.261	0.160	0.004	0.040	0.043
		M2c	0.008	0.022	0.159	0.001	0.927	nd	0.012	0.008	0.201	0.119	0.002	0.036	0.034
		M2d	0.006	0.023	0.237	0.001	0.701	nd	0.014	0.015	0.262	0.171	0.003	0.037	0.066

B. Class of aldehydes

	HD	Batch code	Valeraldehyde (mg/Kg)	Isovaleraldehyde (mg/Kg)	Hexanal (mg/Kg)	2-Methyl-Butanal (mg/Kg)	Trans-2-Pentenal (mg/Kg)	Cis-3-Hexenal (mg/Kg)	Heptanal (mg/Kg)	Trans-2-Hexenal (mg/Kg)	Octanal (mg/Kg)	Trans-2-Heptenal (mg/Kg)	2.4 Hexadienal (mg/Kg)	Trans-2-Octanal (mg/Kg)	Benzaldehyde (mg/Kg)	Trans-2-Nonenal (mg/Kg)	Trans-2-Decenal (mg/Kg)
Frantoio	1	**F1a**	0.128	0.039	0.586	0.062	0.031	1.421	0.025	10.090	0.131	0.038	0.292	0.015	0.031	0.190	0.229
		F1b	0.106	0.027	0.644	0.031	0.035	1.822	0.026	10.217	0.159	0.033	0.313	0.010	0.031	0.196	0.232
		F1c	0.087	0.017	0.596	0.045	0.030	1.569	0.025	9.963	0.134	0.024	0.270	0.010	0.030	0.185	0.237
	2	**F2a**	0.094	0.041	0.481	0.068	0.023	1.085	0.017	7.331	0.063	0.042	0.198	0.010	0.030	nd	0.132
		F2b	0.079	0.031	0.504	0.049	0.028	1.084	0.017	6.938	0.066	0.034	0.189	0.015	0.032	nd	0.125
		F2c	0.070	0.024	0.547	0.043	0.035	1.157	0.023	6.785	0.090	0.031	0.177	0.014	0.033	nd	0.078
		F2d	0.071	0.023	0.535	0.037	0.035	1.050	0.022	6.545	0.103	0.030	0.172	0.016	0.034	nd	0.092
Moraiolo	1	**M1a**	0.092	0.018	0.556	0.034	0.036	1.742	0.028	8.750	0.148	0.013	0.271	0.019	0.028	0.187	0.203
		M1b	0.079	0.022	0.443	0.035	0.036	2.016	0.022	7.684	0.137	0.009	0.262	0.016	0.029	0.197	0.186
		M1c	0.100	0.024	0.489	0.041	0.042	1.937	0.029	7.371	0.152	0.020	0.246	0.014	0.031	0.194	0.204
		M1d	0.087	0.015	0.563	0.028	0.032	2.045	0.025	9.435	0.117	0.023	0.276	0.019	0.028	0.202	0.180
	2	**M2a**	0.053	0.018	0.538	0.033	0.039	1.320	0.027	6.595	0.117	0.024	0.206	0.012	0.036	nd	0.081
		M2b	0.044	0.020	0.474	0.034	0.043	1.803	0.022	6.172	0.093	0.021	0.229	0.009	0.035	nd	0.058
		M2c	0.042	0.021	0.416	0.035	0.045	4.051	0.015	5.121	0.072	0.010	0.348	0.022	0.034	nd	0.059
		M2d	0.070	0.022	0.585	0.036	0.038	1.123	0.025	6.607	0.126	0.036	0.192	0.018	0.039	nd	0.099

C. Class of alcohols

	HD	Batch code	1-Penten-3-ol (mg/Kg)	2-Heptanol (mg/Kg)	Benzene-Ethanol (mg/Kg)	Trans-2-Hexenol (mg/kg)	Pentanol (mg/Kg)	Hexanol (mg/Kg)	Octanol (mg/Kg)	1-Octen-3-ol (mg/Kg)	Trans-3-Hexenol (mg/kg)	Cis-3-Hexenol (mg/Kg)	Cis-2-Pentenol (mg/Kg)	Trans-2-pentenol (mg/Kg)
Frantoio	1	**F1a**	0.446	nd	0.339	0.379	0.005	0.242	0.073	0.002	0.004	0.242	0.337	0.039
		F1b	0.454	nd	0.299	0.273	0.005	0.171	0.058	0.002	0.004	0.260	0.358	0.047
		F1c	0.475	nd	0.303	0.302	0.004	0.168	0.066	0.002	0.003	0.200	0.353	0.051
	2	**F2a**	0.403	0.225	0.251	0.249	0.004	0.223	0.059	0.005	0.004	0.089	0.288	0.038
		F2b	0.491	0.294	0.264	0.243	0.004	0.273	0.065	0.007	0.005	0.259	0.310	0.047
		F2c	0.597	0.351	0.257	0.249	0.005	0.358	0.074	0.008	0.006	0.431	0.382	0.058
		F2d	0.582	0.346	0.269	0.258	0.005	0.369	0.076	0.010	0.007	0.454	0.376	0.059
Moraiol	1	**M1a**	0.554	nd	0.277	0.212	0.006	0.266	0.068	0.002	0.006	0.521	0.428	0.053
		M1b	0.569	nd	0.251	0.229	0.006	0.297	0.053	0.002	0.009	0.695	0.435	0.052
		M1c	0.584	nd	0.271	0.261	0.005	0.312	0.064	0.002	0.008	0.709	0.444	0.052
		M1d	0.571	nd	0.262	0.195	0.006	0.254	0.063	0.002	0.006	0.346	0.447	0.052
	2	**M2a**	0.658	0.375	0.24	0.219	0.005	0.419	0.067	0.009	0.008	0.714	0.420	0.059
		M2b	0.575	0.369	0.216	0.185	0.005	0.355	0.053	0.007	0.005	0.745	0.412	0.057
		M2c	0.603	0.361	0.174	0.162	0.004	0.307	0.049	0.006	0.006	0.853	0.402	0.057
		M2d	0.636	0.381	0.274	0.264	0.006	0.409	0.075	0.012	0.007	0.509	0.394	0.061

D. Class of ketones and phenols

	HD	Batch code	2-Butanone (mg/Kg)	2-Octanone (mg/Kg)	1-Octen-3-one (mg/Kg)	1-Penten-3-one (mg/kg)	Ethyl-vinil-ketone (mg/kg)	6-methyl-5-Hepten-2-one (mg/Kg)	Guaiacol (mg/Kg)	Phenol (mg/Kg)	Ethyl-guaiacol (mg/Kg)	4-Ethyl-phenol (mg/Kg)
Frantoio	1	**F1a**	0.433	0.003	0.001	0.433	0.335	0.004	0.005	0.261	0.131	nd
		F1b	0.477	0.004	0.001	0.477	0.433	0.005	0.005	0.275	0.124	nd
		F1c	0.484	0.004	0.001	0.484	0.477	0.004	0.008	0.253	0.158	0.080
	2	**F2a**	0.360	0.014	0.003	0.360	0.36	nd	0.002	0.198	nd	nd
		F2b	0.481	0.012	0.002	0.481	0.481	nd	0.004	0.200	nd	nd
		F2c	0.708	0.010	0.001	0.708	0.708	nd	0.005	0.193	nd	nd
		F2d	0.712	0.035	0.004	0.712	0.712	nd	0.006	0.203	nd	nd
Moraiolo	1	**M1a**	0.586	0.008	0.002	0.586	0.586	0.004	0.004	0.245	nd	nd
		M1b	0.590	0.004	0.002	0.590	0.59	0.002	0.004	0.251	0.117	nd
		M1c	0.606	0.006	0.002	0.606	0.606	0.004	0.003	0.352	0.114	0.067
		M1d	0.533	0.005	0.001	0.533	0.533	0.003	0.004	0.238	nd	nd
	2	**M2a**	0.715	0.009	0.002	0.715	0.715	nd	0.006	0.208	nd	nd
		M2b	0.711	0.003	0.002	0.711	0.711	nd	0.004	0.210	nd	nd
		M2c	0.720	0.003	0.002	0.720	0.72	nd	0.002	0.196	nd	nd
		M2d	0.753	0.011	0.004	0.753	0.753	nd	0.006	0.205	nd	nd

Table 13: Concentration of volatile compounds in olive oils obtained during 2012 crop season. Samples are encoded in relation to olive cultivar(*Moraiolo*, *Frantoio* or Mixed), harvesting date (1, 2, 3 or 4), batch (a, b, c,…). *A*, *B, C*, *D* and the chemical classes of the compounds analyzed. HD: harvesting date; n.d. not determined. Underlined volatile compounds are intermediate of LOX pathway and they are considered to be responsible for olive oil positive attribute.

A. Class of esters, acids and hydrocarbons

	HD	Batch code	Methyl acetate (mg/Kg)	Ethyl acetate (mg/Kg)	Hexyl acetate (mg/Kg)	Ethyl propionate (mg/Kg)	Cis-3-hexenyl acetate (mg/Kg)	Trans-2-hexenyl acetate (mg/Kg)	Butyric acid (mg/Kg)	Heptane (mg/Kg)	Octane (mg/Kg)	Ocimene (mg/Kg)
Moraiolo	1	**2M1a**	0.004	0.001	0.174	n.d.	0.888	nd	0.201	0.009	0.029	n.d.
		2M1b	0.001	n.d.	0.210	n.d.	0.997	0.061	0.182	0.004	0.014	n.d.
		2M1c	0.002	n.d.	0.208	n.d.	1.193	0.057	0.155	0.002	0.013	0.129
		2M1d	0.002	n.d.	0.182	n.d.	1.152	0.064	0.118	0.003	0.014	0.104
		2M1e	0.001	n.d.	0.187	n.d.	1.363	0.132	0.134	0.002	0.011	n.d.
		2M1f	0.001	n.d.	0.170	n.d.	1.238	0.113	0.114	0.002	0.012	n.d.
Mixed	2	**2V2a**	0.005	0.011	0.053	n.d.	0.277	nd	0.047	0.004	0.023	n.d.
		2V2b	0.006	0.016	0.037	n.d.	0.275	nd	0.024	0.004	0.019	n.d.
Moraiolo	3	**2M3a**	0.008	0.017	0.131	0.016	1.155	0.019	0.032	0.003	0.015	0.022
		2M3b	0.006	0.012	0.134	0.016	1.306	0.024	0.028	0.003	0.011	0.023
		2M3c	0.006	0.017	0.158	0.016	1.072	0.016	0.012	0.003	0.011	n.d.
Frantoio	4	**2F4a**	0.011	0.006	0.131	0.012	0.734	0.024	0.002	0.003	0.057	0.026
		2F4b	0.007	0.007	0.134	0.011	0.763	0.038	0.002	0.003	0.032	0.015
		2F4c	0.003	0.004	0.158	0.009	0.846	0.033	0.002	0.002	0.014	0.024

B. Class of aldehydes

	HD	Batch code	Valeraldehyde (mg/Kg)	Isovaleraldheyd (mg/Kg)	Hexanal (mg/Kg)	2 Methyl-Butanal (mg/Kg)	Trans-2-Pentenal (mg/Kg)	Cis-3-Hexenal (mg/Kg)	Trans-2-Hexenal (mg/Kg)	Octanal (mg/Kg)	2.4 Hexadienal (mg/Kg)	2.4 Heptadienal (mg/Kg)	Trans-2-Octanal (mg/Kg)	Benzaldehyde (mg/Kg)	Trans-2-Decenal (mg/Kg)
Moraiol															
	1	**2M1a**	0.060	0.007	1.036	0.033	0.078	5.958	13.781	0.066	1.623	n.d.	0.065	0.094	n.d.
		2M1b	0.034	0.004	0.816	0.023	0.072	6.621	12.826	0.025	1.507	n.d.	0.035	0.058	n.d.
		2M1c	0.041	0.005	0.79	0.027	0.082	6.305	12.635	0.027	1.448	n.d.	0.022	0.072	n.d.
		2M1d	0.044	0.004	0.719	0.029	0.077	5.978	12.068	0.028	1.419	n.d.	0.034	0.066	n.d.
		2M1e	0.035	0.003	0.578	0.018	0.071	7.004	10.299	0.021	1.484	n.d.	0.022	0.058	n.d.
		2M1f	0.040	0.003	0.572	0.019	0.063	5.652	11.665	0.023	1.285	n.d.	0.020	0.051	n.d.
Mixed															
	2	**2V2a**	0.074	0.012	0.272	0.036	0.069	1.793	8.884	0.024	0.73	n.d.	0.024	0.042	n.d.
		2V2b	0.067	0.017	0.276	0.046	0.077	1.820	9.666	0.022	0.757	n.d.	0.017	0.039	n.d.
Moraiolo															
	3	**2M3a**	0.028	0.007	0.35	0.027	0.081	5.692	8.069	n.d.	1.216	0.01	0.022	0.045	0.091
		2M3b	0.022	0.006	0.312	0.019	0.082	5.693	7.322	n.d.	1.182	0.014	0.036	0.043	0.122
		2M3c	0.031	0.005	0.317	0.018	0.076	6.405	7.6	n.d.	1.289	0.01	0.024	0.046	0.099
Frantoio															
	4	**2F4a**	0.044	0.009	0.44	0.034	0.060	1.591	7.527	n.d.	0.594	n.d.	0.030	0.032	0.234
		2F4b	0.048	0.009	0.401	0.030	0.055	1.439	6.942	n.d.	0.532	0.005	0.028	0.030	0.145
		2F4c	0.037	0.008	0.303	0.029	0.049	1.326	6.452	n.d.	0.535	n.d.	0.025	0.029	0.103

C. Class of alcohols

	HD	Batch code	1-Penten-3-ol (mg/Kg)	Trans-2-Hexenol (mg/Kg)	Benzene-Ethanol (mg/Kg)	Pentanol (mg/Kg)	Hexanol (mg/Kg)	Octanol (mg/Kg)	1-Octen-3-ol (mg/Kg)	Trans-3-Hexenol (mg/kg)	Cis-3-Hexenol (mg/Kg)	Cis-2-Pentenol (mg/Kg)	Trans-2-pentenol (mg/Kg)
Moraiolo													
	1	**2Ma**	0.359	0.462	0.294	0.011	0.634	0.097	0.01	0.017	0.009	0.529	0.054
		2Mb	0.432	0.363	0.201	0.005	0.587	0.048	0.005	0.013	0.004	0.526	0.056
		2M1c	0.549	0.375	0.201	0.006	0.599	0.046	0.007	0.016	0.005	0.614	0.066
		2Md	0.528	0.403	0.193	0.006	0.583	0.052	0.008	0.016	0.005	0.604	0.061
		2Me	0.515	0.521	0.159	0.004	0.604	0.046	0.005	0.015	0.004	0.522	0.061
		2M1f	0.497	0.512	0.167	0.003	0.647	0.050	0.004	0.017	0.006	0.505	0.058
Mixed													
	2	**2V2a**	0.443	0.311	0.133	0.004	0.257	0.035	0.003	0.004	0.002	0.435	0.065
		2V2b	0.533	0.387	0.144	0.004	0.280	0.033	0.004	0.005	0.003	0.528	0.081
Moraiolo													
	3	**2Ma**	0.52	0.278	0.136	0.004	0.367	0.027	0.003	0.012	0.003	0.537	0.064
		2Mb	0.517	0.188	0.122	0.004	0.353	0.027	0.003	0.011	0.002	0.523	0.067
		2M3c	0.489	0.176	0.098	0.003	0.273	0.024	0.003	0.009	0.002	0.528	0.062
Frantoio													
	4	**2F4a**	0.34	0.65	0.183	0.004	0.547	0.037	0.003	0.012	0.006	0.372	0.048
		2F4b	0.329	0.586	0.177	0.004	0.546	0.029	0.004	0.013	0.006	0.368	0.048
		2F4c	0.341	0.339	0.128	0.003	0.449	0.025	0.002	0.009	0.005	0.351	0.044

D. Class of ketones and phenols

	HD	Batch code	2-Butanone (mg/Kg)	2-Octanone (mg/Kg)	Ethyl-vinil-ketone (mg/kg)	6-methyl-5-Hepten-2-one (mg/Kg)	Phenol (mg/Kg)	4-Ethyl-phenol (mg/Kg)
Moraiolo								
	1	**2Ma**	0.017	0.020	0.394	0.015	0.984	0.098
		2Mb	0.001	n.d.	0.459	0.009	0.361	0.069
		2M1c	0.001	n.d.	0.603	0.010	0.353	0.063
		2Md	0.001	n.d.	0.547	0.004	0.350	0.057
		2Me	0.001	n.d.	0.481	0.005	0.333	0.062
		2M1f	0.001	n.d.	0.463	0.007	0.321	0.049
Mixed								
	2	**2V2a**	0.009	n.d.	0.679	0.006	0.266	0.033
		2V2b	0.002	n.d.	0.791	0.004	0.274	0.025
Moraiolo								
	3	**2M3a**	0.001	0.005	0.725	0.010	0.260	0.031
		2M3b	0.001	0.005	0.76	0.009	0.252	0.027
		2M3c	0.001	0.006	0.642	0.009	0.265	0.036
Frantoio								
	4	**2F4a**	0.001	0.003	0.513	0.009	0.203	0.014
		2F4b	0.001	0.002	0.477	0.007	0.197	0.015
		2F4c	0.001	0.011	0.479	0.007	0.192	0.013

Table 14: Concentration of volatile compounds in olive oils obtained during 2013 crop season. Samples are encoded in relation to olive cultivar (Mixed), harvesting date (1, 2 and 3), batch (a, b,…). *A*, *B, C* and the chemical classes of the compounds analyzed. HD: harvesting date; n.d. not determined. Underlined volatile compounds are intermediate of LOX pathway and they are considered to be responsible for olive oil positive attribute.

A. Class of esters, acids and hydrocarbons

	HD	Batch code	Methyl acetate (mg/Kg)	Ethyl acetate (mg/Kg)	Hexyl acetate (mg/Kg)	Cis-3-hexenyl acetate (mg/Kg)	Octane (mg/Kg)	Ocimene (mg/Kg)
Mixed								
	1	**3Va**	0.099	0.0205	0.084	0.425	0.0375	0.129
		3Vb	0.066	0.0145	0.094	0.6075	0.0295	0.127
Mixed								
	2	**3Vc**	0.061	0.0395	0.1185	0.539	0.0225	0.2285
		3Vd	0.047	0.0215	0.0945	0.432	0.0225	0.1205
Mixed								
	3	**3Ve**	0.0265	0.0105	0.1275	0.3985	0.0335	0.1465

B. Class of aldehydes

	HD	Batch code	Valeraldehyde (mg/Kg)	Isovaleraldehyde (mg/Kg)	Hexanal (mg/Kg)	2-Methyl-Butanal (mg/Kg)	Trans-2-Pentenal (mg/Kg)	Cis-3-Hexenal (mg/Kg)	Trans-2-Hexenal (mg/Kg)	2.4 Hexadienal (mg/Kg)	Benzaldehyde (mg/Kg)
Mixed											
	1	**3Va**	0.1015	0.019	0.568	0.0465	0.117	2.9845	15.2585	1.517	0.0805
		3Vb	0.103	0.0245	0.4065	0.058	0.1145	2.432	12.8335	1.336	0.071
Mixed											
	2	**3Vc**	0.0365	0.017	0.8485	0.055	0.086	2.8405	15.65	1.54	0.06
		3Vd	0.028	0.022	0.3935	0.0555	0.0885	2.4055	13.403	1.3815	0.062
Mixed											
	3	**3Ve**	0.046	0.009	0.752	0.027	0.043	2.566	12.974	1.5305	0.066

C. Class of alcohols, ketones and phenols

HD	Batch code	1-Penten-3-ol (m/Kg)	Benzene-Ethanol (mg/Kg)	Pentanol (mg/Kg)	Hexanol (mg/Kg)	Octanol (mg/Kg)	Cis-3-Hexenol (mg/Kg)	Cis-2-Pentenol (mg/Kg)	Trans-2-Hexenol (mg/Kg)	Trans-2-Pentenol (mg/Kg)	2-Butanone (mg/Kg)	3-Pentanone (mg/Kg)	Ethyl-Vinyl-Ketone (mg/Kg)	Phenol (mg/Kg)
Mixed														
1	**3Va**	0.9285	0.4305	0.0075	0.3275	0.088	0.664	0.6785	0.3855	0.105	0.0045	0.3655	1.022	0.3715
	3Vb	0.8375	0.3445	0.006	0.34	0.0725	0.7125	0.6435	0.3265	0.094	0.003	0.2695	0.974	0.3575
Mixed														
2	**3Vc**	0.447	0.2805	0.007	0.7485	0.07	0.5705	0.396	0.8245	0.0515	n.d.	0.2345	0.6055	0.3925
	3Vd	0.623	0.327	0.0055	0.395	0.0665	0.5835	0.618	0.3965	0.0815	n.d.	0.207	0.7325	0.3975
Mixed														
3	**3Ve**	0.215	0.38	0.0075	0.6185	0.084	0.7475	0.2555	0.3695	0.0465	n.d.	0.109	0.2745	0.4455

Table 15: Main volatile compounds determined in standard fusty, mustiness-humidity, winery-vinegary and rancid virgin olive oil (Morales et al., 2005). The main compound contributors to the defect are in yellow, the middle are in blue, the lower are in green.

SENSORY DEFECT	VOLATILE COMPOUND	Odour threshold in oil (mg/Kg)	SENSORY NOTE
FUSTY	Ethyl butanoate	0.03	Sweet, fruity
	Ethyl propanoate	0.10	Fruit, strong
	Butyl acetate	0.30	Green, fruity, pungent
	Propyl butanoate	0.15	Pineapple, sharp
	2-Methylpropyl	0.10	Unpleasant, winery,
	Propanoic acid	0.72	Pungent, sour
	Butanoic acid	0.65	Fusty, strong, cheese
	Octane	0.94	Sweety, alkane
	Butan-2-ol	0.10	Winery
	Acetic acid	0.50	Sour, vinegary
	Pentanoic acid	0.60	Putrid, pungent
	Heptanoic acid	0.10	Rancid, fatty
MUSTINESS-HUMIDITY	1-Octen-3-ol	0.001	Mould, earthy
	1-Octen-3-one	0.01	Mushroom, mould,
	trans-2-Heptenal	0.001	Pungent, soapy
	3-Methyl-butan-1-ol	0.10	Woody, sweet
	5-Methyl-5-hepten-2-	1.00	Herbaceous, pungent
	Heptan-2-ol	0.01	Earthy, sweety
	Guaiacol	0.02	Woody, smoky, spicy
	Octan-2-ol	0.10	Earthy, fatty
WINERY-VINEGARY	Ethyl acetate	0.94	Sticky, sweet
	3-Methyl butan-1-ol	0.10	Woody, whiskey, sweet
	Acetic acid	0.50	Sour, vinegary
	Octane	0.94	Sweety, alcane
	2-Methyl butan-1-ol	0.48	Winey, spicy
	Octan-2-one	0.51	Mould, green
	Propanoic acid	0.72	Pungent, sour
	Butanoic acid	0.65	Rancid, cheese
	Pentanoic acid	0.60	Unpleasant, pungent
	Hexanoic acid	0.70	Pungent, rancid
	Heptanoic acid	0.10	Rancid, fatty
RANCID	trans-2-Heptenal	0.005	Oxidised, tallow,
	trans-2-Octenal	0.004	Herbaceous, spicy
	trans-2-Decenal	0.01	Painty, fishy, fatty
	Hexanoic acid	0.70	Rancid, pungent
	Butanoic acid	0.14	Rancid
	Acetic acid	0.50	Pungent, sour
	Nonanal	0.15	Fatty, waxy, pungent
	Heptanal	0.50	Oily, fatty, woody
	Pentanal	0.24	Woody, bitter, oily
	Octanal	0.32	Fatty, sharp
	6-Methyl-5-epten-2-	1.00	Oily, pungent
	Nonanol	0.28	Fatty
	Heptanoic acid	0.10	Rancid
	Octane	0.94	Sweet, alcane

3.3.3.2 Polyphenolic compounds analysis

The contents of biophenolic compounds of oil samples collected during the three years of study are reported in Tables 17-19.

The distribution of total phenolic compounds in all the oil samples was (Tab. 17-19):

1. 60-70% oleuropein and its derivatives,
2. 20-25% ligstroside aglycone and its derivatives
3. 10% lignans

These results confirmed that the presence of oleuropein and its derivatives was predominant compared to the other classes of phenolic compounds (Migliorini et al., 2008; Oliveras Lopez et al., 2008).

No significant differences were found in the percentage composition of the different classes of phenolic compounds in oils from different cultivar.

The total phenolic compound content was about 600 mg/kg in the oils of 2011 and 2012 (Tab 16 and 17), a concentration that was respectively 50% and 40% higher than the mean value of the Tuscan productions of those years (Migliorini et al., 2013). The phenolic contents in the oils of 2013 was about 500 mg/kg (Tab. 18) a concentration that was 40% higher than the mean value of the selection of PDO and PGI extra virgin olive oil of Tuscany of those years. The dialdehydic form of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA) was the most abundant phenolic compound in 2011 and 2013 oils (about 150 and 142 mg/kg respectively) (Tab. 16 and 18), while in 2012 oils the most abundant phenolic compound was oleuropein (about 130 mg/kg), as showed in Table 17.

Polyphenolic compounds of EVOO, in particular secoiridoids derivatives and lignans, have antioxidant activities and healthy properties (Servili et al., 2004; Servili et al.,2009; Obied et al., 2012; Cicerale et al., 2010; Carrera-Gonzales et al., 2013). Moreover, phenolic fraction has a strong impact on bitterness, astringency and pungency; in particular the dialdehydic form of decarboximethyl ligstroside aglycone (*p*-HPEA-EDA) is responsible for the strong "pungent" attribute, while oleuropeina aglycone (3,4-DHPEA-EA) and ligstroside aglycone (p-HPEA-EA) represent the impact

components for the “bitter” note (Servili et al., 2014). Also the dialdehydic form of decarboximethyl oleuropein aglycone (3,4-DHPEA-EDA) contributes to the sensation of bitter but with a marginal role for the “pungent” note (Andrewes et al., 2003).

Table 16: Concentration of biophenols compounds in olive oils obtained during in 2011 crop season. Samples are encoded in relation to olive cultivar (*Moraiolo* or *Frantoio*), harvesting date (1 or 2), and batch (a, b, c or d). HD: harvesting date.

	HD	Batch code	Hydroxytyrosol (mg_{Tyr}/Kg_{oil})	Tyrosol (mg_{Tyr}/Kg_{oil})	Vanillic Acid+ Caffeic Acid (mg_{Tyr}/Kg_{oil})	Vanillin (mg_{Tyr}/Kg_{oil})	*p*-Coumaric Acid (mg_{Tyr}/Kg_{oil})	Hydroxytyrosil Acetate (mg_{Tyr}/Kg_{oil})	Ferulic Acid (mg_{Tyr}/Kg_{oil})	*o*-Coumaric Acid (mg_{Tyr}/Kg_{oil})	Decarboxymethyloleuropein Aglycone Dialdehydic Oxidized Form (mg_{Tyr}/Kg_{oil})	Decarboxymethyloleuropein Aglycone Dialdehydic Form (mg_{Tyr}/Kg_{oil})	Oleuropein (mg_{Tyr}/Kg_{oil})	Oleuropein aglycone dialdehydic form (mg_{Tyr}/Kg_{oil})
Frantoio	**1**	**F1a**	0	1	0	3	0	1	1	3	16	89	41	32
		F1b	0	2	1	3	1	1	2	2	28	220	87	59
		F1c	0	2	1	2	1	1	1	2	27	166	75	52
		F1d	1	2	1	3	2	2	1	2	31	192	100	63
	2	**F2a**	0	1	0	2	0	1	1	2	23	122	61	39
		F2b	1	1	1	2	1	1	1	2	29	122	86	58
		F2c	1	2	1	2	2	0	1	3	44	134	137	88
		F2d	1	2	1	2	2	0	1	3	41	135	132	80
Moraiolo	**1**	**M1a**	1	1	1	2	1	0	1	2	33	130	136	79
		M1b	1	1	1	2	2	1	1	3	39	144	182	93
		M1c	1	1	1	2	2	0	1	3	44	145	156	93
		M1d	1	2	1	3	1	0	1	3	35	142	128	75
	2	**M2a**	1	1	1	2	2	7	1	2	44	134	171	64
		M2b	1	1	1	3	2	4	1	2	36	96	155	59
		M2c	1	1	1	2	2	2	0	3	39	105	191	107
		M2d	1	2	1	2	2	9	1	1	31	100	122	46

Cultivar	HD	Batch code	Decarboxymkethylligstroside aglycone dialdehydic oxidized form (mg_{Tyr}/Kg_{oil})	Decarboxymethylligstroside aglycone dialdehydic form (mg_{Tyr}/Kg_{oil})	Pinoresinol, 1-acetoxypinoresinol (mg_{Tyr}/Kg_{oil})	Cinnamic Acid (mg_{Tyr}/Kg_{oil})	Ligstroside aglycone dialdehydic form (mg_{Tyr}/Kg_{oil})	Oleuropein aglycone, aldehydic and hydroxyl oxydized form (mg_{Tyr}/Kg_{oil})	Luteolin (mg_{Tyr}/Kg_{oil})	Oleuropein aglycone, aldehydic and hydroxyl form (mg_{Tyr}/Kg_{oil})	Ligstroside aglycone, aldehydic and hydroxyl oxydized form (mg_{Tyr}/Kg_{oil})	Apigenin (mg_{Tyr}/Kg_{oil})	Methyl-luteolin (mg_{Tyr}/Kg_{oil})	Ligstroside aglycone, aldehydic and hydroxyl form (mg_{Tyr}/Kg_{oil})	TOTAL BIOPHENOL (mg_{Tyr}/Kg_{oil})
Frantoio	**1**	**F1a**	5	72	15	7	49	11	15	37	16	9	2	8	**435**
		F1b	7	85	16	10	63	19	29	32	20	12	6	3	**708**
		F1c	7	79	16	8	71	17	33	30	16	13	5	4	**630**
		F1d	6	79	18	10	70	20	36	27	19	13	3	6	**705**
	2	**F2a**	8	107	19	5	32	16	10	46	19	5	11	6	**538**
		F2b	7	85	18	6	46	18	13	46	19	7	10	6	**586**
		F2c	7	52	20	9	56	23	16	40	21	7	7	4	**674**
		F2d	8	46	19	9	58	23	16	46	21	8	8	4	**669**
Moraiolo	**1**	**M1a**	8	52	21	9	56	21	21	34	19	10	4	4	**646**
		M1b	7	41	17	6	51	24	25	34	21	9	5	3	**712**
		M1c	6	43	18	10	45	22	23	30	18	10	3	4	**681**
		M1d	7	61	20	9	57	18	21	27	19	9	6	2	**648**
	2	**M2a**	6	35	17	8	56	23	19	40	24	8	3	4	**672**
		M2b	5	31	17	8	51	20	19	40	20	8	3	4	**588**
		M2c	5	23	17	9	43	25	19	38	22	7	6	2	**670**
		M2d	5	36	16	8	54	22	13	38	30	6	3	6	**555**

Table 17: Concentration of biophenols compounds in olive oils obtained during in 2012 crop season. Samples are encoded in relation to olive cultivar (*Moraiolo*, *Frantoio* or mixed), harvesting date (1, 2, 3 or 4), and batch (a, b,…). HD: harvesting date.

	HD	Batch code	Hydroxytyrosol (mg_{Tyr}/Kg_{oil})	Tyrosol (mg_{Tyr}/Kg_{oil})	Vanillic Acid+ Caffeic Acid (mg_{Tyr}/Kg_{oil})	Vanillin (mg_{Tyr}/Kg_{oil})	*p*-Coumaric Acid (mg_{Tyr}/Kg_{oil})	Hydroxytyrosil Acetate (mg_{Tyr}/Kg_{oil})	Ferulic Acid (mg_{Tyr}/Kg_{oil})	*o*-Coumaric Acid (mg_{Tyr}/Kg_{oil})	Decarboxymethyloleuropein Aglycone Dialdehydic Oxidized Form (mg_{Tyr}/Kg_{oil})	Decarboxymethyloleuropein Aglycone Dialdehydic Form (mg_{Tyr}/Kg_{oil})	Oleuropein (mg_{Tyr}/Kg_{oil})	Oleuropein aglycone dialdehydic form (mg_{Tyr}/Kg_{oil})
Moraiolo														
	1	**2M1a**	3	2	0	3	1	1	0	2	20	94	86	27
		2M1b	2	2	1	3	1	1	0	3	33	85	138	37
		2M1c	2	2	2	3	1	1	1	3	41	111	175	46
		2M1d	2	2	2	3	1	1	0	3	35	101	146	43
		2M1e	1	2	1	2	0	4	0	2	28	62	144	45
		2M1f	2	2	1	2	1	1	0	3	40	105	177	57
Mixed														
	2	**2V2a**	1	2	1	3	1	3	0	2	36	186	105	35
		2V2b	2	2	1	3	1	3	0	2	29	187	96	33
Moraiolo														
	3	**2M3a**	2	2	1	2	1	0	1	2	36	109	135	50
		2M3b	2	2	1	2	1	0	0	3	42	92	150	55
		2M3c	1	2	1	1	1	0	0	3	59	97	178	63
Frantoio														
	4	**2F5a**	1	2	1	2	0	3	0	1	30	98	87	31
		2F5b	1	2	0	2	0	3	1	2	30	100	99	33
		2F5c	2	2	0	1	0	2	0	1	38	100	121	29

	HD	Batch code	Decarboxymmethyl ligstroside aglycone dialdehydic oxidized form (mgTyr/Kgoil)	Decarboxymethylligstroside aglycone dialdehydic form (mgTyr/Kgoil)	Pinoresinol, 1-acetoxypinoresinol (mgTyr/Kgoil)	Cinnamic Acid (mgTyr/Kgoil)	Ligstroside aglycone dialdehydic form (mgTyr/Kgoil)	Oleuropein aglycone, aldehydic and hydroxyl oxydized form (mgTyr/Kgoil)	Luteolin (mgTyr/Kgoil)	Oleuropein aglycone, aldehydic and hydroxyl form (mgTyr/Kgoil)	Ligstroside aglycone, aldehydic and hydroxyl oxydized form (mgTyr/Kgoil)	Apigenin (mgTyr/Kgoil)	Methyl-luteolin (mgTyr/Kgoil)	Ligstroside aglycone, aldehydic and hydroxyl form (mgTyr/Kgoil)	**TOTAL BIOPHENOL (mgTyr/Kgoil)**
Moraiolo															
	1	**2M1a**	7	45	28	15	47	17	14	51	22	9	6	9	**507**
		2M1b	7	33	31	14	53	23	17	99	25	10	8	13	**637**
		2M1c	8	38	32	17	54	29	18	103	31	12	6	13	**747**
		2M1d	7	38	32	15	57	19	17	61	28	12	5	10	**639**
		2M1e	6	27	28	12	78	18	14	55	29	10	5	8	**580**
		2M1f	7	34	30	19	61	19	17	50	29	11	5	7	**681**
Mixed															
	2	**2V2a**	8	132	27	13	58	17	24	51	34	12	9	7	**767**
		2V2b	9	120	24	13	55	17	21	52	31	11	7	8	**728**
Moraiolo															
	3	**2M3a**	6	29	21	11	41	28	21	94	28	11	6	9	**647**
		2M3b	6	24	21	12	42	20	17	38	29	11	4	7	**581**
		2M3c	7	30	25	16	44	21	21	54	30	11	4	8	**678**
Frantoio															
	4	**2F5a**	6	52	16	12	48	14	18	70	21	14	7	17	**554**
		2F5b	6	52	18	12	48	18	18	59	20	16	7	16	**561**
		2F5c	9	57	20	12	46	18	24	75	27	13	8	16	**621**

Table 18: Concentration of biophenols compounds in olive oils obtained during in 2013 crop season. Samples are encoded in relation to olive cultivar (Mixed), harvesting date (1, 2 or 3), and batch (a, b, c, d or e). HD: harvesting date.

	HD	Batch code	Hydroxytyrosol (mg_{Tyr}/Kg_{oil})	Tyrosol (mg_{Tyr}/Kg_{oil})	Vanillic Acid+ Caffeic Acid (mg_{Tyr}/Kg_{oil})	Vanillin (mg_{Tyr}/Kg_{oil})	*p*-Coumaric Acid (mg_{Tyr}/Kg_{oil})	Hydroxytyrosil Acetate (mg_{Tyr}/Kg_{oil})	Ferulic Acid (mg_{Tyr}/Kg_{oil})	*o*-Coumaric Acid (mg_{Tyr}/Kg_{oil})	Decarboxymethyloleuropein Aglycone Dialdehydic Oxidized Form (mg_{Tyr}/Kg_{oil})	Decarboxymethyloleuropein Aglycone Dialdehydic Form (mg_{Tyr}/Kg_{oil})	Oleuropein (mg_{Tyr}/Kg_{oil})	Oleuropein aglycone dialdehydic form (mg_{Tyr}/Kg_{oil})
Mixed														
	1	**3Va**	1	2	2	5	2	3	1	0	29	157	90	20
		3Vb	1	2	2	5	1	2	0	1	28	170	104	17
Mixed														
	2	**3Vc**	1	1	1	4	1	2	0	1	13	126	35	15
		3Vd	1	2	3	5	1	2	1	1	28	189	85	35
Mixed														
	3	**3Ve**	2	2	4	4	0	1	1	1	12	70	45	17

	HD	Batch code	Decarboxymkethylligstroside aglycone dialdehydic oxidized form (mg$_{Tyr}$/Kg$_{oil}$)	Decarboxymethylligstroside aglycone dialdehydic form (mg$_{Tyr}$/Kg$_{oil}$)	Pinoresinol, 1-acetoxypinoresinol (mg$_{Tyr}$/Kg$_{oil}$)	Cinnamic Acid (mg$_{Tyr}$/Kg$_{oil}$)	Ligstroside aglycone dialdehydic form (mg$_{Tyr}$/Kg$_{oil}$)	Oleuropein aglycone, aldehydic and hydroxyl oxydized form (mg$_{Tyr}$/Kg$_{oil}$)	Luteolin (mg$_{Tyr}$/Kg$_{oil}$)	Oleuropein aglycone, aldehydic and hydroxyl form (mg$_{Tyr}$/Kg$_{oil}$)	Ligstroside aglycone, aldehydic and hydroxyl oxydized form (mg$_{Tyr}$/Kg$_{oil}$)	Apigenin (mg$_{Tyr}$/Kg$_{oil}$)	Methyl-luteolin (mg$_{Tyr}$/Kg$_{oil}$)	Ligstroside aglycone, aldehydic and hydroxyl form (mg$_{Tyr}$/Kg$_{oil}$)	TOTAL BIOPHENOL (mg$_{Tyr}$/Kg$_{oil}$)
Mixed															
	1	**3Va**	4	45	17	7	63	18	9	64	17	11	6	13	**587**
		3Vb	4	66	17	9	70	19	12	80	15	12	8	18	**662**
Mixed															
	2	**3Vc**	4	47	11	4	41	11	15	57	12	11	6	10	**428**
		3Vd	6	58	16	10	51	21	12	48	16	10	5	9	**612**
Mixed															
	3	**3Ve**	4	24	10	7	40	8	18	33	15	14	4	6	**342**

3.3.3.3 Correlation studies.

Some statistically significant correlations were found between yeast cell densities at the different steps of the extraction process and some aromatic and polyphenolic compounds of olive oil in the three years of study. The significant correlations between yeast concentrations and volatile compound content of the final olive oil samples are reported in Table 19. In particular, the two chosen correlation coefficients (i.e. Pearson and Spearman) agreed on indicating significant positive and negative correlations between yeast counts in crushed pastes, oil from decanter and pomaces, and some volatile compounds; among the latter, the highest significance correlations were related to methyl acetate, ethyl acetate, trans-2-hexenyl acetate, cis-3-hexenyl acetate, butyric acid, cis-3-hexenal, cis-3-hexenol contents (Tab. 19). 24, 13 and 12 volatile compounds were significantly correlated with the yeast concentrations respectively of one, two and all steps of the extraction process (Tab 19).

The highest significance correlations between yeast counts in crushed pastes and some volatile compounds were related to acid aromatic compounds, ocimene, benzene-ethanol and 6-methyl-5-hepten-2-one contents. The highest significance correlations between yeast counts in oil from decanter and some volatile compounds were related to octane, trans-2-hexenal, 2,4 hexadienal, 2-pentanol and iso-butanol contents. Finally, the highest significance correlations between yeast counts in pomaces and some volatile compounds were related to isovaleraldheyde, cis-2-hexenol, trans-3-hexenol, 4-ethyl-phenol and 6-methyl-5-hepten-2-one contents (Tab. 19). Since most of these compounds in literature are related to sensory defects in olive oil, as described in the previous paragraph (Tab. 15), yeast contamination may have been responsible for those sensory defects. In this contest, a recent study demonstrated the capability of some oil born strains of *Candida spp*. to induce defects such as "musty" and/or "rancid" in oil (Zullo et al., 2013). However, further studies are needed to confirm these findings.

Table 19: Correlation between aromatic compounds and yeast concentrations of the 3 years of study at the different steps of the extraction process; CP: crushed pastes, OfD: oil from decanter, P: pomaces. Underlined volatile compounds are intermediate of LOX pathway and they are considered (DI GIACINTO *et al*., 2010; KOTTI *et al.*, 2011; APARICIO *et al*., 2012) to be responsible for olive oil "fruity", "grassy" and other positive attribute. Underlined numbers indicate statistically significant correlations.

	CP		**OfD**		**P**	
	Spearman r	**Pearson r**	**Spearman r**	**Pearson r**	**Spearman r**	**Pearson r**
Esters, acids and hydrocarbons						
Methyl acetate	0,4594	0,3699	0,3457	0,08771	0,7382	0,4937
Ethyl acetate	0,5569	0,4989	0,4885	0,4744	0,4792	0,6826
Butil acetate	0,4233	0,3720	0,2048	0,1946	0,01220	0,2253
Hexyl acetate	0,1937	0,1458	-0,06740	-0,1671	-0,4947	-0,4089
trans-2-hexenyl acetate	-0,3903	-0,4087	-0,5392	-0,6349	-0,5926	-0,5566
cis-3-hexenyl acetate	-0,3407	-0,3707	-0,3445	-0,3961	-0,6569	-0,5471
Butyric acid	-0,6255	-0,6158	-0,1634	-0,5810	-0,4839	-0,7761
Propionic acid	0,4507	0,4155	0,2994	0,3057	-0,1070	0,2068
Pentanoic acid	0,4703	0,4164	0,3024	0,2919	-0,01141	0,2242
Hexanoic acid	0,4451	0,4170	0,2233	0,2584	-0,02387	0,2296
Heptanoic acid	0,4297	0,4096	0,2372	0,2734	-0,1071	0,2187
Octanoic acid	0,4448	0,3639	0,3392	0,3541	-0,02739	0,2059
Octane	0,4872	0,5075	0,4676	0,4621	0,3769	0,4554
Ocimene	0,5653	0,5971	0,02983	-0,0519	0,3881	0,2860
Limonene	0,4531	0,4282	0,3010	0,2041	0,06008	0,2398
Copaene	0,3882	0,3785	0,2028	0,2248	0,08263	0,2594
Aldheyds						
Benzaldheyde	-0,2970	-0,2453	-0,3104	-0,4267	0,04888	-0,2988
Isovaleraldheyde	0,5396	0,4785	0,4248	0,3796	0,4873	0,5259
Hexanal	0,09862	0,1132	-0,3877	-0,4189	-0,2511	-0,4106
trans-2-hexenal	-0,2106	-0,0267	-0,5143	-0,4542	0,1312	-0,0997
Heptanal	0,4026	0,4059	0,2031	0,2455	-0,04520	0,2229
2-methyl-butanal	0,3977	0,3827	0,4328	0,3132	0,6091	0,5037
2.4 hexadienal	-0,3369	-0,3453	-0,4576	-0,5184	-0,03970	-0,2849
trans-2-heptenal	0,4092	0,3707	0,2599	0,2483	0,08715	0,2701
trans-2-pentenal	-0,3760	-0,3833	-0,2241	-0,2292	0,009615	-0,0738
trans-2-octanal	-0,3670	-0,4085	-0,2732	-0,2708	-0,1985	-0,5002
cis-3-hexenal	-0,4601	-0,5940	-0,5862	-0,6425	-0,3632	-0,5807

	CP		OfD		P	
	Spearma	**Pearso**	**Spearma**	**Pearson**	**Spearma**	**Pearso**
Alcohols, ketons,						
Phenol	-0,2511	-0,2331	-0,4742	-0,3166	0,05934	-0,3507
2-pentanol	0,1993	0,1328	0,4640	0,4411	0,2105	0,2268
2-octanol	-0,2309	-0,2679	-0,1529	-	-0,2551	-0,3755
2-heptanol	0,3418	0,2537	0,4403	0,3965	0,01716	0,1396
Octanol	0,4371	0,4253	0,07323	0,02378	0,2678	0,1487
Guaiacol	0,4583	0,4250	0,2537	0,2169	0,004142	0,2298
1-octen-3-ol	-0,2506	-0,2530	0,09489	0,05640	-0,4216	-0,4057
cis-2-hexenol	-0,3754	-0,4342	-0,1711	-0,1931	-0,6183	-0,6701
cis-2-pentenol	-0,5344	-0,5033	-0,3575	-0,3792	-0,2196	-0,2675
Iso-butanol	0,3856	0,3543	0,5050	0,4582	0,02667	0,2528
Ethyl-vinil-ketone	-0,04522	-0,1060	0,3590	0,3094	0,2039	0,3082
2-butanone	0,09238	-0,1611	0,4325	0,3209	0,01960	-
trans-2-pentenol	-0,4362	-0,2631	-0,07552	0,00304	-	0,1642
trans-3-hexenol	-0,4931	-0,5790	-0,3186	-0,4240	-0,7568	-0,7500
cis-3-hexenol	-0,4111	-0,5163	-0,4157	-0,5256	-0,5260	-0,6388
2-e-3-methyl-1-butanol	0,3207	0,3434	0,4202	0,3936	-0,03710	0,2146
Nonanol	0,000635	0,0414	-0,04995	0,00016	-0,3494	-
Hexanol	-0,06935	-	-0,2200	-0,3655	-0,2693	-0,4282
Benzene-ethanol	0,4735	0,5643	-0,05002	0,01894	0,3638	0,3327
4-ethyl-phenol	-0,5779	-0,4683	-0,3113	-0,3935	-0,4185	-0,5342
3-pentanone	0,3166	0,3092	-0,04351	0,00864	0,5736	0,4000
6_methyl_5_hepten_2	-0,6067	-0,5889	-0,3295	-0,2957	-0,4591	-0,5497
1-octen-3-one	0,4204	0,3251	0,2976	0,3339	-0,02076	0,1956
2_heptanone	-0,2309	-0,2679	-0,1529	-	-0,2551	-0,3755

The significant correlations between yeast concentrations and polyphenolic compound content of olive oil samples in the three years of study are reported in Table 20. In particular, correlation coefficients (i.e. Pearson and Spearman) agreed on indicating significant correlations between yeast counts in crushed pastes, oil from decanter and pomaces, and some polyphenolic compounds. In particular, the highest significance correlations were related to pinoresinol, 1 acetoxypinoresinol and cinnamic acid contents (Tab. 20). 10, 5 and 2 polyphenolic compounds were significantly correlated with the yeast concentrations respectively of one, two and all steps of the extraction process (Tab 20). The highest significance correlations between yeast counts in crushed pastes and some polyphenolic compounds were related to oleuropein aglycone, aldehydic and hydroxyl form and ligstroside aglycone, aldehydic and hydroxyl oxydized form contents (Tab. 20). On the contrary, the highest significance correlations between yeast counts in pomaces and some volatile compounds were related to hydroxytyrosol, oleuropein and ligstroside aglycone, aldehydic and hydroxyl oxydized form contents (Tab. 20). Finally, Pinoresinol, 1 acetoxypinoresinol and cinnamic acid were negatively significantly correlated with all steps of the extraction process (Tab. 20). Pinoresinol and 1 acetoxypinoresinol are lignans present in the olive pulp and in the woody portion of the seed; they are relased in oil during the extraction process without biochemical modification (Servili et al., 2009). Cinnamic acid has shown to be effective against growth of *S. enterica* and *E. coli* in apple cider and orange juice (Truong et al., 2010; Zheng et al., 2013), with a minimum inhibitory concentration between 200 mg/L, for *Z. bailii,* and 500 mg/L for *T.delbrueckii* and *C. krusei* (Audra Ann Wallis, 2013). Some yeast strains could have enzymatic capabilities to modify the polyphenolic compounds of the olive oils. Indeed, yeasts with ß-glucosidase activity could have an important role in the degradation of the main phenol compound in olives, oleuropeine, into simpler and no longer bitter compounds characterized by a high antioxidant activity (Ciafardini et al., 1994; Ciafardini and Zullo, 2002b). However, further studies are needed to confirm these findings.

Table 20: Correlation between polyphenolic compounds and yeast concentration of the 3 years of study at the different steps of the extraction process; CP: crushed pastes, OfD: oil from decanter, P: pomaces. Underlined numbers indicate statistically significant correlations.

	CP		OfD		P	
	Spearman r	**Pearson r**	**Spearman r**	**Pearson r**	**Spearman r**	**Pearson r**
Hydroxytyrosol	-0,3861	-0,4109	-0,3110	-0,3285	-0,4227	-0,5916
Tyrosol	-0,4794	-0,4238	-0,2148	-0,2468	-0,09408	-0,2237
Vanillin	0,1069	0,2178	-0,2484	-0,1727	0,4514	0,2935
p-Coumaric acid	0,1538	0,07617	0,3530	0,3198	-0,1440	0,09725
Ferulic acid	0,4489	0,4544	0,2629	0,2632	0,2098	0,3218
o-coumaric acid	-0,2385	-0,2800	-0,2344	-0,2448	-0,4867	-0,3513
Decarboxymethyloleuropein Aglycone Dialdehydic Oxidized Form	-0,1865	-0,3632	0,1482	0,06604	-0,4465	-0,2213
Decarboxymethyloleuropein Aglycone Dialdehydic Form	0,07919	0,06173	0,2268	0,2022	0,3522	0,4355
Oleuropein	-0,2555	-0,3439	-0,1449	-0,1538	-0,6379	-0,4239
Oleuropein aglycone dialdehydic form	0,08244	0,06221	0,1992	0,2560	-0,3879	-0,03685
Decarboxymmethylligstroside aglycone dialdehydic oxidized form	-0,4260	-0,4654	-0,2599	-0,2057	-0,3516	-0,4197
Decarboxymethylligstroside aglycone dialdehydic form	0,07224	-0,1038	0,1386	0,1630	0,4660	0,3463
Pinoresinol, 1 acetoxypinoresinol	-0,7763	-0,8123	-0,5750	-0,7551	-0,5683	-0,7174
Cinnamic acid	-0,7143	-0,6821	-0,3938	-0,5537	-0,4920	-0,6145
Oleuropein aglycone aldehydic form	-0,04943	-0,3255	0,02698	-0,05457	-0,4919	-0,3366
Oleuropein aglycone, aldehydic and hydroxyl form	-0,4597	-0,7184	-0,1576	-0,3003	0,1595	-0,3010
Ligstroside aglycone, aldehydic and hydroxyl oxydized form	-0,6497	-0,7461	-0,1979	-0,3345	-0,5079	-0,4643

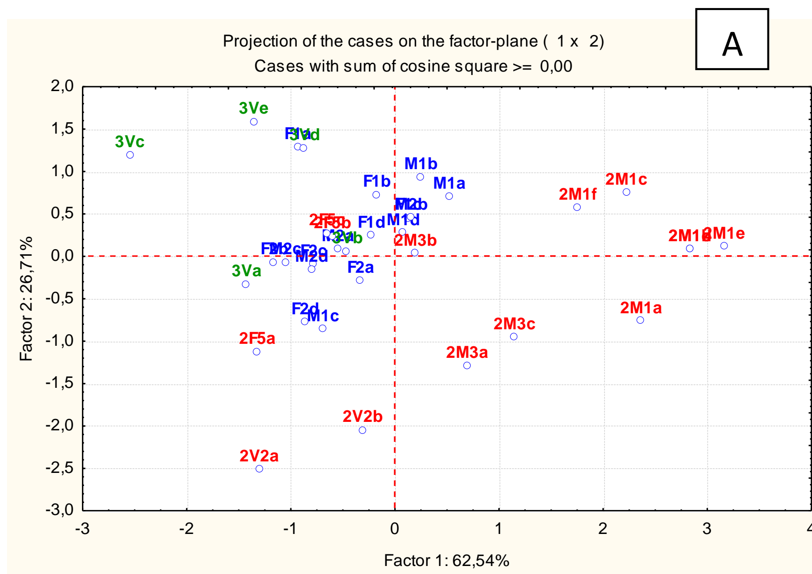
3.3.3.4 Principal Component Analysis

A multidimensional map of all samples related to yeast counts in crushed pastes, oil from decanter and pomaces was obtained by PCA. The relevant sample loading and score plots are reported in Figure 18. The model explained 89% of data variability along the first (Factor 1) and second (Factor 2) principal components. A comparison between the score plot and the loading plot showed that olive oil samples extracted from yeast contaminated processes were all positioned on the left side of the plot. Moreover, even if not so well defined, samples tended to be distributed on the plot depending on the harvesting year: 2013 on the top left side, those of 2011 on the center, while 2012 predominantly on the right side of the plot (Fig. 18). Finally, the samples were not grouped according to the cultivar of olives (Fig. 18).

A multidimensional map of all samples related to volatile compounds was obtained by PCA. The relevant sample loading and score plots are reported in Figure 19. The model explained 55% of data variability along the first (Factor 1) and second (Factor 2) principal components. A comparison between the score plot and the loading plot showed that olive oil samples were grouped depending of the different harvesting year. Indeed, the oils of the first year were all positioned on the left side of the plot, the oils of the second year were positioned on the top right side of the plot, while the oils of the third year were positioned on the bottom right side of the plot (Fig. 19).

A multidimensional map of all samples related to phenolic compounds was obtained by PCA. The relevant sample loading and score plots are reported in Figure 20. The model explained 44 % of data variability along the first (Factor 1) and second (Factor 2) principal components. A comparison between the score plot and the loading plot showed that olive oil samples were grouped depending of the different harvesting year. Indeed, the oils of the first year were all positioned on the top side of the plot; almost all oils of the second year were positioned on the bottom right side of the plot, while the oils of the third year were positioned on the bottom left side of the plot (Fig. 20).

Finally, in order to generalize, the results showed that the elaborations made for the yeast contamination and the content of aromatic and phenolic compounds leaded to group the samples depending on the harvesting year and not according to the cultivar of olives.



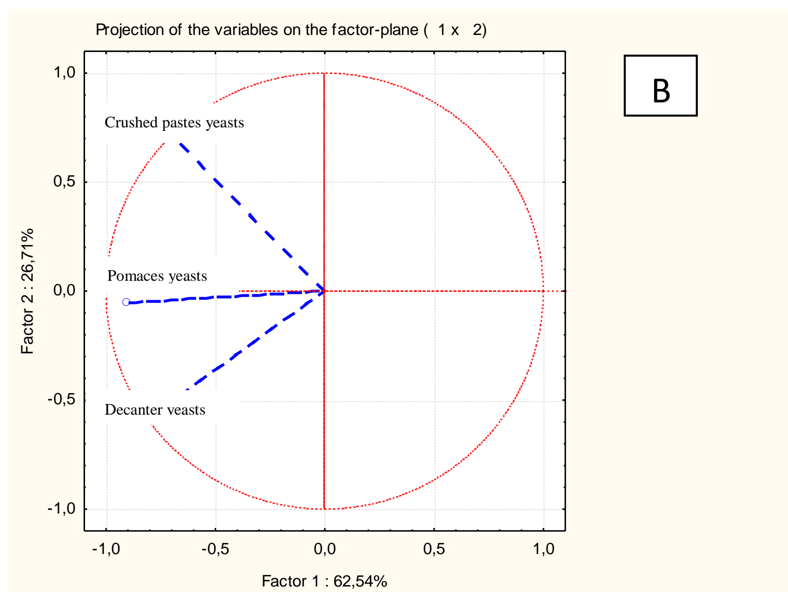
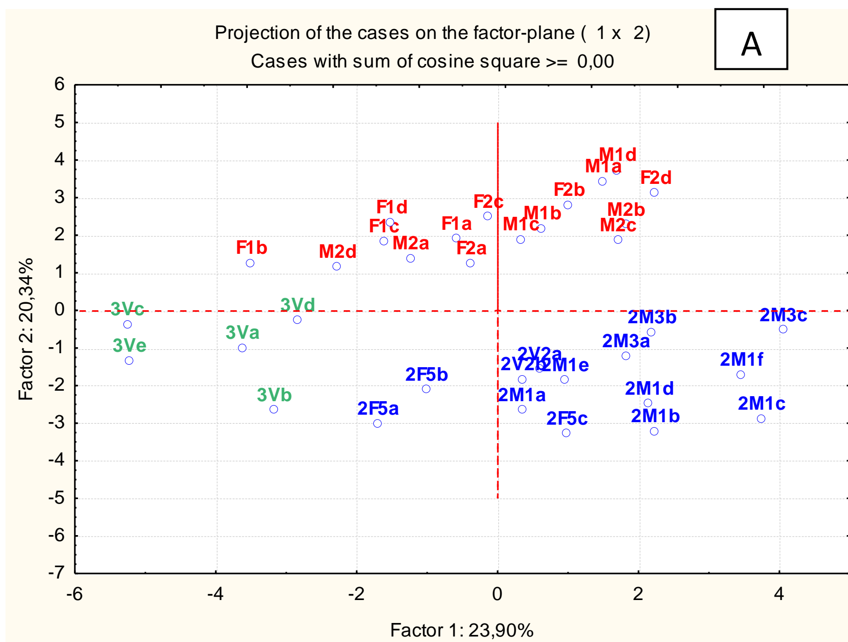


Figure 18: Principal Component Analysis of the various olive batches analyzed considering as variables the yeast cell concentrations during various extraction process steps: crushed pastes, oil from decanter and pomaces. Oils of 2011 are in blue, oils of 2012 are in red, oils of 2013 are in green; samples are encoded in relation to olive cultivar (*Moraiolo*: M, *Frantoio:* F or mixed: V), harvesting date (1, 2, 3,…)and batch (a, b, c,…). A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane.

Figure 19: Principal Component Analysis carried out on volatile compounds content of olive oil samples of the three years of study. Oils of 2011 are in blue, oils of 2012 are in red, oils of 2013 are in green; samples are encoded in relation to olive cultivar (*Moraiolo:* M, *Frantoio*: F or mixed: V), harvesting date (1, 2, 3,…), and batch (a, b, c,…). A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane.



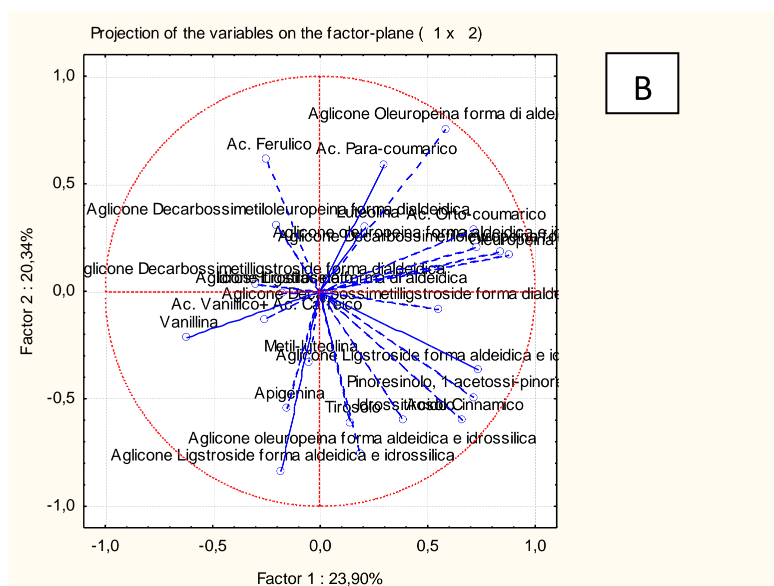


Figure 20: Principal Component Analysis carried out on phenolic compounds content of olive oil samples of the three years of study. Oils of 2011 are in blue, oils of 2012 are in red, oils of 2013 are in green; samples are encoded in relation to olive cultivar (*Moraiolo*: M, *Frantoio*: F or mixed: V), harvesting date (1, 2, 3,…), and batch (a, b, c,…). A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane.

3.3.4 Conclusion

During the extractive process, the environmental conditions promote a selective enrichment of specific yeast species that could affect olive oil quality. Volatile compounds can be correlated with common sensory attributes, both positive and negative (Di Giacinto *et al.*, 2010; Morales *et al.*, 2005). Even the phenolic component may be subject to changes, which will also impact on various sensory properties, first of all the spicy and bitter (Zanoni, 2014). Trans-2-hexenal, considered with other compounds to be responsible for olive oil "fruity", "grassy" and other positive attribute (Aparicio *et al*., 2012) was the most abundant compound present in all three years of study with concentration ranged between 5.12 and 15.65 mg/Kg oil. Concerning the aromatic compounds related to olive oil defects (Morales et al., 2005), all the oils of the 2011 showed a content of trans-2-heptenal, trans-2-decenal and 1-octen-3-ol well above the odour threshold. In 2012 only 1-octen-3-ol was detected above the odor threshold in all oils extracted, while butyric acid and trans-2-decenal were detected above the odor threshold only in three oils from the first harvesting date and in the oils from the third and fourth harvesting date. On the contrary, in the oils of the 2013, compounds associated with defect were not detected or detected below the odor threshold. The results regarding the polyphenolic compounds of oils confirmed that the presence of oleuropein and its derivatives were predominant compared to the other classes of phenolic compounds. The most abundant phenolic compound in 2011 and 2013 oils was 3,4-DHPEA-EDA, while in 2012 oils the most abundant phenolic compound was oleuropein. Correlation studies between yeast concentrations in crushed pastes, oil from decanter and pomaces and some volatile compounds showed that 24, 13 and 12 volatile compounds were significantly correlated with the yeast concentrations respectively of one, two and all steps of the extraction process. The highest significant correlations were related to methyl acetate, ethyl acetate, trans-2-hexenyl acetate, cis-3-hexenyl acetate, butyric acid, cis-3-hexenal and cis-3-hexenol contents. Since most of these compounds in literature are related to sensory defects in olive oil, yeast contamination could be responsible for

those sensory defects. In this contest, a recent study demonstrated the capability of some oil born strains of *Candida spp*. to induce defects such as “musty” and/or “rancid” in oil (Zullo *et al.*, 2013). Moreover, correlation studies between yeast concentrations in crushed pastes, oil from decanter and pomaces and some polyphenolic compounds showed that 10, 5 and 2 polyphenolic compounds were significantly related with the yeast concentrations respectively of one, two and all steps of the extraction process. In particular, the highest significance negative correlations were related to pinoresinol, 1 acetoxypinoresinol and cinnamic acid contents. Pinoresinol and 1 acetoxypinoresinol are lignans present in the olive pulp and in the woody portion of the seed; they are relased in oil during the extraction process without biochemical modification (Servili et al, 2009). Cinnamic acid has shown to be effective against growth of *S. enterica* and *E. coli* in apple cider and orange juice (Truong et al., 2010; Zheng et al., 2013), with a minimum inhibitory concentration between 200 mg/L, for *Z. bailii,* and 500 mg/L for *T.delbrueckii* and *C. krusei* (Audra Ann Wallis, 2013).

Finally, Principal Component Analysis of the various analyzed olive batches was made, considering as variables the yeast cell concentrations during various extraction process steps (crushed pastes, oil from decanter and pomaces), the volatile compounds and the polyphenolic compounds.

Considering the first variable, the result showed that the olive oil samples were grouped depending on the contamination of the process, while for the last two variables the samples were grouped depending on the harvesting year.

REFERENCES

Andrewes P., Busch J.L., de Joode T., Groenewegen A., Alexandre H. 2003. Sensory properties of virgin olive oil polyphenols: Identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. Journal of Agricultural and Food Chemistry, 51, 1415–1420

Aparicio M., Morales M.T. and Garcìa-Gonzàles D.L. 2012. Towards new analyses of aroma and volatiles to understand sensory perception of olive oil. European Journal of Lipid Science and Technology, 114, 1114-1125.

Aparicio R. and Morales M.T. 1998. Characterization of olive ripeness by green aroma compounds of virgin olive oil. Journal of Agricultural and Food Chemistry, 46, 1116-1122.

Wallis A. A. 2013. Inhibition of Spoilage Yeasts using Spice Essential Oils and Their Components. Master's Thesis, University of Tennessee, 2013.

Bendini A., Valli E., Barbieri S. and Gallina Toschi T. 2012. Sensory Analysis of Virgin Olive Oil. Ch. 6. In "Olive Oil Constituents, Quality, Health Properties and Bioconversions". D. Boskou (Ed.), p. 109. InTech Open Access Publisher

Carrera-Gonzales M.P, Ramìrez-Esposito M.J., Mayas M.D., Martìnez-Martos J.M. 2013. Protective role of oleuropein and its metabolita hydroxytyrosol on cancer. Trends in Food Science & Technology, 31, 92–99.

Ciafardini G., Marsilio V., Lanza B., Pozzi N. 1994. Hydrolysis of oleuropein by Lactobacillus plantarum strains associated with olive fermentation. Applied and Environmental Microbiology, 60(11), 4142-4147.

Ciafardini, G., Zullo, B.A., 2002a. Survival of microorganisms in extra virgin olive oil during storage. Food Microbiology, 19, 105-109.

Ciafardini G., Zullo B.A. 2002b. Microbiological activity in stored olive oil. International Journal of Food Microbiology, 75, 111–118.

Ciafardini G., Zullo B.A., Iride A. 2006a. Lipase production by yeasts from extra virgin olive oil. Food Microbiology, 23, 60–67.

Ciafardini G., Zullo B.A., Cioccia G., Iride A. 2006b. Lipolytic activity of Williopsis californica and Saccharomyces cerevisiae in extra virgin olive oil. International Journal of Food Microbiology, 107, 27–32.

Cicerale S., Lucas L., Keast R. 2010. Biological activities of phenolic compounds present in virgin olive oil International Journal of Molecular Sciences, 11, 458–479.

Di Giacinto L., Di Loreto G., Di Natale C., Gianni G., Guasti S., Migliorini M., Pellegrino M., Perri E. and Santonico M. 2010 Caratterizzazione analitica degli attributi sensoriali degli oli vergini di oliva - AROMOLIO. Camera di Commercio di Firenze, Nuova Grafica Fiorentina, Firenze.

European Community, Commission Regulation 1348/2013, 2013. Amending Regulation No 2568/91/EEC. Official Journal of the European Communities, December 16, L338, 31-67.

IOC/T.20/Doc No 29. Official methods of analysis. Determination of biophenols in olive oils by HPLC. COI. Madrid (2009).

ISO 9936:2006. Animal and vegetable fats and oils – Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography. ISO. Geneva (2006).

Kotti F., Cerretani L., Gargouri M., Chiavaro, E., Bendini A. 2011.Evaluation of volatile fraction of commercial virgin olive oils from Tunisia and Italy: relation with olfactory attributes. Journal of Food Biochemistry, 35, 681–698.

Migliorini M., Cherubini C., Cecchi L., Mori G., e Sartori N. 2013. Quaderni operativi del progetto OLEOTEKINNOVA. Libro pubblicato da Metropoli Azienda Speciale della Camera di Commercio di Firenze.

Migliorin, M., Zanoni B., Berti A., Cherubini C., Cini E., Daou M., e Mugelli M. 2008. Protocolli innovativi per la filiera olivo-oleicola toscana. Libro pubblicato dalla Camera di Commercio di Firenze.

Migliorini M., Cecchi L., Cherubini C., Trapani S., Cini E. and Zanoni B. 2012. Understanding degradation of phenolic compounds during olive oil processing by inhibitor addition. European Journal of Lipid Science *and* Technology, 114, 942-950.

Morales M.T., Luna G. and Aparicio R. 2005. Comparative study of virgin olive oil sensory defects. Food Chemistry, 91, 293–301.

Obied H.K., Prenzler P.D., Omar S.H., 2012. Pharmacology of Olive Biophenols. In: Fishbein JC and Heilman JM. eds.Advances in molecular toxicology, Vol. 6.

Oliveras Lopez M. J., Innocenti M., Ieri F., Giaccherini C., Romani A., Mulinacci N. (2008). HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian *Olea europaea L*. fruits. Journal of Food Composition and Analysis, 21, 62-70.

Romo-Sánchez S., Alves-Baffi M., Arévalo-Villenaa M., Úbeda-Iranzoa J., Briones-Pérez A., 2010. Yeast biodiversity from oleic ecosystems: Study of their biotechnological properties. Food Microbiology 27, 487-492.

Servili M., Selvaggini R., Esposto S., Taticchi A., Montedoro G., Morozzi G. 2004. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. Journal of Chromatography A, 1054, 113–127.

Servili M., Esposto S., Fabiani R., Urbani S., Taticchi A., Mariucci F., Selvaggini R., Montedoro G.F. 2009. Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure. Inflammopharmacology. 17, 76-84.

Truong Vy. R., Boyer Renee R., McKinney Julie M.,O'Keefe Sean F., Williams Robert C. 2010. Effect of α-Cyclodextrin–Cinnamic Acid Inclusion Complexes on Populations of *Escherichia coli* O157:H7 and *Salmonella enterica* in Fruit Juices. Journal of Food Protection, 73, 92-96

Vichi S., Castellote A.I., Pizzale L., Conte L.S., Buxaderas S. and Lòpez-Tamames E. 2003. Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. Journal of. Chromatography A. 983, 19-33.

Vichi S., Romero A., Tous J. and Caixach J. 2011. The activity of healthy olive microbiota during virgin olive oil extraction influences oil chemical composition. Journal of Agricultural and Food Chemistry, 59, 4705.

Zanoni B. 2014. Which processing markers are recommended for measuring and monitoring the transformation pathways of main components of olive oil? Italian Journal of Food Science, 26, 1-11.

Zheng L., Bae Y.M, Jung K.S, Heu S., Lee S.Y. 2013. Antimicrobial activity of natural antimicrobial substances against spoilage bacteria isolated from fresh produce. Food Control, 32, 665-672.

Zullo B.A. and Ciafardini G. 2008. Lypolitic yeast distribution in commercial extra virgin olive oil. Food Microbiology, 25, 970-977.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2013. Effects of some oil-born yeasts on the sensory characteristics of Italian virgin olive oil during its storage. Food Microbiol. 36, 70-78.

PAPER

INVESTIGATION ON MICROBIOLOGY OF OLIVE OIL EXTRACTION PROCESS

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ABSTRACT

Several batches of approx. 200 kg olives from *Frantoio* and *Moraiolo* cultivars were processed in an oil mill at two dates of harvesting. Samples were collected in several steps of extraction process for sensory, chemical and microbial analyses.
All extracted olive oil from the second olive harvesting date was affected by sensory defects and hence classified as being "non-extra virgin". A distinction between extra virgin olive oil and non-extra virgin olive oil obtained from both harvesting dates was explained by the volatile compounds content of olive oil samples and by yeast and mould counts collected at different processing steps.

- Keywords: moulds, sensory defects, virgin olive oil, volatile compounds, yeasts -

INTRODUCTION

The absence of sensory defects is necessary for olive oil to be marketed as "extra virgin" in the EU.

Extra virgin olive oil (EVOO) is characterized by pleasant sensory notes. They are mainly originated by aldehydes, esters, alcohols and ketones, which are responsible for oil sensory attributes such as "green" and "fruity" (APARICIO and MORALES, 1998; MORALES *et al.*, 2005, BENDINI *et al.*, 2012). However, several phenomena can alter the initial pleasant flavour, giving rise to unpleasant sensory notes.

The current olive oil regulations (EU Reg. 1348/2013) classify the most frequent sensory defects into four groups as follows: "fusty", "musty", "winey–vinegary", and "rancid".

Storage of olive fruits in piles before being processed is a cause of sensory alterations in EVOO. Olive transpiration during storage is known to increase pile temperature, enabling microbial cells to grow and to affect the chemical composition of olives (MORALES *et al.*, 2005). Both biogenesis of volatile compounds and transformation phenomena of phenolic compounds can be significantly influenced by microbial contamination of olives. Effects of olive microbiota on oil characteristics are considered even greater than time-temperature conditions of malaxation (VICHI *et al.*, 2011).

Oil quality may be affected by microorganisms, according to their metabolic activities. During olive crushing, microorganisms might migrate into oil through both solid particles of olive fruit and micro-drops of vegetation water (CIAFARDINI and ZULLO, 2002). Some microorganisms do not survive a long time, but others may persist and become a typical microbiota of olive oil. For example, yeasts may remain metabolically active during olive oil storage and thus modify olive oil characteristics (ZULLO *et al.*, 2010).

Enzymatic activities of yeasts and moulds isolated from either olives or EVOO have been reported to include β-glucosidase, β-glucanase, polyphenoloxidases, peroxidase and, in some cases, lipase and cellulase activities (CIAFARDINI and ZULLO, 2002; CIAFARDINI *et al.*, 2006; ZULLO and CIAFARDINI, 2008; ROMO-SANCHEZ *et al.*, 2010). Enzymes such as β-glucosidase are known to improve oil quality by increasing phenolic compound extractability, while others such as lipase, polyphenoloxidases and peroxidase are known to cause detrimental effects (PALOMARES *et al.*, 2003; ROMO-SANCHEZ *et al.*, 2010; VICHI *et al.*, 2011; MIGLIORINI *et al.*, 2012). *Penicillium* and *Fusarium* spp. isolates have been shown to produce amounts of exogenous lipoxygenase (FAKAS *et al.*, 2010) that, together with endogenous lipoxygenase, is the key enzyme of LOX pathway (ANGEROSA *et al.*, 2004).

Extraction process control should include monitoring activities on microbial contamination in olives and EVOO, as associated with sensory and chemical analyses. The study of the microbial populations occurring at different steps of EVOO extraction process, as well as their role in affecting oil characteristics, appears to be increasingly useful.

The aim of this work was to investigate both microbial ecology throughout olive oil processing and a possible relationship between EVOO volatile compound content and microbial contamination.

MATERIALS AND METHODS

Experimental design

During 2011 crop season, several batches of approx. 200 kg olives from *Frantoio* and *Moraiolo* cultivars were processed in an oil mill (Azienda Agricola Buonamici, Fiesole, Florence, Italy).

Plant for oil extraction (TEM, Florence, Italy) consisted of a cleaning and water washing system, an olive grinding cutter crusher (mod. FR350), a controlled-temperature vertical axis malaxation equipment (500 kg capacity) (mod. V500), a "decanter" (two-step mod. D1500) with 1500 kg/h maximum capacity and a cardboard filter press (15 µm cut-off). Plastic residue or "alperujo" from decanter was subjected to separation by centrifugation of stone fragments to obtain destoned pomace (Fig. 1).

Olives were processed within 12 h from harvest at two dates (HD): November 16, 2011 (HD1) and November 23, 2011 (HD2). Oil extraction trials were carried out in quadruple.

Olives were crushed at 2,500 rpm (crusher holes 6.5 mm in diameter); malaxation was carried out at half capacity under vacuum (residual pressure of 20 kPa) at 22 ± 1°C for a mean time of 15 min to work under low oxidative stress impact conditions; decanter worked with a screw conveyor rotating at a slower speed than that of the bowl.

Samples were collected in several steps of extraction process for sensory, chemical and microbial analyses, as shown in Fig. 1.

Chemical analyses

Olives

A homogeneous olive sample was crushed with a laboratory crusher, and resulting olive paste was used for chemical analyses.

The water content (g kg^{-1} of dry matter) was measured on olive paste by gravimetric method (CHERUBINI *et al.*, 2009).

The total sugar content was determined by the UNI 22608 method, modified as described in a previous study (CHERUBINI *et al.*, 2009). Results for sugar content obtained from the equipment (Compact Titrator, Crison, Modena, Italy) were expressed as g kg^{-1} of dry matter.

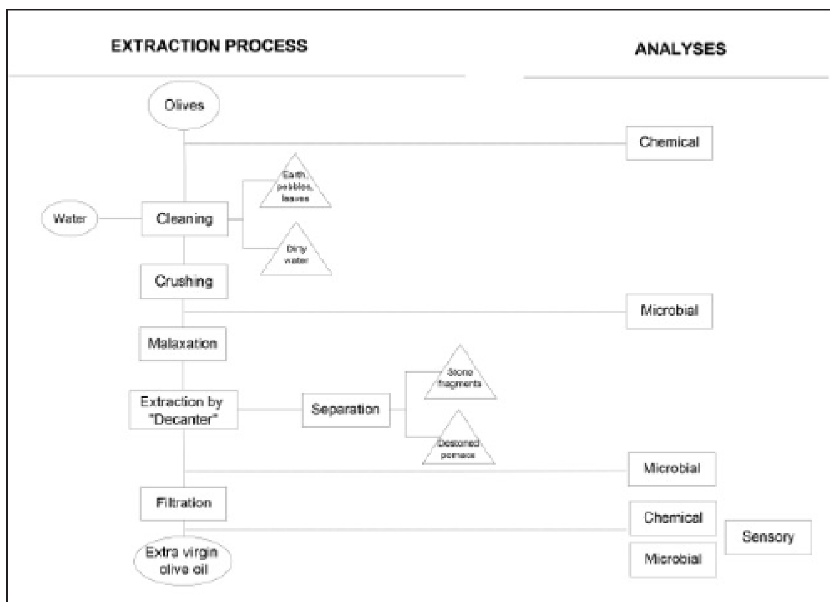

Fig. 1 - Overview of extraction process and analyses carried out.

The total oil content was determined with hexane in an automatic Randall extractor (mod. 148, Velp Scientifica, Milan, Italy), following the analytical technique described in a previous study (CHERUBINI *et al.*, 2009). Results were expressed as g kg^{-1} of dry matter.

The total phenolic compounds content was determined by weighing 4 g crushed olives and adding 80 mL Methanol:Water (60:40) solution; two series of stirring for 30 min and centrifugation at 4,000 rpm for 15 min were performed, and the supernatant was collected. The phenolic extract was adjusted to volume of 200 mL by Methanol:Water (60:40) and placed in the freezer for 2 h. After thawing, the phenolic extract was filtered. One mL filtered extract, 5 mL Folin Ciocalteu reagent, and 20 mL sodium carbonate were placed into a 100 mL flask and adjusted to volume with distilled water. Sixty minutes were waited for colour development; after one hour, UV reading (UV/VIS, Varian model Cary 1E, The Netherlands) was performed at 765 nm wavelength. The total phenolic compounds content was expressed as mg kg^{-1} of dry matter on a calibration curve.

Olive oil

Acidity (% oleic acid), peroxide value (meq O_2 kg^{-1}) and spectroscopic indices were measured according to EU official method (EC Reg. 1989/2003).

Extraction, identification and determination of phenolic compounds were performed in agreement with IOC Official Method (IOC, 2009) by an HPLC equipment consisting of a Hewlett Packard 1200 diode-array detector system and a Hewlett Packard model 1200 autosampler (Agilent Technologies, Santa Clara, California, USA). Secoiridoids, lignans, flavonoids and phenolic acids were quantified in $mg_{tyrosol} kg_{oil}^{-1}$. The total phenolic compounds content ($mg_{tyrosol} kg_{oil}^{-1}$) was determined using the sum of the peak areas of phenols recorded at 280 nm.

The tocopherol content was determined according to ISO 9936:2006 (ISO, 2006) using a Hewlett Packard mod. 1050 liquid chromatograph with quaternary pump and fluorescence detector, provided with Hewlett Packard mod.1100 autosampler (Agilent Technologies, Santa Clara, California, USA). Quantitative analysis was carried out using the external standard method. Results were expressed as mg of total tocopherols per kg of oil.

The volatile compound content was determined according to the literature (VICHI *et al.*, 2003), using HS-SPME-GC-MS technique (solid phase microextraction of the head space, cou-

pled with a gas chromatograph with a mass spectrometer as a detector). Analysis was performed using the Trace CG instrument combined with a Trace DSQ Thermo Finnigan instrument (Fisher Scientific SAS, Illkirch, France). Quantitative analysis was performed using 4-methyl-2-pentanol as an internal standard. Results were expressed as mg of volatile compound per kg of oil.

Sensory analyses

Sensory evaluation of olive oil was performed by a panel test according to the EU official method (EU Reg. 1348/2013). Samples were analyzed by a panel of professional tasters (8 tasters and a panel leader) of CCIAA (Chamber of Commerce, Industry, Handcraft and Agriculture) of Florence. The panel has been recognized by MIPAAF (Ministry of Agricultural Policies, Food and Forestry) since 2002. Intensity of both sensory defects and "fruity", "bitter" and "pungent" attributes was assessed and expressed as the median of tasters score on a scale range from 0 to 10.

Microbiological analysis

Paste and oil samples from each batch were sterilely withdrawn and then transported to the laboratory under refrigerated conditions (4°C). Ten g of olive paste or 10 mL of unfiltered oil were transferred into 90 mL of sterile saline and homogenized for 10 min with a Stomacher Lab Blender 400 (Seward Ltd, Worthing, West Sussex, UK) and a magnetic stirrer, respectively. After decimal dilutions, 100 µL suspension was plated on specific growth media for cell enumeration in triplicate using the spread plating technique. Yeasts were counted on MYPG agar (ZULLO and CIAFARDINI, 2008) integrated with ampicillin and sodium propionate in order to inhibit growth of bacteria and moulds, respectively (ROMO-SANCHEZ, 2010). The plates were incubated at 30°C for 48-72 h. Moulds were counted on MYPG agar without inhibitors (KAWAI *et al.*, 1994) after incubation at 30°C for 24-48 h. Finally, total mesophilic microorganisms were counted on Plate Count Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) after incubation at 30°C for 48 h.

Filtered oil samples (100 mL) were microfiltered through nitrocellulose filters with a porosity of 0.45 µm (Minisart NML-Sartorius, Göttingen, Germany), which was able to retain yeasts and moulds. Then the nitrocellulose filters containing the microorganisms were washed with 10 mL saline and placed onto the specific media described above.

Data processing

Chemical, sensory and microbiological determinations were processed according to one-way ANOVA followed by Tukey's test (significance level: $p = 0.05$).

Principal Component Analysis (PCA) was used to classify samples by Statistica 7.0 software package (Stasoft GmbH, Hamburg, Germany). Correlation studies between microbial cell density and the volatile compounds content of oil samples were carried out by calculating both Pearson and Spearman rank correlation coefficients (significance level: $\alpha = 0.05$).

RESULTS

Characteristics of olive and olive oil samples

Chemical characteristics of processed olives are given in Table 1. They show a slight increase in olive ripening level between the two dates of harvesting. As reported in the literature (RYAN *et al.*, 2002; SERVILI *et al.*, 2004; CHERUBINI *et al.*, 2009), a significant decrease in phenolic compounds content occurred, and a decrease in sugar content, even if significant only for *Frantoio* cultivar, was also observed. No significant variations were measured in both water and olive oil contents during the harvesting interval.

Sensory and chemical characteristics of extracted olive oil are given in Table 2, while their volatile compounds content is reported in Table 3. Samples are encoded in relation to olive cultivar, harvesting date and batch.

Table 2 shows that all olive oil samples extracted from olives of the first harvesting date were extra virgin. They had much lower values

Table 1 - Olive characteristics on two harvesting dates (HD1 and HD2). SD: standard deviation; different letters in the same row indicate significant differences ($p < 0.05$) for the same cultivar; dm: dry matter.

	Frantoio Cultivar				*Moraiolo* Cultivar			
	HD1		HD2		HD1		HD2	
	Mean value	SD	Mean value	SD	Mean value	SD	Mean value	SD
Phenolic Compounds (mg/kg dm)	33000 ^{a}	2285	26000 ^{b}	1811	37000 ^{a}	2579	30000 ^{b}	2097
Sugar Content (g/kg dm)	75 ^{a}	5	54 ^{b}	4	77 ^{a}	5	69 ^{a}	5
Water Content (g/kg)	391 ^{b}	20	437 ^{a}	22	411 ^{a}	21	430 ^{a}	22
Oil Content (g/kg dm)	440 ^{a}	31	460 ^{a}	32	500 ^{a}	35	450 ^{a}	31

Table 2 - Chemical and sensory characteristics of extracted olive oil.

		Frantoio cultivar									*Moraiolo* cultivar										
		HD1				HD2					HD1					HD2					
	Batch code[1]	F1a	F1b	F1c	Mean value ± SD	F2a	F2b	F2c	F2d	Mean value ± SD	M1a	M1b	M1c	M1d	Mean value ± SD	M2a	M2b	M2c	M2d	Mean value ± SD	
EU legal characteristics	Free acidity (% oleic acid)	0.20	0.20	0.22	**0.21^{a}±0.01**	0.19	0.19	0.23	0.24	**0.21^{a}±0.03**	0.20	0.22	0.22	0.22	**0.22^{a}±0.01**	0.25	0.25	0.27	0.27	**0.26^{b}±0.01**	
	Peroxide value (meq O_2/kg oil)	3.6	3.6	3.5	**3.8^{b}±0.1**	6.2	4.9	5.3	4.6	**5.2^{a}±0.7**	3.9	4.0	4.3	4.6	**4.2^{b}±0.3**	5.3	4.5	5.2	4.9	**5.0^{a}±0.4**	
	K_{232}	1.76	1.71	1.74	**1.74^{b}±0.03**	1.77	1.80	1.87	1.85	**1.82^{a}±0.05**	1.80	1.74	1.82	1.80	**1.79^{a}±0.03**	1.79	1.86	1.79	1.83	**1.82^{a}±0.03**	
	K_{270}	0.15	0.15	0.16	**0.15^{b}±0.01**	0.19	0.19	0.23	0.24	**0.21^{a}±0.03**	0.17	0.16	0.17	0.17	**0.17^{a}±0.01**	0.15	0.17	0.17	0.17	**0.17^{a}±0.01**	
	ΔK	0.00	0.00	0.00	**0.00^{a}±0.00**	0.00	0.00	0.00	0.00	**0.00^{a}±0.00**	0.00	0.00	0.00	0.00	**0.00^{a}±0.00**	0.00	0.00	0.00	0.00	**0.00^{a}±0.00**	
	P.A.: Fruity* (0-10)	3.5	3.4	4.1	**3.7^{a}±0.4**	n.d.	2.9	3.3	4.0	**3.4^{a}±0.6**	3.8	3.9	4.4	3.7	**4.0^{a}±0.3**	2.9	3.2	3.4	4.1	**3.4^{b}±0.5**	
	P.A.: Bitter* (0-10)	2.6	3.6	3.6	**3.3^{a}±0.6**	n.d	3.6	3.3	5.1	**4.0^{a}±1.0**	2.6	3.6	4.6	3.7	**3.6^{a}±0.8**	3.8	3.5	3.5	3.5	**3.6^{a}±0.1**	
	P.A.: Pungent* (0-10)	3.7	4.5	4.9	**4.4^{a}±0.6**	4.1	4.1	4.2	5.0	**4.4^{a}±0.4**	4.0	4.2	4.7	4.6	**4.4^{b}±0.3**	4.9	5.8	5.6	5.6	**5.5^{a}±0.4**	
	N.A.: Rancid* (0-10)	0.0	0.0	0.0	**0.0^{b}±0.0**	0.0	1.9	1.6	1.6	**1.3^{a}±0.9**	0.0	0.0	0.0	0.0	**0.0^{b}±0**	1.2	1.4	1.4	1.1	**1.3^{a}±0.2**	
	N.A.: Fusty* (0-10)	0.0	0.0	0.0	**0.0^{b}±0.0**	2.1	1.6	1.3	1.0	**1.5^{a}±0.5**	0.0	0.0	0.0	0.0	**0.0^{b}±0**	1.1	1.2	0.8	1.0	**1.0^{a}±0.2**	
	N.A.: Winey-Vinegary* (0-10)	0.0	0.0	0.0	**0.0^{b}±0.0**	1.7	1.7	0.6	1.0	**1.3^{a}±0.5**	0.0	0.0	0.0	0.0	**0.0^{b}±0**	1.2	1.4	1.4	1.1	**1.3^{a}±0.2**	
Biocompounds (mg/kg)	Total phenol content	720	640	720	**690^{a}±46**	560	610	700	690	**640^{a}±68**	690	670	730	720	**700^{a}±30**	570	700	610	700	**640^{b}±65**	
	Oleuropein	39	32	32	**34^{a}±4**	30	40	50	50	**40^{a}±11**	40	50	70	60	**55^{a}±11**	40	50	60	60	**50^{a}±10**	
	3,4 DHPEA-EDA[2]	230	170	170	**190^{a}±32**	130	130	150	140	**140^{a}±10**	153	142	152	157	**151^{a}±6**	100	140	100	110	**110^{b}±19**	
	p-HPEA-EDA[3]	92	84	84	**83^{a}±4**	110	90	60	50	**80^{a}±27**	67	59	46	47	**55^{a}±10**	40	38	34	26	**34^{b}±6**	
	3,4 DHPEA-EA[4]	31	29	26	**29^{a}±2**	41	38	58	51	**47^{a}±9**	26	36	33	33	**32^{b}±4**	48	48	40	49	**46^{a}±4**	
	pHPEA-EA[5]	8.4	8.1	8.0	**8.1^{a}±0.2**	8.0	8.0	19.0	18.0	**13^{a}±6**	8.6	8.2	7.7	7.4	**8.0^{a}±0.5**	17	15	11	12	**14^{b}±2**	
	3,4 DHPEA[6]	0.5	0.5	0.7	**0.6^{a}±0.1**	0.4	0.6	0.8	0.7	**0.6^{a}±0.2**	0.8	0.6	0.7	0.6	**0.7^{a}±0.1**	0.6	0.7	1.2	0.9	**0.9^{a}±0.3**	
	Tocopherols	290	250	240	**260^{a}±25**	292	292	287	288	**290^{a}±3**	249	256	252	257	**254^{b}±4**	280	280	250	250	**270^{a}±17**	
	Fruity volatile compounds[7] (mg/kg)	13.4	14.0	13.4	**13.6^{a}±0.4**	9.8	10.2	10.9	10.5	**10.3^{b}±0.4**	13.6	13.4	13.1	14.2	**13.6^{a}±0.5**	11.7	11.5	13.0	10.9	**11.8^{b}±0.9**	

HD: harvesting date; SD: standard deviation; a-b different letters in the same row indicate significant differences ($p < 0.05$) for the same cultivar; n.d.: not determined; *median of the tasters score; P.A. and N.A.: Positive and Negative Attributes.
(1) F: *Frantoio*; M: *Moraiolo*; 1: First harvesting date; 2: Second harvesting date; a, b, c, d: olive batches.
(2) Dialdehydic form of decarboxymethyl oleuropein aglycone; (3) Dialdehydic form of decarboxymethyl ligstroside aglycone; (4) Oleuropein aglycone; (5) Ligstroside aglycone; (6) Hydroxytyrosol; (7) Sum of the underlined volatile compound contents in Table 3.

than EU legal chemical limits, no sensory defects and a value of "fruity" attribute with a medium intensity of perception, as reported in EU Reg. 1348/2013.

Conversely, all olive oil samples extracted from olives of the second harvesting date were not extra virgin, as they had significant sensory defects. Despite this, they were in compliance with all legally established (EU Reg. 1348/2013) chemical characteristics and "fruity" attribute.

As a result of malaxation operating conditions at low oxidative stress impact, olive oil resulted in high phenolic compounds content and a phenolic profile characterized by slightly degraded phenolic compounds (SERVILI *et al.*, 2004; GOMEZ-RICO *et al.*, 2009). The total phenolic compound content was approx. 670 mg/kg; the dialdehydic form of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA) was the most abundant phenolic compound, and its content was approx. 150 mg/kg; low (approx. 0.7 mg/kg) hydroxytirosol content (3,4-DHPEA) was found. No significant differences were observed between samples at the two harvesting dates; the medium intensity of "bitter" and "pungent" attribute perception can be explained by phenolic compounds values (EU Reg. 1348/2013).

Volatile compounds content of olive oil samples were subdivided into chemical classes, as reported in Table 3. Compounds that have been shown (KALUA *et al.*, 2007; DI GIACINTO *et al.*, 2010; APARICIO *et al.*, 2012) to be significantly related to oil defects are reported. Underlined volatile compounds are intermediate of LOX pathway and they are considered (DI GIACINTO *et al.*, 2010; KOTTI *et al.*, 2011; APARICIO *et al.*, 2012) to be responsible for olive oil "fruity" positive attribute.

A sum of underlined compound contents is reported in Table 2 as "Fruity volatile compounds"; "fruity" attribute, measured by panel test, can be explained by these values.

Table 3 - Volatile compounds content of extracted olive oil. HD: harvesting date; n.d. not determined.

A. Class of esters, acids and hydrocarbons

	HD	Batch code	Methyl acetate (mg/Kg)	Ethyl acetate (mg/Kg)	Butyl acetate (mg/Kg)	Cis-3-hexenyl acetate (mg/Kg)	Trans-2-hexenyl acetate (mg/Kg)	Butyric acid (mg/Kg)	Pentanoic acid (mg/Kg)	Hexanoic acid (mg/Kg)	Octanoic acid (mg/Kg)	Heptan (mg/Kg)	Octan (mg/Kg)
Frantoio	1	F1a	0.036	0.019	0.002	0.144	0.003	nd	0.010	0.274	0.070	0.007	0.048
		F1b	0.022	0.017	0.002	0.131	0.002	nd	0.013	0.333	0.089	0.006	0.043
		F1c	0.015	0.020	0.002	0.134	0.001	nd	0.011	0.255	0.047	0.004	0.038
	2	F2a	0.015	0.042	0.002	0.070	nd	0.011	0.007	0.210	0.121	0.005	0.053
		F2b	0.007	0.035	0.002	0.409	nd	0.010	0.008	0.243	0.135	0.002	0.039
		F2c	0.008	0.027	0.001	0.614	nd	0.012	0.013	0.237	0.170	0.003	0.035
		F2d	0.006	0.023	0.001	0.619	nd	0.010	0.010	0.235	0.166	0.003	0.036
Moraiolo	1	M1a	0.005	0.018	0.001	0.747	0.003	nd	0.004	0.236	0.095	0.003	0.032
		M1b	0.005	0.016	0.002	1070	0.027	nd	0.007	0.259	0.083	0.005	0.031
		M1c	0.006	0.019	0.003	1.173	0.004	nd	0.008	0.277	0.065	0.003	0.033
		M1d	0.005	0.015	0.001	0.480	0.006	nd	0.004	0.214	0.065	0.005	0.028
	2	M2a	0.006	0.021	0.001	1032	nd	0.013	0.012	0.277	0.185	0.003	0.035
		M2b	0.005	0.020	0.002	0.858	nd	0.013	0.011	0.261	0.160	0.004	0.040
		M2c	0.008	0.022	0.001	0.927	nd	0.012	0.008	0.201	0.119	0.002	0.036
		M2d	0.006	0.023	0.001	0.701	nd	0.014	0.015	0.262	0.171	0.003	0.037

B. Class of aldehydes

	HD	Batch code	Valeraldheyde (mg/Kg)	Hexanal (mg/Kg)	Trans-2-Pentenal (mg/Kg)	Cis-3-Hexenal (mg/Kg)	Heptanal (mg/Kg)	Trans-2-Hexenal (mg/Kg)	Octanal (mg/Kg)	Trans-2-Heptenal (mg/Kg)	2,4 Hexadienal (mg/Kg)	Trans-2-Octanal (mg/Kg)	Benzaldehyde (mg/Kg)	Trans-2-Nonenal (mg/Kg)	Trans-2-Decenal (mg/Kg)
Frantoio	1	F1a	0.128	0.586	0.031	1.421	0.025	10.090	0.131	0.038	0.292	0.015	0.031	0.190	0.229
		F1b	0.106	0.644	0.035	1.822	0.026	10.217	0.159	0.033	0.313	0.010	0.031	0.196	0.232
		F1c	0.087	0.596	0.030	1.569	0.025	9.963	0.134	0.024	0.270	0.010	0.030	0.185	0.237
	2	F2a	0.094	0.481	0.023	1.085	0.017	7.331	0.083	0.042	0.198	0.010	0.030	nd	0.132
		F2b	0.079	0.504	0.028	1.084	0.017	6.938	0.086	0.034	0.189	0.015	0.032	nd	0.125
		F2c	0.070	0.547	0.035	1.157	0.023	6.785	0.090	0.031	0.177	0.014	0.033	nd	0.078
		F2d	0.071	0.535	0.035	1.050	0.022	6.545	0.103	0.030	0.172	0.016	0.034	nd	0.092
Moraiolo	1	M1a	0.092	0.556	0.036	1.742	0.028	8.750	0.148	0.013	0.271	0.019	0.028	0.187	0.203
		M1b	0.079	0.443	0.036	2.016	0.022	7.684	0.137	0.009	0.262	0.016	0.029	0.197	0.18
		M1c	0.100	0.489	0.042	1.937	0.029	7.371	0.152	0.020	0.246	0.014	0.031	0.194	0.204
		M1d	0.087	0.563	0.032	2.045	0.025	9.435	0.117	0.023	0.276	0.019	0.028	0.202	0.180
	2	M2a	0.053	0.538	0.039	1.320	0.027	6.595	0.117	0.024	0.206	0.012	0.036	nd	0.081
		M2b	0.044	0.474	0.043	1.803	0.022	6.172	0.093	0.021	0.229	0.009	0.035	nd	0.058
		M2c	0.042	0.416	0.045	4.051	0.015	5.121	0.072	0.010	0.348	0.022	0.034	nd	0.059
		M2d	0.070	0.585	0.038	1.123	0.025	6.607	0.126	0.036	0.192	0.018	0.039	nd	0.099

C. Class of alcohols, ketones and phenols

	HD	Batch code	1-Penten-3-ol (mg/Kg)	2-Heptanol (mg/Kg)	Pentanol (mg/Kg)	1-Octen-3-ol (mg/Kg)	Trans-3-Hexenol (mg/kg)	Cis-3-Hexenol (mg/Kg)	Cis-2-Pentenol (mg/Kg)	2-Butanone (mg/Kg)	2-Octanone (mg/Kg)	1-Octen-3-one (mg/Kg)	1-Penten-3-one (mg/kg)	6-methyl-5-Hepten-2-one (mg/Kg)	Guaiacol (mg/Kg)	Phenol (mg/Kg)	Ethyl-guaiacol (mg/Kg)	4-Ethyl-phenol (mg/Kg)
Frantoio																		
	1	F1a	0.446	nd	0.005	0.002	0.004	0.242	0.337	0.433	0.003	0.001	0.433	0.004	0.005	0.261	0.131	nd
		F1b	0.454	nd	0.005	0.002	0.004	0.260	0.358	0.477	0.004	0.001	0.477	0.005	0.005	0.275	0.124	nd
		F1c	0.475	nd	0.004	0.002	0.003	0.200	0.353	0.484	0.004	0.001	0.484	0.004	0.008	0.253	0.158	0.080
	2	F2a	0.403	0.225	0.004	0.005	0.004	0.089	0.288	0.360	0.014	0.003	0.360	nd	0.002	0.198	nd	nd
		F2b	0.491	0.294	0.004	0.007	0.005	0.259	0.310	0.481	0.012	0.002	0.481	nd	0.004	0.200	nd	nd
		F2c	0.597	0.351	0.005	0.008	0.006	0.431	0.382	0.708	0.010	0.001	0.708	nd	0.005	0.193	nd	nd
		F2d	0.582	0.346	0.005	0.010	0.007	0.454	0.376	0.712	0.035	0.004	0.712	nd	0.006	0.203	nd	nd
Moraiolo																		
	1	M1a	0.554	nd	0.006	0.002	0.006	0.521	0.428	0.586	0.008	0.002	0.586	0.004	0.004	0.245	nd	nd
		M1b	0.569	nd	0.006	0.002	0.009	0.695	0.435	0.590	0.004	0.002	0.590	0.002	0.004	0.251	0.117	nd
		M1c	0.584	nd	0.005	0.002	0.008	0.709	0.444	0.606	0.006	0.002	0.606	0.004	0.003	0.352	0.114	0.067
		M1d	0.571	nd	0.006	0.002	0.006	0.346	0.447	0.533	0.005	0.001	0.533	0.003	0.004	0.238	nd	nd
	2	M2a	0.658	0.375	0.005	0.009	0.008	0.714	0.420	0.715	0.009	0.002	0.715	nd	0.006	0.208	nd	nd
		M2b	0.575	0.369	0.006	0.007	0.005	0.745	0.412	0.711	0.003	0.002	0.711	nd	0.004	0.210	nd	nd
		M2c	0.603	0.361	0.004	0.006	0.006	0.853	0.402	0.720	0.003	0.002	0.720	nd	0.002	0.196	nd	nd
		M2d	0.636	0.381	0.006	0.012	0.007	0.509	0.394	0.753	0.011	0.004	0.753	nd	0.006	0.205	nd	nd

A multidimensional map of all samples related to volatile compounds was obtained by PCA. The relevant sample loading and score plots are reported in Fig. 2. The model explained 60% of data variability along the first (Factor 1) and second (Factor 2) principal components.

A comparison between the score plot and the loading plot showed that olive oil samples extracted from olives of the second harvesting date were all positioned on the left side of the plot. They were characterized by high values of benzaldehyde, 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol, 1-octen-3-one and 2-octanone.

All these compounds are related to olive oil defects: These compounds have been associated with "musty", "winey–vinegary" and "fusty" defects by some literature data (KALUA *et al.*, 2007; APARICIO *et al.*, 2012), whereas they have been associated with "rancid" defect by DI GIACINTO *et al.* (2010).

Microbial ecology of oil extraction process

Cell concentrations of dominant microbial populations at different steps of oil extraction process from *Frantoio* and *Moraiolo* cultivar olives are shown in Tables 4 and 5, respectively.

Yeasts and/or moulds were always the dominant populations, independently of the sampling point. Cell density of bacteria only accounted for 1% of the total microbial counts on PCA plates.

The cell concentrations in olive paste after crushing (P) and in extracted olive oil (D) ranged between values below 10 and above 10^4 CFU/g or mL. These values were higher than that obtained from filtered olive oil (O), which, in most cases, was < 10^2 CFU/100 mL.

Microbial counts of each olive batch were often affected by high standard deviation values, as it typically occurs in manufacturing processes of raw materials (such as olives) at industrial scale. A rough general pattern for microbial evolution during olive processing could nonetheless be drawn.

Mould counts in olive paste after crushing (PM) were always significantly higher than those in extracted olive oil (DM), while yeast counts showed a different behaviour.

In most olive batches (from both *Frantoio* and *Moraiolo* cultivars) of the first harvesting date, yeast counts decreased by about one order of magnitude from olive paste after crushing (PY) to extracted olive oil (DY), as expected on the basis of olive oil yield. At the second harvesting date, yeast counts remained almost unchanged from olive paste (PY) to olive oil (DY), or even increased in extracted olive oil (DY), suggesting a progressive yeast colonization of the malaxation equipment and/or "decanter". Indeed, at the second harvesting date, olive paste (PY) harboured almost the same yeast concentration as that at the first harvesting date, with values ranging be-

Fig. 2 - Principal Component Analysis carried out on volatile compounds content of olive oil samples. A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane. Samples are coded as reported in Table 2.

tween 10^2 and above 10^3 CFU/g. On the contrary, the extracted olive oil of the second harvesting date (DY) harboured yeast concentrations, in most cases, of about one or two orders of magnitude higher than the extracted olive oil of the first harvesting date.

Correlation studies demonstrated that mould counts in olive paste after crushing (PM) and in extracted olive oil (DM) were positively related to each other, suggesting that mould contamination of unfiltered oil could be affected by the hygienic level of olives (Table 6). On the contrary, yeast cell densities in olive paste (PY) and in olive oil (DY) were statistically unrelated, sug-

Table 4 - Microbial cell counts at different steps of oil extraction process on two harvesting dates (HD) for *Frantoio* cultivar. P = olive paste after crushing; D = olive oil after extraction by "decanter"; O = olive oil after filtration; TMC = total microbial count; different letters indicate significant differences between different extractive steps of the same olive batch ($p < 0.05$); when no letter is reported, no significant difference was found.

HD	Batch code	Sampling point	Yeasts		Moulds		TMC	
			Mean	SD	Mean	SD	Mean	SD
1	F1a	P (CFU/g)	1.60×10^{3a}	1.40×10^{2}	1.00×10^{2}	0	1.40×10^{3a}	1.40×10^{2}
		D (CFU/mL)	4.50×10^{1b}	7.07	<10	-	4.00×10^{1b}	2.82
		O (CFU/100mL)	<1	-	<1	-	<1	-
	F1b	P (CFU/g)	8.50×10^{2a}	2.12×10^{2}	<10	-	1.60×10^{3a}	5.66×10^{2}
		D (CFU/mL)	1.00×10^{2b}	2.80×10^{1}	<10	-	4.00×10^{1b}	0
		O (CFU/100mL)	<1	-	<1	-	<1	-
	F1c	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	8.00×10^{2}	0	2.00×10^{3}	1.41×10^{3}
		D (CFU/ml)	3.25×10^{2b}	3.54×10^{1}	<10	-	5.00×10^{2}	2.83×10^{2}
		O (CFU/100mL)	<1	-	<1	-	<1	-
2	F2a	P (CFU/g)	1.00×10^{2a}	1.40×10^{1}	4.20×10^{4a}	2.82×10^{3}	4.80×10^{4a}	2.83×10^{3}
		D (CFU/mL)	3.00×10^{2b}	2.80×10^{1}	4.00×10^{1b}	2.82	3.00×10^{2b}	2.88×10^{1}
		O (CFU/100mL)	5.00×10^{1c}	1.41	<1	-	5.00×10^{1b}	2.82
	F2b	P (CFU/g)	2.70×10^{3}	1.84×10^{3}	2.85×10^{4}	2.32×10^{4}	8.75×10^{3a}	3.18×10^{3}
		D (CFU/ml)	2.92×10^{3}	1.99×10^{3}	3.33×10^{1}	3.27×10^{1}	1.00×10^{2b}	0
		O (CFU/100mL)	5.50×10^{1}	1.41	<1	-	6.50×10^{1b}	1.41
	F2c	P (CFU/g)	2.30×10^{3}	9.90×10^{2}	2.50×10^{4}	2.25×10^{4}	1.10×10^{3a}	1.41×10^{2}
		D (CFU/ml)	3.26×10^{3}	1.60×10^{3}	9.67×10^{1}	8.96×10^{1}	1.81×10^{3a}	7.66×10^{2}
		O (CFU/100mL)	5.50×10^{1}	2.82	<1	-	1.00×10^{1b}	2.82
	F2d	P (CFU/g)	4.00×10^{2a}	2.83×10^{1}	3.45×10^{4}	3.32×10^{4}	7.00×10^{3a}	1.41×10^{2}
		D (CFU/ml)	1.38×10^{4b}	6.36×10^{2}	1.20×10^{2}	2.83×10^{1}	1.35×10^{4b}	9.90×10^{2}
		O (CFU/100mL)	1.50×10^{1a}	1.40	5.00	0	4.00×10^{1c}	2.82

Table 5 - Microbial cell counts at different steps of olive oil extraction process on two harvesting dates (HD) for cultivar *Moraiolo*. P = olive paste after crushing; D = olive oil after extraction by "decanter"; O = olive oil after filtration; TMC = total microbial count; different letters indicate significant differences between different extraction steps of the same olive batch ($p < 0.05$); when no letter is reported, no significant difference was found.

HD	Batch code	Sampling point	Yeasts		Moulds		TMC	
			Mean	SD	Mean	SD	Mean	SD
1	M1a	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	4.00×10^{2a}	0	1.45×10^{3}	6.36×10^{2}
		D (CFU/mL)	4.50×10^{1b}	7.07	<10	-	4.00×10^{1}	1
		O (CFU/100mL)	<1	-	4.00×10^{1b}	1.41	1.00×10^{1}	1
	M1b	P (CFU/g)	3.75×10^{3a}	3.54×10^{2}	5.50×10^{2}	5.36×10^{2}	5.35×10^{3}	2.33×10^{3}
		D (CFU/mL)	5.00×10^{1b}	1.40	<10	-	<10	-
		O (CFU/100mL)	<1	-	2.00×10^{1}	1.00	2.00×10^{1}	1.40
	M1c	P (CFU/g)	1.10×10^{3a}	1.41×10^{2}	4.00×10^{2a}	0	2.35×10^{3a}	9.19×10^{2}
		D (CFU/mL)	6.90×10^{2b}	2.82×10^{2}	<10	-	1.50×10^{4b}	3.54×10^{3}
		O (CFU/100mL)	<1	-	1.00×10^{1b}	2.82	1.00×10^{1a}	1.40
	M1d	P (CFU/g)	1.10×10^{3}	1.41×10^{2}	4.00×10^{2a}	1.41×10^{1}	1.45×10^{3}	7.78×10^{2}
		D (CFU/ml)	3.20×10^{2}	3.11×10^{2}	<10	-	3.50×10^{1}	2.12×10^{1}
		O (CFU/100mL)	<1	-	2.00×10^{1b}	0	2.00×10^{1}	1.40
2	M2a	P (CFU/g)	1.70×10^{3}	2.83×10^{2}	2.60×10^{3a}	1.98×10^{3}	2.70×10^{3a}	4.24×10^{2}
		D (CFU/mL)	1.04×10^{3}	7.45×10^{2}	6.00×10^{1a}	5.66×10^{1}	9.73×10^{2ab}	8.60×10^{2}
		O (CFU/100mL)	<1	-	5.50×10^{1b}	2.82	5.50×10^{1a}	1.41
	M2b	P (CFU/g)	2.45×10^{3a}	7.78×10^{2}	6.00×10^{2}	5.66×10^{2}	2.35×10^{3a}	9.19×10^{2}
		D (CFU/ml)	3.27×10^{2b}	1.42×10^{2}	3.50×10^{1}	2.12×10^{1}	3.80×10^{2b}	2.31×10^{2}
		O (CFU/100mL)	1.00×10^{1c}	0	1.60×10^{2}	2.82×10^{1}	7.50×10^{1b}	3.53
	M2c	P (CFU/g)	7.45×10^{3}	2.19×10^{3}	1.00×10^{3}	2.82×10^{2}	4.15×10^{3a}	2.12×10^{2}
		D (CFU/mL)	9.72×10^{3}	5.04×10^{3}	4.00×10^{1}	3.66×10^{1}	1.16×10^{3b}	1.07×10^{3}
		O (CFU/100mL)	8.00×10^{1}	14.00	<1	-	1.65×10^{2b}	3.00
	M2d	P (CFU/g)	1.65×10^{3}	1.61×10^{3}	6.75×10^{3}	1.77×10^{3}	5.80×10^{3}	3.11×10^{3}
		D (CFU/mL)	3.08×10^{3}	3.02×10^{3}	6.00×10^{1}	5.66×10^{1}	2.53×10^{3}	2.04×10^{3}
		O (CFU/100mL)	1.10×10^{3}	2.00	<1	-	5.50×10^{1}	1.41

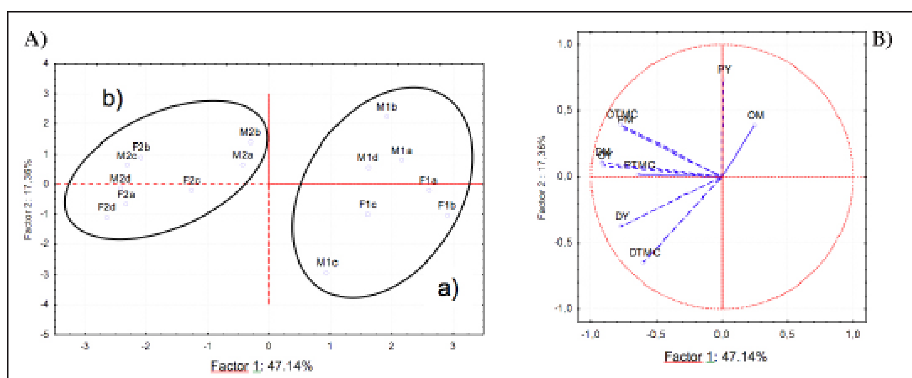

Fig. 3 - Principal Component Analysis of the various olive batches tested by considering as variables the microbial cell concentrations during various extraction process steps. Samples are coded by combination of letters which identify both samples at processing steps (P = olive paste after crushing; D = olive oil after centrifugation by "decanter"; O = olive oil after filtration) and microorganisms (TMC = total microbial count; Y = yeasts; M = moulds). A: similarity map determined by Principal Component (Factor) 1 and 2; B: projection of the variables on the factor plane.

gesting that yeast growth could be encouraged by malaxation and/or "decanting" steps. Finally, no correlation was found between yeast and mould concentrations in both olive paste (PY and PM, respectively) and filtered oil (OY and OM, respectively).

According to PCA of all microbiological data (Fig. 3), processed olive batches clustered into two different groups, independently of the olive cultivar: The samples of the first harvesting date, harboring the lowest microbial cell densities, clustered in group a), while all batches of the second harvesting date resulted to be included in group b). It is worth noting that both the PCA resulting from all microbiological data (Fig. 3) and the PCA resulting from volatile compounds (Fig. 2) are in full agreement, as olive batches from both statistical analyses are clustered in the same way.

Finally, some statistically significant correlations were found between microbial cell densities at the different steps of oil processing and some volatile compounds of olive oil. The significant correlations between yeast (Y) and mould (M) counts, in both extracted (D) and filtered olive oil (O), and volatile compounds content of the final olive oil samples are reported in Table 7. In particular, correlation coefficients (i.e. Pearson and Spearman) agreed on indicating significant positive correlations between yeast and mould counts in olive oil, both before and after filtration, and some volatile compounds; among the latter, the highest significance was related to ethyl acetate, 2-butanone, butyric acid, pentanol, 2-heptanol, octanoic acid and 1-octen-3-ol contents.

Since most of these compounds were identical to those correlated to olive oil batches with sensory defects, as described in the previous paragraph, yeast and mould contamination may have been responsible for those sensory defects. Which specific sensory defects were associated with the above-mentioned compounds could not be explained, as in the literature "rancid", "fusty", "winey–vinegary" and "musty" defects have been associated with both yeasts and moulds. As an example, a recent study demonstrated the capability of some oil born strains of *Candida*

Table 6 - Correlation coefficients calculated between microbial contaminations (Y = yeasts; M = moulds) of olive paste after crushing (P) and microbial contaminations of extracted (D) and filtered olive oil (O). Statistically significant correlations ($p<0.05$) are underlined.

	DY		DM		OY		OM	
	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r
PM			<u>0.8304</u>	<u>0.7347</u>			-0.1575	-0.2485
PY	0.08641	0.05563			0.2841	0.1241		

Table 7 - Correlation coefficients calculated between yeast (Y) and mould (M) counts of extracted and filtered olive oil (D) and volatile compounds of the final olive oil samples (O). Statistically significant correlations ($p<0.05$) are underlined.

	DY		DM		OY		OM	
	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r	Spearman r	Pearson r
Esters, acids and hydrocarbons								
Methyl acetate	-0.006737	-0.3253	-0.08564	-0.4042	0.01485	-0.2926	-0.8317	-0.5387
Ethyl acetate	0.4464	0.2619	0.6978	0.5603	0.7348	0.6665	-0.4953	-0.4413
Butyl acetate	-0.3046	-0.27	-0.5824	-0.5091	-0.3481	-0.3366	-0.1901	-0.1524
Cis-3-hexenil acetate	0.3194	0.325	0.1589	0.2075	-0.0114	0.01915	0.652	0.6335
Trans-2-hexenil acetate	-0.5621	-0.4382	-0.862	-0.4494	-0.7775	-0.3811	0.243	0.2902
Butyric acid	0.5532	0.5918	0.8694	0.9727	0.7434	0.8125	0.03821	-0.0028
Pentanoic acid	-0.0685	-0.1613	-0.1662	-0.1045	-0.3671	-0.331	0.5344	0.5813
Hexanoic acid	-0.1944	-0.3239	-0.337	-0.3943	-0.4902	-0.5172	-0.01623	-
Octanoic acid	0.4624	0.4945	0.8818	0.9251	0.6282	0.6242	0.1469	0.1633
Heptan	-0.6824	-0.5102	-0.5535	-0.4601	-0.5756	-0.4022	-0.0887	-0.218
Octan	-0.1092	-0.2505	0.0174	-0.04	0.205	0.0699	-0.6035	-0.5001
Aldehydes								
Valeraldheyde	-0.5583	-0.4607	-0.708	-0.7013	-0.5681	-0.5144	-0.291	-0.388
Hexanal	-0.3341	-0.3271	-0.3477	-0.3659	-0.4185	-0.4153	-0.3585	-0.315
Trans-2-Pentenal	0.3599	0.3423	0.2134	0.1599	0.06842	0.02669	0.5254	0.464
Cis-3-Hexenal	-0.2487	0.1017	-0.59	-0.1577	-0.4412	0.03461	0.3068	0.07831
Heptanal	-0.3023	-0.3369	-0.4689	-0.5529	-0.7091	-0.7582	0.2273	0.2932
Trans-2-Hexenal	-0.6784	-0.6681	-0.8182	-0.8242	-0.6991	-0.7192	-0.2628	-0.1959
Octanal	-0.405	-0.4349	-0.6645	-0.7347	-0.7142	-0.7745	0.1745	0.2041
Trans-2-Heptenal	-0.0552	-0.01303	0.1559	0.1205	0.2061	0.1596	-0.6411	-0.5767
2.4 Hexadienal	-0.4503	-0.3867	-0.7002	-0.6627	-0.4882	-0.42	-0.0957	-0.0823
Trans-2-Octanal	-0.0244	0.1057	-0.0918	-0.1199	0.08817	0.08237	-0.0718	-0.1306
Benzaldehyde	-0.5041	0.3715	0.5785	0.4958	0.4283	0.3689	-0.1509	0.1584
Trans-2-Nonenal	-0.6304	-0.6281	-0.8605	-0.9655	-0.7762	-0.8358	0.07103	0.04037
Trans-2-Decenal	-0.5946	-0.615	-0.8119	-0.895	-0.6682	-0.7092	-0.2806	-0.2243
Alcohols, ketones and phenols								
1-Penten-3-ol	0.6681	0.4847	0.5822	0.4303	0.3147	0.1095	0.5055	0.5417
2-Heptanol	0.6178	0.6498	0.8857	0.9774	0.7328	0.7846	0.06237	0.0213
Pentanol	0.4111	0.4182	0.8213	0.8118	0.7221	0.7422	-0.1372	-0.2154
Cis-3-Hexenol	0.3252	0.2959	0.2486	0.216	0.07623	0.05016	0.6277	0.5872
Trans-3-Hexenol	0.3032	0.2647	0.2875	0.1636	0.0247	-	0.5309	0.4904
1-Octen-3-ol	0.6212	0.6381	0.9304	0.9199	0.7286	0.7176	-0.0491	-0.0339
Cis-2-Pentenol	0.0486	0.08362	-0.1142	-0.16	-0.2467	-0.2997	0.685	0.618
2-Butanone	0.5204	0.5477	0.782	0.7111	0.5529	0.5283	0.00517	-0.1297
1-Penten-3-one	0.6461	0.5539	0.6247	0.5717	0.412	0.3304	0.3666	0.4029
2-Octanone	0.264	0.4949	0.5565	0.5658	0.4097	0.3878	-0.06146	-0.0623
1-Octen-3-one	0.2545	0.3958	0.5142	0.5573	0.5459	0.5804	0.1314	0.0085
6-methyl-5-Hepten-2-one	-0.5882	-0.5785	-0.8678	-0.9186	-0.7828	-0.779	-0.1013	-0.1062
Guaiacol	-0.0316	-0.1852	0.08201	-0.1711	-0.2404	-0.3518	-0.1757	-0.1935
Phenol	-0.4724	-0.258	-0.8245	-0.8028	-0.8142	-0.7034	0.1395	0.0637
Ethyl-guaiacol	-0.3957	-0.4097	-0.6943	-0.7573	-0.6262	-0.6423	-0.3215	-0.2771
4-Ethyl-phenol	-0.0820	-0.1033	-0.4472	-0.4672	-0.3997	-0.3962	-0.1999	-0.2132

spp. to induce defects such as "musty" and/or "rancid" in oil (ZULLO *et al.*, 2013).

CONCLUSIONS

This study was carried out on several olive oil samples extracted by olive batches from *Frantoio* and *Moraiolo* cultivars, harvested on two different dates. All extracted olive oil samples from the second olive harvesting date were classified as "non extra virgin", as they were affected by sensory defects.

By combining chemical, sensory, and microbiological data, it can be assumed that the olive oil samples with sensory defects were significantly correlated with specific volatile compounds (i.e., 2-butanone, butyric acid, 2-heptanol, octanoic acid, 1-octen-3-ol). The same volatile compounds were correlated to both yeast and mould counts. It could not be evidenced whether a specific sensory defect might result from specific volatile compounds, which in turn can be produced by specific yeasts and moulds.

Different processing steps were also identified, which resulted to be the most critical steps to cause the measured sensory defects: (i) the

mould contamination of olives; (ii) the two central steps of olive oil processing (i.e. malaxation and extraction by "decanter"), which were likely to have enabled some yeast species to grow. A study on identification of yeast isolates and determination of their enzymatic properties is being carried out to further investigate the incidence of yeast populations during olive oil extraction process.

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REFERENCES

Angerosa F., Servili M., Selvaggini R., Taticchi A., Esposto S. and Monteodoro G. 2004. Volatile compounds in virgin olive oil: occurrence and their relationship with the quality. J. Chromatogr. A. 1054:17.

Aparicio M., Morales M.T. and Garcia-Gonzàles D.L. 2012. Towards new analyses of aroma and volatiles to understand sensory perception of olive oil. *Eur. J. Lipid Sci Technol.* 114:1114.

Aparicio R. and Morales M.T. 1998. Characterization of olive ripeness by green aroma compounds of virgin olive oil. *J Agric Food Chem.* 46: 1116.

Bendini A., Valli E., Barbieri S. and Gallina Toschi T. 2012. Sensory Analysis of Virgin Olive Oil. Ch. 6. In "Olive Oil - Constituents, Quality, Health Properties and Bioconversions". D. Boskou (Ed.), p. 109. InTech Open Access Publisher.

Cherubini C., Migliorini M., Mugelli M., Viti P., Berti A., Cini E. and Zanoni B. 2009. Towards a technological ripening index for olive oil fruits. J. Sci Food Agric. 89:671.

Ciafardini G. and Zullo B.A. 2002. Microbiological activity in stored olive oil. Int. *J Food Microbiol.* 75:111.

Ciafardini G., Zullo B.A., Cioccia G. and Iride A. 2006. Lipolytic activity of *Williopsis californica* and *Saccharomices cerevisiae* in extra virgin olive oil. Int. *J Food Microbiol.* 107: 27.

Commission Regulation (EC) No 1989/2003. OJEU. L295:57.

Commission Implementing Regulation (EU) No 1348/2013. OJEU. L338:31.

Di Giacinto L., Di Loreto G., Di Natale C., Guianni G., Guasti S., Migliorini M., Pellegrino M., Perri E. and Santonico M. 2010. Caratterizzazione analitica degli attributi sensoriali degli oli vergini di olive (AROMOLIO). Camera di Commercio di Firenze. Florence.

Fakas S., Kefalogianni I., Makri A., Tsoumpeli G., Rouni G., Gardeli C., Papanikolaou S. and Agelis G. 2010. Characterization of olive fruit microflora and its effect on olive oil volatile compounds biogenesis. *Eur. J. Lipid Sci Technol.* 112:1024.

Gomez-Rico A., Salvador M.D. and Fregapane G. 2009. Virgin olive oil and olive fruit minor constituent as effected by irrigation management based on SWP and TDF as compared to ETc in medium-density young olive orchards (*Olea Europea* L. cv. Cornicabra and Morisca). *Food Res. Int.* 42:1067.

IOC/T.20/Doc No 29. Official methods of analysis. Determination of biophenols in olive oils by HPLC. COI. Madrid (2009).

ISO 9936:2006. Animal and vegetable fats and oils – Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography. ISO. Geneva (2006).

Kalua C.M., Allen M.S., Bedgood Jr. D.R., Bishop A.G, Prenzler P.D. and Robards K. 2007. Olive oil volatile compounds, flavour development and quality: A critical review. Food Chem. 100:273.

Kawai Y., Otaka M., Kaki M., Oeda Y., Inoue N. and Shinano H. 1994. Screening of Antioxidant-Producing Fungi in *Aspergillus niger* Group for Liquid- and Solid-State Fermentation. Bull. Fac. Fish. Hokkaido Univ. 45:26.

Migliorini M., Cecchi L., Cherubini C., Trapani S., Cini E. and Zanoni B., 2012. Understanding degradation of phenolic compounds during olive oil processing by inhibitor addition. *Eur. J. Lipid Sci Technol.* 114:942.

Morales M.T., Luna G. and Aparicio R. 2005. Comparative study of virgin olive oil sensory defects. Food Chem. 91:293.

Palomares O., Villalba M.M. and Rodriguez R. 2003. The C-terminal segment of the 1,3-ß-glucanase Ole e 9 from olive (*Olea europea*) pollen is an indipendent domain with allergenic activity: expression in *Pichia pastoris* and characterization. Biochem. J. 369:593.

Romo-Sànchez S., Alves-Baffi M., Arèvalo-Villena M., Ubeda-Iranzo J. and Briones-Pèrez A. 2010. Yeast biodiversity from oleic ecosystems: study of their biotechnological properties. Food Microbiol. 75:487.

Ryan D., Antolovich M., Prenzler P., Robards K. and Lavee S. 2002. Biotranformations of phenolic compounds in *Olea Europea L.*. Hort. *Sci.* 92:147.

Servili M., Selvaggini R., Esposto S., Taticchi A., Montedoro G.F. and Morozzi G. 2004. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurence in the oil. . J. Chromatogr. A. 1054:113.

Vichi S., Castellote A.I., Pizzale L., Conte L.S., Buxaderas S. and Lòpez-Tamames E. 2003. Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. *J.* J. Chromatogr. A. 983:19.

Vichi S., Romero A., Tous J. and Caixach J. 2011. The activity on healthy olive microbiota during virgin olive oil extraction influences oil chemical composition. *J. Agric. Food Chem.* 59:4705.

Zullo B.A. and Ciafardini G. 2008. Lypolitic yeast distribution in commercial extra virgin olive oil. Food Microbiol. 25:970.

Zullo B.A., Cioccia G. and Ciafardini G. 2010. Distribution of dimorphic yeast species in commercial extra virgin olive oil. Food Microbiol. 27:1035.

Zullo B.A., Cioccia G. and Ciafardini G. 2013. Effects of some oil borne yeasts on the sensory characteristics of Italian virgin olive oil during storage. Food Microbiol. 36:70.

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Capitolo 4. Microrganismi nel processo di estrazione

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Premessa

I microrganismi presenti nella carposfera delle olive possono passare nell'olio durante il processo di estrazione insieme alle particelle solide ed alle microgocce di acqua di vegetazione (Ciafardini e Zullo, 2002). I lieviti presenti nell'olio appena prodotto possono rimanere attivi durante il periodo di conservazione e possono migliorare o peggiorare la qualità dell'olio extra vergine d'oliva in base alle loro capacità metaboliche (Ciafardini *et al.*, 2006a,b; Zullo e Ciafardini, 2008; Romo-Sanchez *et al.*, 2010; Zullo *et al.*, 2010). Studi recenti hanno dimostrato, infatti, come le qualità organolettiche e le capacità antiossidanti dell'olio possano essere incrementate dall'attività esterasica e β-glucosidasica di alcuni lieviti (Ciafardini e Zullo, 2002). Allo stesso tempo però, altri studi hanno evidenziato come l'acidità dell'olio possa essere incrementata dall'attività lipasica di questi microrganismi attraverso la degradazione dei trigliceridi (Ciafardini *et al.*, 2006b). Inoltre, la quasi totalità dei ceppi di lievito isolati da olio, presi in considerazione in uno studio condotto da Romo-Sanchez *et al.* (2010), ha dimostrato di possedere attività perossidasica, un'attività che può compromettere la qualità dell'olio attraverso la degradazione ossidativa di alcuni composti fenolici (Gomez-Rico *et al.*, 2008). Tutti gli studi condotti sulla microbiologia dell'olio riguardano la presenza di microrganismi, soprattutto lieviti, durante la conservazione, mentre non è conosciuta l'ecologia microbica del processo di estrazione. Considerando la mancanza di informazioni su questo argomento, la partecipazione al progetto OLEOSALUSISTEM ha fornito l'opportunità di:

1. effettuare uno studio sulla presenza di microrganismi durante nelle varie fasi del processo di estrazione e durante la conservazione dell'olio extra vergine d'oliva;
2. avviare uno studio per comprendere il ruolo di questi microrganismi nella definizione delle caratteristiche aromatiche dell'olio finito.

4.1 Risultati e discussione

Per conoscere l'ecologia microbica del processo estrattivo dell'olio extra vergine d'oliva, la presenza di lieviti, muffe e batteri è stata monitorata dalla frangitura delle olive fino alla filtrazione dell'olio. L'indagine è stata condotta nel corso di due successive campagne olearie (2011 e 2012) prendendo in esame un totale di 30 processi estrattivi diversi. I campioni sono stati prelevati in doppio da ciascuna fase del processo. Infine, è stata valutata la stabilità microbiologica degli oli filtrati (campagna olearia 2011) durante un intero anno di conservazione.
Durante la **campagna olearia 2011** sono state analizzate microbiologicamente le paste appena frante, l'olio in uscita dal decanter a due fasi, le sanse e l'olio filtrato. Le paste frante, le sanse, l'olio in uscita da decanter sono risultate contaminate da lieviti e muffe, mentre la presenza di batteri è risultata inferiore al limite di rilevabilità

(<100 UFC/g). In Figura 31 (A) e (B) sono riportate le concentrazioni rispettivamente di lieviti e muffe nelle varie fasi del processo.

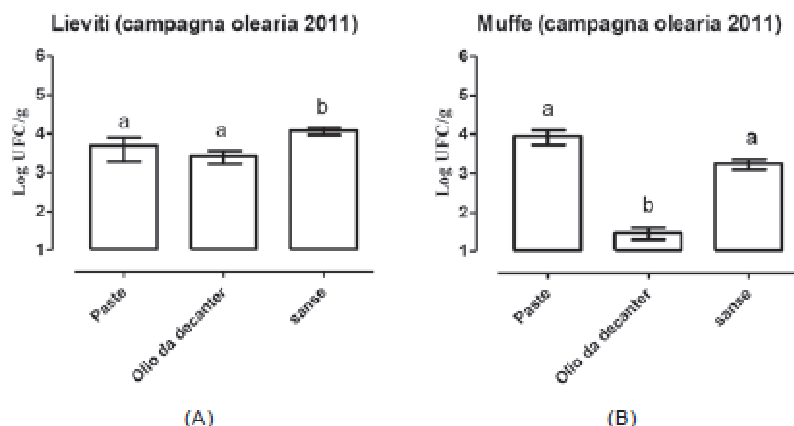

Figura 31. Concentrazione dei lieviti (A) e muffe (B) presenti nelle varie fasi dei processi di estrazione condotti durante la campagna olearia 2011; lettere diverse indicano differenze statisticamente significative (ANOVA; p<0,05).

Nonostante l'olio in uscita dal decander corrisponda in peso più o meno al 15% delle paste (e di conseguenza le sanse corrispondono all'85%), è possibile osservare come i lieviti, a differenza delle muffe, siano presenti nell'olio alle stesse concentrazioni di quelle riscontrate nelle paste e che quest'ultime si siano rivelate addirittura inferiori a quelle presenti nelle sanse (Figure 31A e 31B). Queste osservazioni lascerebbero supporre un fenomeno di arricchimento per i lieviti, ma non per le muffe. Studi di correlazione hanno rafforzato questa ipotesi:

1. la presenza di muffe nell'olio in uscita da decanter è risultata positivamente correlata con la presenza di questi microrganismi nelle paste, ovvero, paste d'oliva più contaminate da muffe hanno dato oli più contaminati da questi microrganismi;
2. la presenza di lieviti nell'olio in uscita da decanter non è risultata statisticamente correlata con la presenza di questi microrganismi nelle paste, ovvero, paste d'oliva meno contaminate da lieviti non necessariamente hanno dato oli meno contaminati da questi microrganismi, confermando il fenomeno di arricchimento lungo il processo di estrazione.

Pertanto, i quesiti aperti sull'argomento sono risultati i seguenti:

QUESITO 1: In quale fase si realizza l'arricchimento dei lieviti? Durante la gramolatura, durante la separazione o in entrambe le fasi?

QUESITO 2: L'arricchimento è soltanto numerico oppure anche speciologico? E' influenzato dalla cultivar delle olive? Le specie coinvolte potrebbero modificare chimicamente l'olio?

QUESITO 3: Quale impatto può avere l'arricchimento della popolazione dei lieviti sulla composizione aromatica dell'olio?

QUESITO 1

Per rispondere al primo quesito, in occasione della **campagna olearia 2012** è stato inserito un punto di campionamento in più e precisamente è stato prelevato un campione di paste non solo al termine della frangitura, ma anche al termine della gramolatura. Questo è stato possibile grazie all'inserimento di un rubinetto sulla linea di produzione tra gramola e decanter.

A differenza della campagna olearia 2011, le analisi condotte durante la **campagna olearia 2012** hanno mostrato non solo una contaminazione da parte di lieviti e muffe, ma anche di batteri (prevalentemente bastoncini Gram positivi, spesso sporigeni) (figure 32 A, B e C).

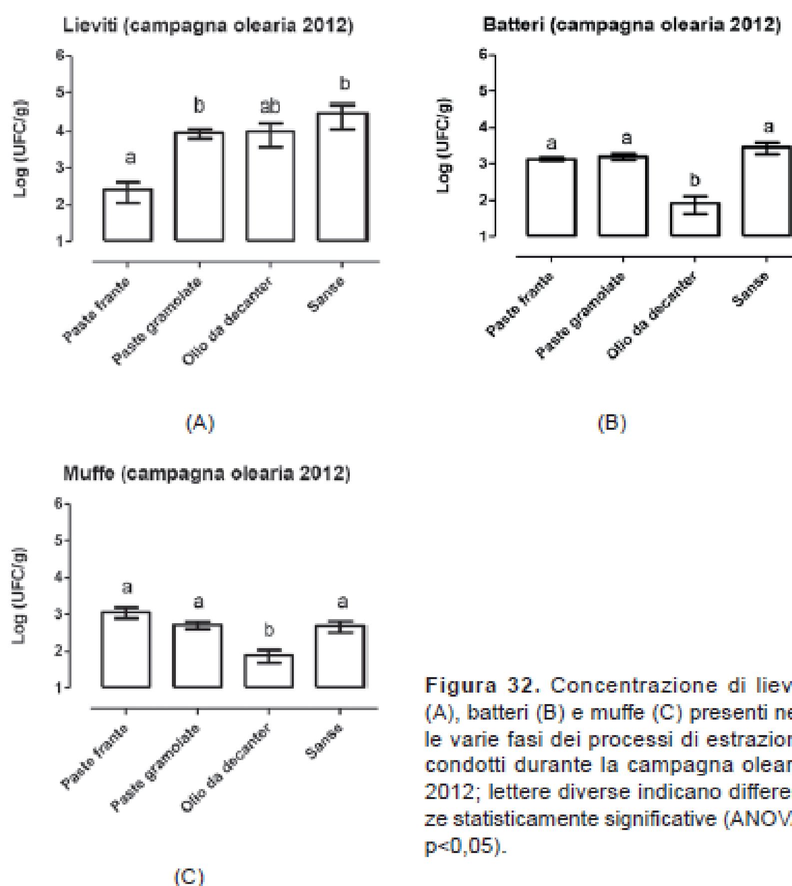

Figura 32. Concentrazione di lieviti (A), batteri (B) e muffe (C) presenti nelle varie fasi dei processi di estrazione condotti durante la campagna olearia 2012; lettere diverse indicano differenze statisticamente significative (ANOVA; $p<0,05$).

L'ecologia dei batteri nel processo di estrazione è risultata del tutto simile a quella delle muffe nel senso che entrambi i gruppi microbici non mostravano alcun feno-

meno di arricchimento (Figura 32B e 32C). I lieviti invece hanno mostrato nel 2012 (Figura 32A) un arricchimento addirittura superiore rispetto a quello osservato nel 2011 (Figura 31A). Grazie all'inserimento del punto di campionamento in più, è stato anche possibile osservare come questo fenomeno si realizzi sia nella gramola, sia nel decanter. Nella gramola l'incremento medio rispetto alle paste frante è risultato di oltre un ordine di grandezza (Figura 32A). Per quanto riguarda l'olio da decanter, il fatto di riscontrare la stessa concentrazione di lieviti delle paste gramolate presuppone un ulteriore arricchimento visto che questo rappresenta il 15% delle paste gramolate (Figura 32A). A conferma di ciò le sanse hanno dimostrato di avere la stessa concentrazione di lieviti delle paste gramolate (Figura 32A).
Confermato quindi il fenomeno di arricchimento numerico dei lieviti nel processo, la ricerca è proseguita con lo scopo di valutare se tale arricchimento fosse o meno dipendente dalle specie di lievito coinvolte (quesito 2).

QUESITO 2
Per quanto riguarda il secondo quesito, un numero significativo di isolati di lievito, provenienti dalle diverse fasi di tutti i processi saggiati, sono stati purificati e identificati a livello di specie utilizzando metodiche molecolari (analisi dei profili di restrizione della regione spaziatrice dell'interno trascritto del rDNA e conferma mediante sequenziamento della regione D1/D2 del 26S rDNA). Nelle Figure 33 e 34 sono riportate le specie riscontrate nelle diverse fasi delle due campagne olearie prese in considerazione (2011 e 2012 rispettivamente).

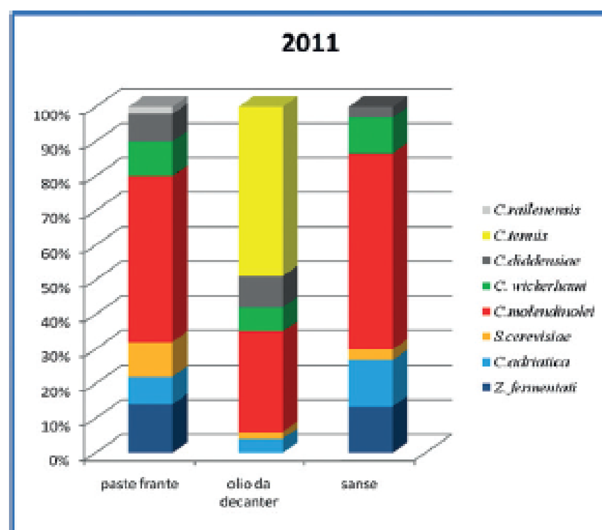

Figura 33. Frequenze di isolamento dei lieviti presenti nelle varie fasi del processo di estrazione della campagna olearia 2011.

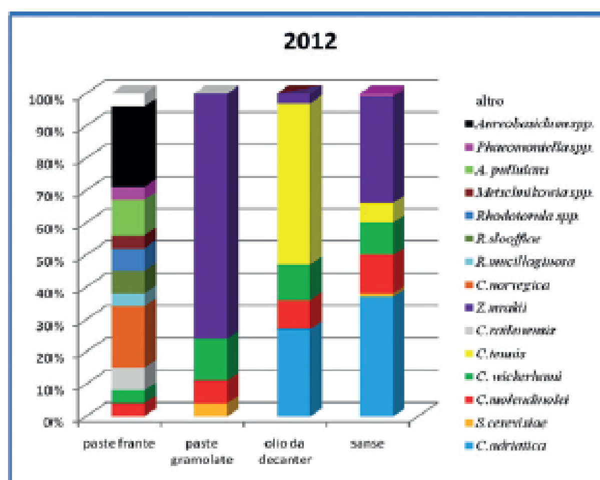

Figura 34. Frequenze di isolamento dei lieviti presenti nelle varie fasi del processo di estrazione della campagna olearia 2012.

Durante la campagna olearia 2011 sono state riscontrate 6-7 specie diverse di lievito in ciascuna delle fasi del processo prese in considerazione (Figura 33). *Candida molendinolei* è risultata presente in tutte le fasi con una frequenza di isolamento nelle paste e nelle sanse del 50% e del 30% nell'olio da decanter. Anche le specie *Candida whickerami*, *Candida diddensiae*, *Candida adriatica*, e *Saccharomyces cerevisiae* sono risultate presenti in tutte le fasi, ma a concentrazioni inferiori o uguali al 10%. *Zygosaccharomyces fermentati* era presente solo nelle paste e nelle sanse, mentre *Candida tenuis* solo nel decanter a concentrazioni intorno al 50%. Nel 2012 la situazione è risultata piuttosto diversa, soprattutto nelle paste frante dove sono state riscontrate un numero notevolmente maggiore di specie diverse di lievito (Figura 34). In questa fase erano, infatti, presenti ben 13 specie diverse, mentre nelle altre fasi un numero compreso tra 5 e 7 come osservato nel 2011. *C. molendinolei* e *C. wickerhamii* erano presenti in tutte le fasi prese in esame con frequenze di isolamento comprese tra il 4 e il 13%. *Zygosaccharomyces mrakii*, non riscontrata in nessuna fase nel 2011, è risultata presente nelle paste gramolate con una frequenza di isolamento del 76%, nell'olio del 3% ed infine nelle sanse del 33% anche se era assente nelle paste frante. *C. adriatica*, a differenza di quanto osservato nel 2011, è stata riscontrata solo nell'olio da decanter e nelle sanse con frequenze di isolamento del 27 e del 37% rispettivamente. Infine *C. tenuis* è stata riscontrata nell'olio da decanter con la stessa frequenza di isolamento riscontrata nel 2011 (50%) e soltanto al 6% nelle sanse.

Da quanto detto è possibile formulare le seguenti ipotesi:

1. La presenza di specie diverse sulle paste frante del 2011 rispetto a quelle del 2012 potrebbe essere una conseguenza della stagionalità;
2. *C. whickeramii* e *C. adriatica* sono presenti in tutte le fasi di entrambe le campagne

olearie confermando quanto riportato in letteratura riguardo la presenza frequente di questi lieviti nell'olio;

3. La gramolatura sembra aver arricchito selettivamente la pasta della campagna olearia 2012 con la specie *Z. mrakii*;
4. La centrifugazione sembra aver arricchito selettivamente l'olio di entrambe le campagne olearie con la specie *C. tenuis*.

Pertanto, è possibile affermare che gramolatura e centrifugazione non sono soltanto in grado di arricchire quantitativamente il contenuto di lieviti, ma anche di selezionare alcune specie a discapito di altre. A questo punto resta da chiarire se la tipologia di cultivar delle olive impiegate nel processo possa avere o meno un ruolo in questo fenomeno di arricchimento selettivo. Per comprendere ciò, i risultati sono stati elaborati in modo da distinguere i processi estrattivi a carico della varietà Moraiolo da quelli della varietà Frantoio nelle due campagne olearie prese in considerazione, escludendo quindi dall'elaborazione tutti quei processi condotti con olive miste. I risultati ottenuti per la cultivar Moraiolo e Frantoio sono riportati nelle Figure 35 e 36.

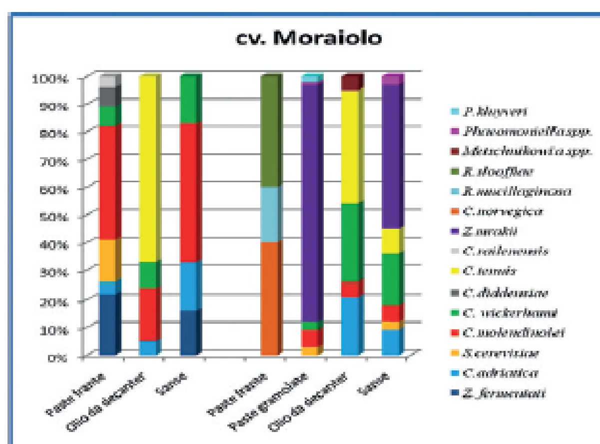

Figura 35. Frequenze di isolamento dei lieviti presenti nelle varie fasi del processo di estrazione di olive della cultivar (cv.) Moraiolo.

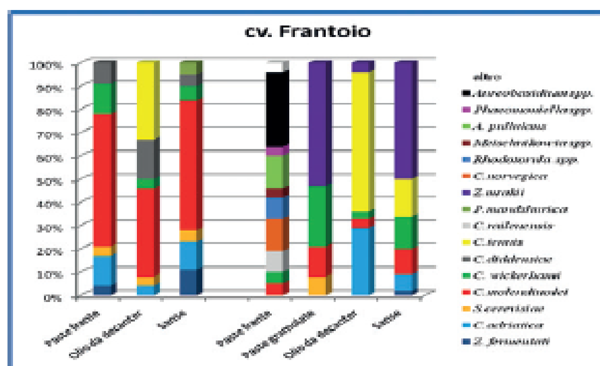

Figura 36. Frequenze di isolamento dei lieviti presenti nelle varie fasi del processo di estrazione di olive della cultivar (cv.) Frantoio.

Confrontando le specie di lievito riscontrate sulle paste frante nel 2011 rispetto a quelle del 2012 a parità di cultivar, è possibile osservare la presenza di specie completamente diverse lasciando supporre che non ci sia alcuna relazione tra cultivar e tipologia di specie presente sulle paste frante. Andando poi a confrontare, a parità di campagna olearia, le specie presenti nell'olio da decanter e nelle sanse ottenute da cultivar diverse, è possibile osservare una quasi completa omologia di composizione speciologica, anche se in rapporti percentuali molto diversi, come se nel frantoio si fosse stabilizzata una popolazione di lieviti piuttosto stabile, indipendente dalla tipologia di oliva impiegata nel processo.
Infine, per valutare la possibilità che le specie di lievito individuate nelle paste gramolate, nell'olio da decanter e nelle sanse siano potenzialmente in grado di modificare chimicamente l'olio, un numero significativo di isolati appartenenti a ciascuna di queste specie sono stati saggiati per le attività glucosidasica, lipasica e esterasica utilizzando un kit miniaturizzato (APIZYM, Biomerieux). I risultati ottenuti sono riportati in Tabella 6 e mostrano come quasi il 50% delle specie possieda tutte le attività enzimatiche saggiate (la presenza dell'attività è indicata dal rettangolo colorato). Degna di nota è soprattutto la specie *C. tenuis* la quale, oltre ad essere stata riscontrata con una frequenza di isolamento nell'olio da decanter intorno al 50% in entrambi i processi, ha dimostrato di possedere tutte le attività enzimatiche prese in esame. Solo due specie, *Z. mrakii* e *P. kluyveri*, si sono dimostrate prive di tutte le attività enzimatiche prese in esame.

Tabella 6. Attività enzimatiche delle specie di lievito isolate dalle paste gramolate, dall'olio da decanter e dalle sanse; il rettangolo colorato indica la presenza della attività.

	Esterasi	Lipasi	β-glucosidasi
Candida adriatica	■	■	■
Candida diddensiae	■	■	■
Candida molendinolei	■	■	■
Candida norvegica			■
Candida railenensis	■	■	■
Candida tenuis	■	■	■
Candida wickerhamii	■	■	■
Pichia kluyveri			
Pichia mandshurica	■	■	
Rhodotorula slooffiae	■		■
Saccharomyces cerevisiae	■		
Zygosaccharomyces fermentati	■	■	
Zygosaccharomyces mrakii			

QUESITO 3

Per rispondere al terzo quesito, ovvero quale impatto può avere l'arricchimento della popolazione dei lieviti sulla composizione aromatica dell'olio, è stato condotto uno studio statistico di correlazione tra contenuto dei lieviti nell'olio da decanter e nelle sanse con i vari composti aromatici quantificati da Metropoli (Azienda Speciale della

Camera di Commercio di Firenze). Lo studio è stato condotto prendendo in esame entrambe le campagne olearie. Per il calcolo delle correlazioni sono stati utilizzati due coefficienti, quello di Pearson e quello di Sperman poiché il primo assume che i dati siano distribuiti secondo una Gaussiana, il secondo no. I risultati sono riportati in Tabella 7 dove con un rettangolo colorato è indicata l'esistenza di una correlazione statisticamente significativa tra la concentrazione della molecola aromatica e la quantità di lieviti presenti nelle due fasi considerate.

Tabella 7. Risultati dello studio di correlazione (coefficienti di Sperman e di Pearson; $p<0,05$) condotto tra le concentrazioni di lievito presenti nell'olio da decanter e nelle sanse con alcuni composti aromatici; i rettangoli colorati indicano l'esistenza di una correlazione statisticamente significativa.

		Lieviti nell'olio da decanter	Lieviti nelle sanse
Aldeidi	2-metil-butanale		
	isovaleraldeide		
	E-2-eptenale		
	valeraldeide		
	esanale		
	E-2-pentenale		
	Z-3-Esenale		
	E-2-esenale		
	Benzaldeide		
	E-2-decenale		
	2,4-esadienale		
	E-2-ottanale		
Chetoni	2-butanone		
	etil vinil chetone		
	6-metil-5-epten-2-one		
Alcoli	propanolo		
	iso-butanolo		
	2-metil-1-but.olo + 3-metil-1-but.olo		
	2-eptanolo		
	Z-2-pentenolo		
	E-3-esenolo		
	Z-3-esenolo		
	Z-2-esenolo		
	2-pentanolo		
	esanolo		
Esteri	metil-acetato		
	etil-acetato		
	E-2-esenil-acetato		
	Z-3-esenil-acetato		
	Butil acetato		

Acidi	**acido-butirrico**		
	acido pentanoico		
	acido esanoico		
	acido ottanoico		
	acido eptanoico		
Altro	**ottano**		
	copaene		
	guaiacolo		
	4-etil-fenolo		
	fenolo		

positiva con entrambi i coefficienti — positiva con un solo coefficiente

negativa con entrambi i coefficienti — negativa con un solo coefficiente

Da quanto riportato in tabella è possibile osservare come, a fronte di 72 composti aromatici identificati, 21 mostrassero una correlazione positiva ed altri 18 una correlazione negativa. In sostanza, oltre il 50% delle componenti aromatiche risulta correlata con la presenza di lieviti nell'olio da decanter e nella sansa. Sulla base dei risultati fin qui ottenuti, non è stato possibile ipotizzare relazioni di causa e effetto tra composti aromatici e tanto meno tra questi e precise attività metaboliche dei lieviti. Per fare ciò ulteriori studi saranno necessari e dovranno tener conto della grande biodiversità riscontrata all'interno delle popolazioni di lievito presenti nelle due campagne olearie oggetto di studio.
Per finire, lo studio condotto sulla stabilità microbiologica dell'olio in conservazione ha mostrato come la filtrazione sia un metodo efficace per eliminare lieviti, muffe e batteri da questa matrice. Bisogna però tener presente che i microrganismi possono rilasciare enzimi in due modi diversi: volontariamente, se sono enzimi che svolgono la loro funzione all'esterno della cellula (enzimi extracellulari), oppure per effetto di autolisi. Se uno di questi due fenomeni si verifica durante il processo di estrazione, la filtrazione non garantirà di eliminare completamente il possibile contributo dei microrganismi alla composizione chimica dell'olio.

4.2 Conclusioni

L'olio appena prodotto possiede un'apparenza opalescente dovuta alla presenza di particelle solide e microgocce di acqua di vegetazione che contengono microrganismi costituiti da lieviti, muffe e batteri. Questi microrganismi derivano non solo dalla carposfera delle olive, ma anche, per lo meno nel caso dei lieviti, da un fenomeno di arricchimento selettivo che si realizza durante il processo di estrazione (in particolare durante le fasi di gramolatura e centrifugazione). Infatti, studi condotti nell'ambito del presente progetto hanno dimostrato come i lieviti presenti nelle paste al momento della frangitura non solo siano numericamente inferiori rispetto a quelli presenti nelle fasi successive, ma anche diversi da un punto di vista speciologico. Questo lascerebbe supporre che il processo estrattivo, con il passare delle ore, e soprattutto dei giorni di attività del frantoio, determini un arricchimento "selettivo" cioè un arricchimento di certe specie di lievito a scapito di altre. Tale arricchimento è risultato indipendente dalla tipologia di cultivar delle olive impiegate nel processo e soprattutto piuttosto

diverse tra una campagna olearia e l'altra. Alcune specie, però, sono risultate comuni alle due campagne olearie e probabilmente quella più interessante è *C. tenuis* riscontrata sempre nell'olio da decanter, ma mai nelle paste e solo raramente nelle sanse. La presenza di alcune specie di lievito nell'olio ma non nelle sanse e viceversa potrebbe essere spiegata non solo dall'effetto meccanico della centrifugazione che può trasferire all'olio più o meno sansa e/o acqua di vegetazione, ma anche dalle caratteristiche chimico-fisiche delle pareti dei lieviti che possono variare molto da specie a specie. Tali caratteristiche, infatti, possono conferire alla cellula una diversa affinità per l'acqua di vegetazione o per l'olio, oppure una diversa capacità di adesione alle particelle solide. In ogni caso, ulteriori studi sarebbero necessari per comprendere il fenomeno e soprattutto controllarlo.

Molte delle specie di lievito riscontrate hanno anche dimostrato di possedere capacità enzimatiche potenzialmente in grado di modificare chimicamente l'olio (attività lipasica, esterasica, ß-glucosidasica) ed effettivamente studi statistici hanno dimostrato lesistenza di correlazioni positive o negative statisticamente significative tra quantità di lieviti nelle fasi del processo in cui si realizza l'arricchimento selettivo e le concentrazioni di ben 39 componenti aromatiche nell'olio finito su un totale di 72 prese in esame.

In conclusione, da quanto fin qui descritto, è possibile ipotizzare che ogni frantoio selezioni un proprio microbiota potenzialmente in grado di conferire note aromatiche tipiche oppure difetti al prodotto finito. Pertanto, la produzione di oli di qualità non dovrebbe prescindere dal conoscere, e soprattutto dal controllare, il microbiota presente in frantoio.

Bibliografia

Ciafardini, G., Zullo, B.A., 2002. Int.J. Food Microbiol. 75, 111-118.
Ciafardini, G., Zullo, B.A., Iride, A., 2006a. Food Microbiol. 23, 60-67.
Ciafardini, G., Zullo, B.A., Cioccia, G., Iride, A., 2006b. Int. J. Food Microbiol. 107, 27-32.
Gomez-Rico, A., Fregatane, G., Salvador, M.D., 2008 Food Res. Int. 41, 433-440.
Romo-Sanchez, S., Alves-Baffi, M., Arevalo-Villena, M., Ubeda-Iranzo, J., Briones-Pérez, A., 2010. Food Microbiol. 27, 487-492.
Zullo, B.A., Ciafardini, G., 2008. Food Microbiol. 25, 970-977.
Zullo, B.A., Cioccia, G., Ciafardini, G., 2010. Food Microbiol. 27, 1035-1042.

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Extra Virgin Olive Oil: Microbial Ecology of the Extractive Processes and its Effect on the Aromatic Composition of the Final Products

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Newly produced olive oils might harbor viable microbial cells which could affect, according to their metabolic capability, the oil quality. Indeed, β-glucosidase and esterase capabilities can improve the taste and the antioxidant capability of the oil, while the lipase capability can worsen the oil quality by hydrolyzing triglycerides. Usually, microbial contamination of oils originates from raw material (olives) and/or from the oil-mill. However, information on the microbial occurrence in the different steps of the extra virgin olive oil production and their influence on the aromatic composition of the final product are lacking. Therefore, a study was carried out to investigate on the presence of microorganisms in the pastes after crushing, in the oil after centrifugation in a two phase decanter, in the oil after filtration, and in the oil after three months of conservation in dark-green bottles. A total of 16 extraction processes, carried out in the same manufacture located in Tuscany, were considered. The microbial populations were manly constituted by yeasts and moulds, while in most cases bacteria occurred at very low concentrations. The yeast and mould densities in the pastes and in the oil before the filtration step ranged between values below 1 and of about 10^4 CFU/g, while in the filtrated oil below 10^2 CFU/100 mL. The 16 oils after filtration were also analyzed for their aromatic composition. Correlation studies showed some positive or negative correlations between microbial densities in the different productive steps and some aromatic compounds in the oil. In any case, principal component analysis, carried out on the microbiological and chemical data, demonstrated that all the productive processes showing the highest microbial contaminations were positively associated with the same aromatic compounds. This work may be considered a preliminary study to understand the microbial ecology of the extra virgin olive oil and its effective impact on the aromatic composition of this product.

Extra virgin olive oil: microbial ecology of the extractive processes and its effect on the aromatic composition of the final products.

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Introduction

1. Newly produced olive oils might harbor viable microbial cells which could affect, according to their metabolic capability, the oil quality [1].
2. β-glucosidase and esterase capabilities can improve the taste and the antioxidant capability of the oil, while the lipase capability can worsen the oil quality by hydrolyzing triglycerides [2].
3. Usually, microbial contamination of oils originates from raw material (olives) and/or from the oil-mill. However, information on the microbial occurrence in the different steps of the extra virgin olive oil production and their influence on the aromatic composition of the final product are lacking.

AIM

to investigate on the presence of microorganisms in the pastes after crushing, in the oil after centrifugation in a two phase decanter, in the oil after filtration, and in the oil after three months of conservation in dark-green bottles → A total of 16 extraction processes, carried out in the same manufacture located in Tuscany, were considered.

Materials and methods

- Olive cultivars: Frantoio and Moraiolo
- [illegible]
- [illegible]
- [illegible]
- [illegible]
- [illegible]

Fig. 1

Results

The microbial populations were manly constituted by yeasts and moulds, while in most cases bacteria occurred at very low concentrations. The yeast and mould densities in the pastes and in the oil after the centrifugation ranged between values below 1 and of about 10^4 CFU/g, while in the filtrated oil below 10^2 CFU/100 mL (Fig. 2). Yeast and mould occurred at very low concentrations in the oils after three months of conservation (data not shown).

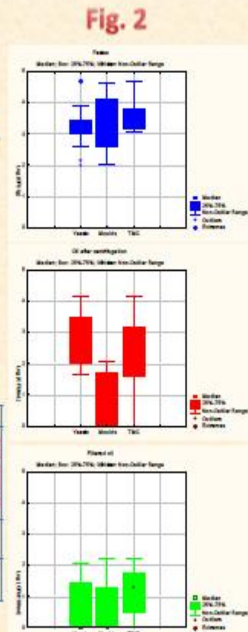


Fig. 2

The 16 oils after filtration were also analyzed for their aromatic composition:

1. Correlation studies showed some positive or negative correlations between microbial densities in the different productive steps and some aromatic compounds in the oil (Table 1).

Tab. 1

	Esters		Alchols									Aldheydes									Ketons	Acids	
	ethyl acetate	metyl acetate	2 and 3 metyl-1-butanol	isobutanol	2-pentanol	2-eptanol	hexanol	1-octen-3-olo	nonanol	phenol	etilguaiacol	heptanal	valeraldeide	E-2-hexenal	Octanal	nonanal	2,4-esadienal	decanal	E-2-nonenal	E-2-decenal	6 Metyl 5 hepten 2 one	Butirric acid	octanoic acid
Yeasts	+	no	+	+	+	+	no	+	-	-	-*	-	-*	-	-	-	no	-	-	-	-	+	+*
Moulds	+	-	+*	+	+	+	+	+	-	-	-	-*	-	-	-	-	-	-	-	-	-	+	+

"+" and "-" positively or negatively correlated respectively → Pearson and Sperman r > 0.7 (p<0.05); "<+" or "<-" positively or negatively related respectively → Pearson and Sperman 0.6< r < 0.7 (p<0.05); no: no correlated.

2. Principal component analysis (Fig. 3), carried out on the microbiological and chemical data, demonstrated that all the productive processes showing the highest microbial contaminations were positively associated with the same aromatic compounds (circulated with ellipses).

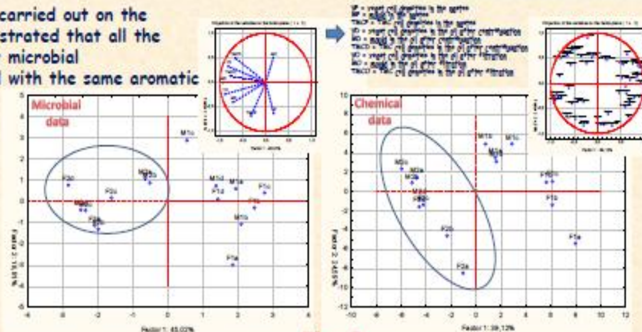


Fig. 3

Conclusion

This work may be considered a preliminary study to understand the microbial ecology of the extra virgin olive oil and its effective impact on the aromatic composition of this product.

References
[1] [illegible] (2002). International Journal of Food Microbiology 75: 111-118.
[2] [illegible] Food Microbiology 27: [illegible]
[3]

3.4 INVESTIGATION ON THE METABOLIC BEHAVIOUR OF THE YEAST SPECIES

Abstract

The olive oil microbiota is mainly composed by yeasts. Some olive oil yeasts are considered useful, as they are able to hydrolyze the bitter tasting secoiridoid compound of the oil, whereas others are considered harmful, as they can damage the quality of the oil (Zullo et al., 2013). To assess the incidence of these abilities in oil-born yeasts, 117 yeast isolates coming from pastes, centrifuged oil and pomaces, collected during 35 olive oil extraction processes carried out in the same oil mill during three different harvest years, were taken into consideration. The isolates were assayed for β-glucosidase, cellulase, polygalacturonase, peroxidase and lipase activities. All of the isolates were peroxidase positive and cellulase negative, while β-glucosidase, lipase and polygalacturonase activities were found in 66, 22 and 2% of the assayed yeasts, respectively. Moreover, three strains, with different enzymatic activities, were separately inoculated in crushed pastes and filtered olive oil to investigate their influence on the oil quality. The oils obtained from crushed pastes after 1 hour of incubation and the oils after two months of storage were analyzed (acidity level, peroxide value, total polyphenols, yeast concentrations) and statistically compared with the control (oil without yeast inoculation). The results showed that the strains with lipase activity were able to increase of about 25% (in oils from pastes) and 20% (in inoculated oils) the acidity of the oils compared to the samples without inoculation and the samples inoculated with the strain without lipase activity. Furthermore, in both tests, all the assayed strains showed a decrease in polyphenols concentrations compared to the control. Moreover, as regards the number of peroxides, no statistically significant difference was found between oils from inoculated and not inoculated pastes, while, in the inoculated oils, peroxide values increased only in the presence of the strain that survived in a significant concentration (10^5 CFU/mL) after 30 days of incubation in oil. Finally, inoculated oils showed values of the aromatic compounds related to olive oil positive attribute (cis-3-hexenyl

acetate, hexenal, trans-2-hexenal and trans-2-hexenol) significantly lower than to the control. These findings show that enzymatic activities of oil-born yeasts may negatively affect the chemical composition of olive oil during the storage.

3.4.1 Introduction

Microorganisms, according to their metabolic activities, may affect oil quality. During olive crushing, microorganisms might migrate into oil through both solid particles of olive fruit and micro-drops of vegetation water (Ciafardini and Zullo, 2002a). Some microorganisms do not survive a long time, but others may persist and become a typical microbiota of olive oil. For example, yeasts may remain metabolically active during olive oil storage and thus modify olive oil characteristics (Zullo *et al*., 2010). Enzymatic activities of yeasts and moulds isolated from either olives or extra virgin olive oil have been reported to include β-glucosidase, β-glucanase, polyphenoloxidases, peroxidase and, in some cases, lipase and cellulase activities (Ciafardini and Zullo, 2002b; Ciafardini *et al*., 2006; Zullo and Ciafardini, 2008; Romo-Sanchez *et al*., 2010). Enzymes such as β -glucosidase are known to improve oil quality by increasing phenolic compound extractability, while others such as lipase, polyphenoloxidases and peroxidase are known to cause detrimental effects (Palomares *et al*., 2003; Romo-Sanchez *et al*., 2010; Vichi *et al*., 2011; Migliorini *et al*., 2012). Therefore, the aim of the study was to investigate on the enzymatic capabilities of the yeast isolates belonging to species most frequently isolated from crushed pastes, kneaded pastes, oil from decanter and pomaces. The yeasts were isolated during 35 extra virgin olive oil extraction processes carried out in three consecutive years and to assess their abilities to modify the chemical composition of the olive oil.

3.4.2 Materials and methods

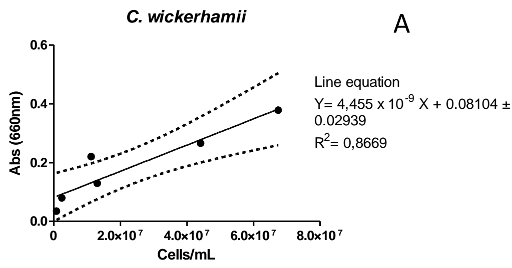
Zymogram screening for specific enzymatic activities

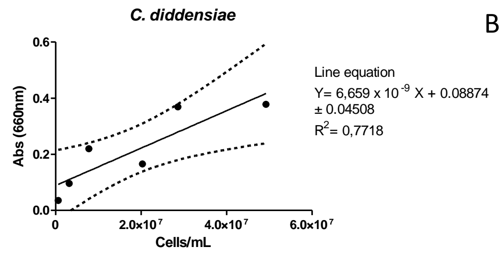
117 yeast isolates from different samples (crushed pastes, kneaded pastes, oil from decanter and pomaces) collected during the 35 extraction processes studied (paragraph 3.1), as reported in table 21, were screened for enzymatic activities relevant in the processing of olives and oils, and of potential interest in terms of product quality. Isolates (identified in paragraph 3.2) belonged to the following species: *Candida molendinolei, Candida wickeramii, Yamadazima terventina, Saccharomyces cerevisiae, Pichia kluyveri, Candida norvegica, Candida adriatica, Candida railenensis, Zygosaccharomyces fermentati, Zygotorulaspora mrakii, Candida diddensiae*. The enzymatic activities screened were: cellulase, polygalacturonase, ß-glucosidase, lipase and peroxidase; the substrates used were, respectively, carboxymethylcellulose (CMC), polygalacturonic acid, cellobiose, $CaCl_2$/Tween 80 and H_2O_2. All of them were purchased to Sigma Aldrich. As described by Romo-Sànchez et al. (2010), each isolate was grown in YEPD broth (yeast extract 10g/L; peptone 20g/L, D-glucose 20g/L) at 30 C° for 24 h. To check for lipase activity, cultures were inoculated into 0.1% virgin olive oil containing 0.01% Tween 80 broth. Cultures for checking cellulase and ß-glucosidase activity were then grown in a yeast nitrogen base (YNB) broth at 30 C° for 6 h under shaking conditions (100 rpm) for consumption of residual carbon source. Aliquots of 5 ml, containing 10^6 cells/mL each one, were used to inoculate agar plates containing YP (yeast extract 10g/L; peptone 20g/L, agar 15g/L)and 1% of each specific substrate as single carbon source. All inoculated substrate agar plates were incubated at 30° C for 2-3 days, except for lipase activity (5-7 days). The activity was detected for clear halo (for polygalactunorase, Fernández González et al., 2004), appearance of white precipitation areas (for lipase, Nuero et al., 1994), or growth (for cellulase and ß-glucosidase, Arévalo Villena et al., 2005). Peroxidase activity was evaluated by bubble formation from H_2O_2. An inoculated YP plates without any substrate was used as negative control.

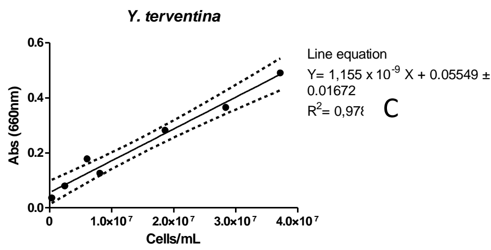
In order to standardize the yeast inoculums (aliquots containing 10^6 cells/mL) on the plates, a linear correlation between optical density and cell concentration of each species was calculated. The spectrophotometric measures was carried out at 660 nm, while cell concentration was obtained by microscope counting with the Thoma chamber. In Figure 21 are showed the linear correlations obtained for the 11 different yeast species examined.

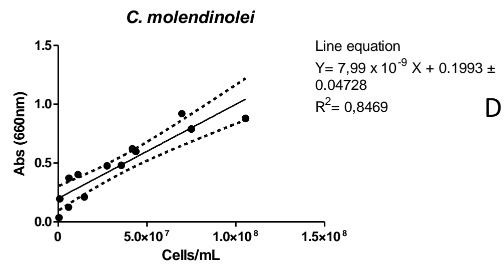
Table 21: Origin of the 117 yeast isolates from 35 olive oil extractive processes carried out during the harvest time in three consecutive years. CP: crushed pastes, KP: Kneaded pastes, OfD: oil from decanter, P: pomaces.

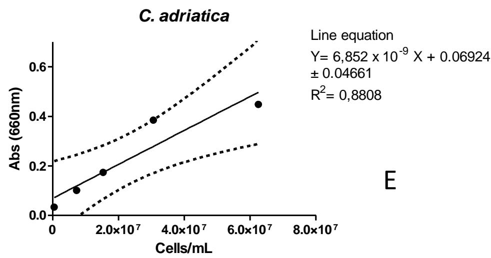
Yeast species	Origin	Crop season			Total isolates	
		2011	2012	2013		
C.molendinolei	CP	4	1	2	7	23
	KP		3	2	5	
	OfD	3	5	3	11	
Y.terventina	KP			1	1	15
	OfD	4	6	4	14	
C.diddensiae	CP	4			4	11
	KP		1	2	3	
	OfD	4			4	
C.wickerhamii	CP	4	1	2	7	21
	KP		2	2	4	
	OfD	2	5	3	10	
C.adriatica	CP	3		2	5	13
	OfD	1	4	3	8	
Z.mrakii	CP		1		1	4
	KP		3		3	
S.cerevisiae	CP	2			2	11
	KP		3	2	5	
	OfD	1		3	4	
Z.fermentati	CP	3			3	6
	P		1	2	3	
C.railenensis	CP	2			2	4
	KP			2	2	
P.kluyveri	KP		3	3	6	6
C.norvegica	CP		3		3	3

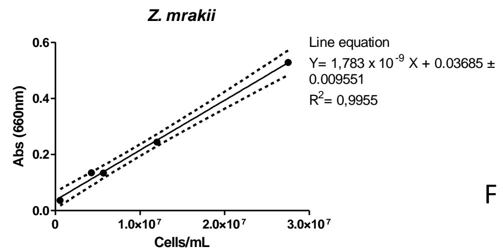

C. wickerhamii
A
Abs (660nm)
0.0
0.2
0.4
0.6
0
2.0x10^7
4.0x10^7
6.0x10^7
8.0x10^7
Cells/mL
Line equation
Y= 4,455 x 10^-9 X + 0.08104 ± 0.02939
R^2= 0,8669


C. diddensiae
B
Abs (660nm)
0.0
0.2
0.4
0.6
0
2.0x10^7
4.0x10^7
6.0x10^7
Cells/mL
Line equation
Y= 6,659 x 10^-9 X + 0.08874 ± 0.04508
R^2= 0,7718


Y. terventina
C
Abs (660nm)
0.0
0.2
0.4
0.6
0
1.0x10^7
2.0x10^7
3.0x10^7
4.0x10^7
Cells/mL
Line equation
Y= 1,155 x 10^-9 X + 0.05549 ± 0.01672
R^2= 0,97


C. molendinolei
D
Abs (660nm)
0.0
0.5
1.0
1.5
0
5.0x10^7
1.0x10^8
1.5x10^8
Cells/mL
Line equation
Y= 7,99 x 10^-9 X + 0.1993 ± 0.04728
R^2= 0,8469


C. adriatica
E
Abs (660nm)
0.0
0.2
0.4
0.6
0
2.0x10^7
4.0x10^7
6.0x10^7
8.0x10^7
Cells/mL
Line equation
Y= 6,852 x 10^-9 X + 0.06924 ± 0.04661
R^2= 0,8808


Z. mrakii
F
Abs (660nm)
0.0
0.2
0.4
0.6
0
1.0x10^7
2.0x10^7
3.0x10^7
Cells/mL
Line equation
Y= 1,783 x 10^-9 X + 0.03685 ± 0.009551
R^2= 0,9955

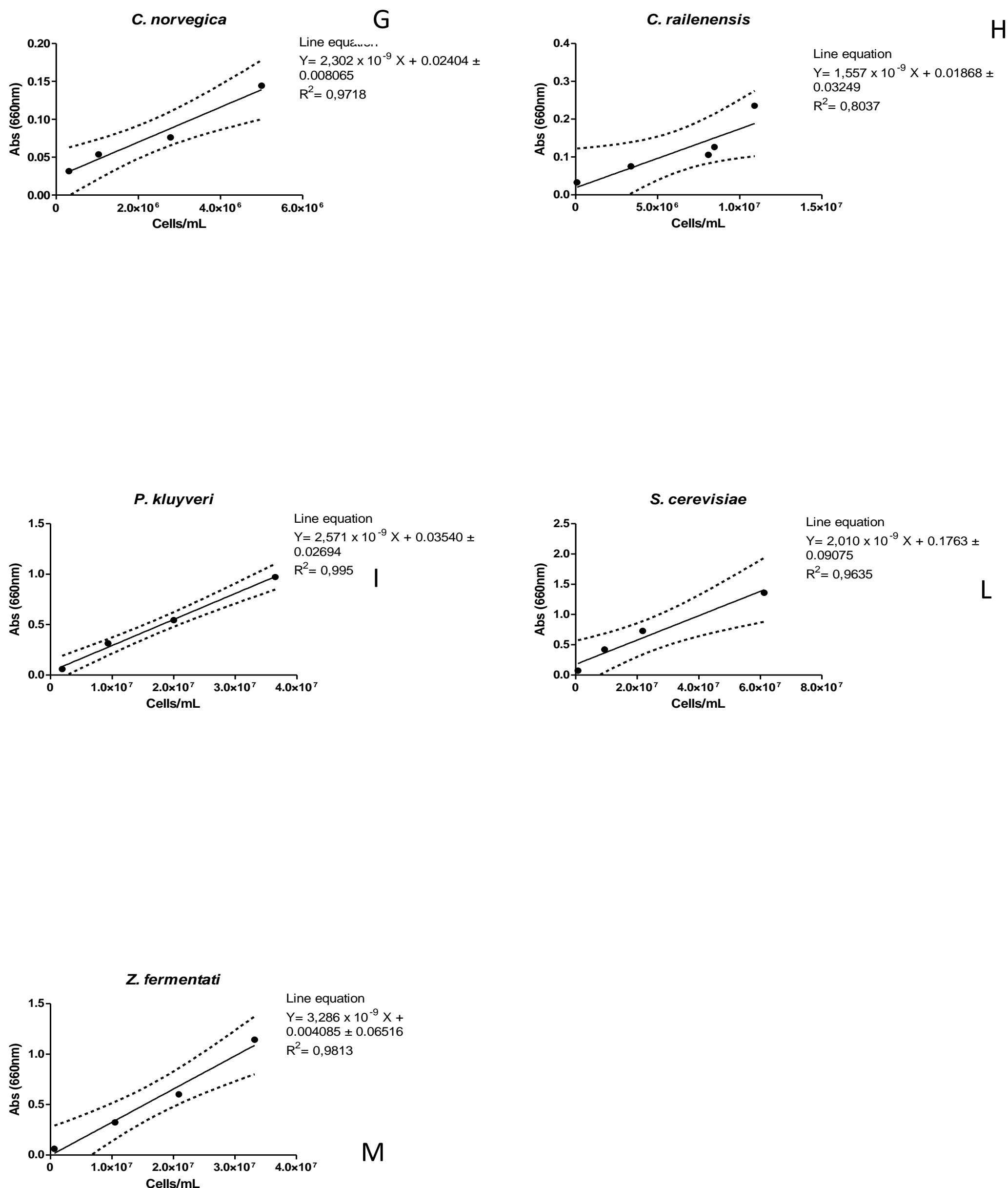


Figure 21.: Linear regressions between cells/mL, and optical density (spectrophotometric measures at 660 nm) measured during growth on YEPD liquid medium at 30 ° C of the various species of yeast. *A: Candida wickerhamii; B: Candida diddensiae*; C: *Yamadazyma terventina*; D: *Candida molendinolei: E: Candida adriatica; F: Zygotorulaspora mrakii; G: Candida norvegica*; H: *Candida railenensis*; I: *Pichia kluyveri ; L: Saccharomyces cerevisiae;* M: *Zygosaccharomyces fermentati*. For each yeast species is shown the line equation and its value of R^2 to evaluate the goodness of fit.

Yeast inoculation into pastes and commercial EVO

The pastes of olives used in the study were collected from a mill situated in Tuscany. The production process and the characteristics of the pastes complied with the olive oil Regulations 61-2011 EU. Crushed pastes were collected during a single production process of extra virgin olive oil. The olives belonged to *Frantoio* and *Moraiolo cultivars*. The pastes were immediately frozen and stored at -20 ° C.

The oil used for the study was a filtered extra virgin olive oil from the 2013 crop season, of a well known Italian brand of supermarkets.

Three yeast strains were chosen for the inoculation into crushed pastes and virgin olive oil: *Candida molendinolei* PG194-2013, *Candida wickeramii* DM15-2012 and *Yamadazima terventina* DFX3-2011.

Each pure isolate was grown in YEPD media until the early stationary phase (Fig. 22). Cells were separately inoculated into 280 g of crushed pastes, in order to have a final concentration of 10^6 cell/g. The pastes inoculated were placed in sterile bags, saturated with nitrogen and incubated at 30° C for 60 minutes (Fig. 23). In order to consider only changes due to the enzymatic action of microorganisms, two negative controls (pastes without any inoculums) were prepared: one before and one after the inoculation of the three strains. After incubation, the pastes were microbiologically analyzed, and then centrifuged at 4200 Xg for 10 minutes at a temperature of 20° C to obtain the oil which was subjected to chemical analysis (Fig. 24).The experiment was repeated three times and chemical analysis of each sample were performed in duplicate. Moreover, results obtained from the oils of the paste inoculated were compared with those of the pastes not inoculated.

The yeasts inoculation in commercial OEVO was performed with the procedure previously described. The final concentration of the yeasts inoculated was of 10^7 cell/mL. The oil inoculated (and the samples without inoculums as control) was placed in sterile glass tubes and bottles, in the dark at a temperature of 15° C and maintained in these conditions for 180 days (Fig. 25). After 3 hours, 30, 60 and 180days of

incubation, the oil was microbiologically analyzed and then centrifuged to remove impurities, collected into centrifuge tubes and then subjected to chemical analysis.

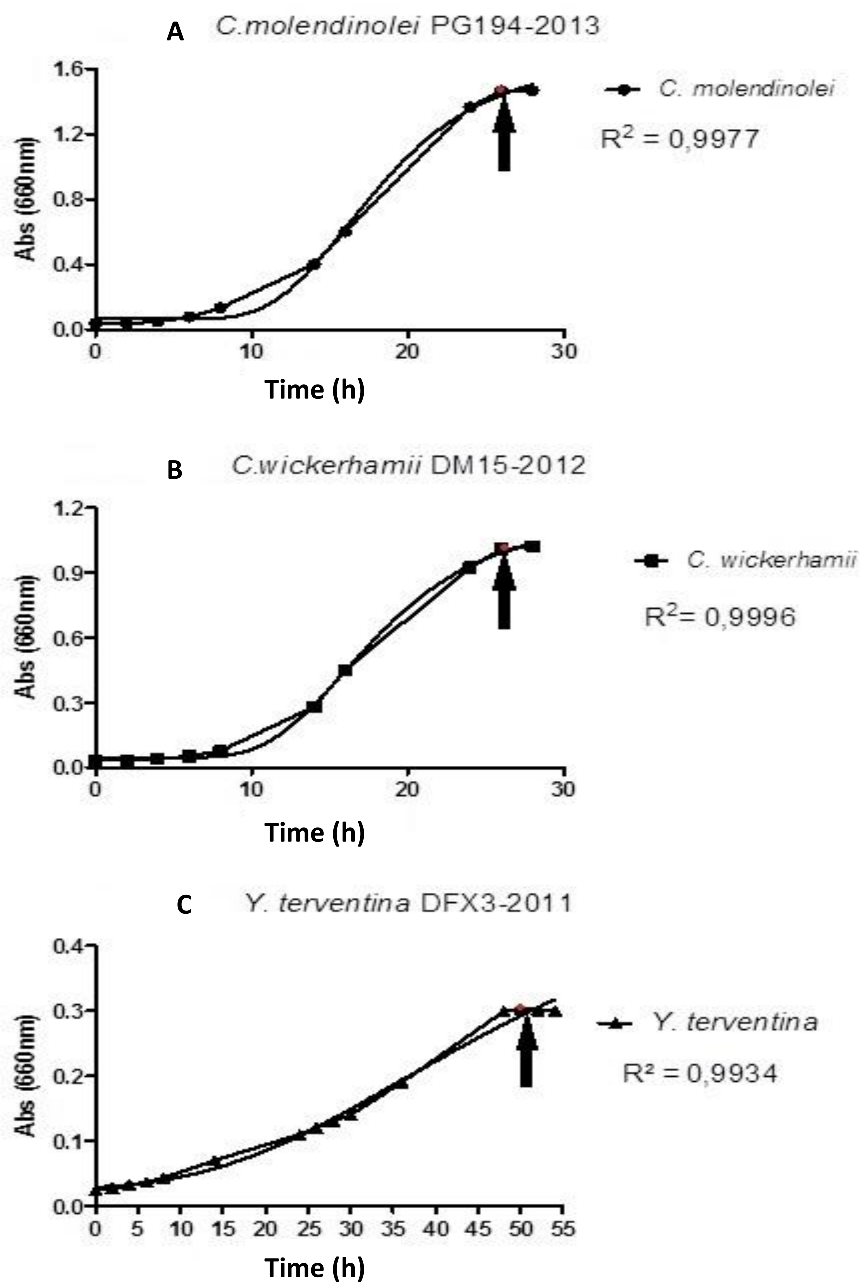


Figure 22: Growth curves of the strains in YEPD medium: A) *C. molendinolei* PG194-2013, B) *C. wickerhamii* DM15-2012, C) *Y.terventina* DFX3-2011. The horizontal axis shows the time in hours, on the ordinate the absorbance measured at 660 nm. The statistical value of R2 indicates the goodness of fit of Gompertz equation. The arrow indicates the time of collection of the culture for the inoculation.

Figure 23: The olive pastes inoculated in sterile bags and saturated with nitrogen before incubation.

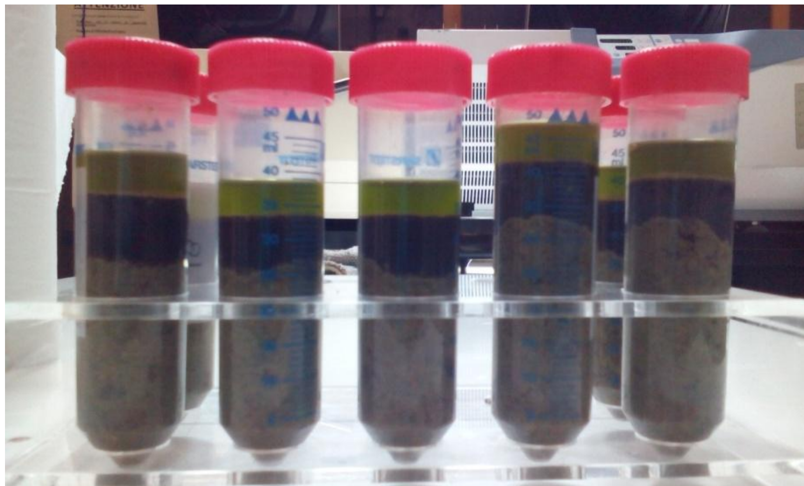


Figure 24: Pastes centrifuged in Falcon at 4200 Xg for 10 minutes at a temperature of 20° C. The oil is visible on the top, below the vegetable water and on the bottom the pomaces.

Figure 25: Commercial OEVO inoculated with the three yeast strains, placed in sterile glass tubes and bottles before storage in the dark.

Microbiological and chemical analysis of oils

Yeasts in pastes and oil inoculated were quantified on MYPG agar (malt extract 5g/L, yeast extract 3g/L; beef extract 5g/L, D-glucose 10g/L, agar 20g/L) plated after decimal dilutions in physiological solution (NaCl, 0.86 g/L).

Acidity, number of peroxides and determination of total polyphenols were performed on the oils samples.

The content of free fatty acids in olive oils was performed by dissolving 10g of oil in a mixture of ethanol-diethyl ether 1: 2. The mixture was then titrated with an ethanol solution of 0.1 N sodium hydroxide, using phenolphthalein as indicator. The result was expressed in g of oleic acid / 100 g of sample.

The number of peroxides was carried out by titration of a mixture consisting of an aliquot of 3g of sample dissolved in acetic acid and chloroform (3: 2), added of a solution of potassium iodide saturated, with standardized sodium thiosulphate solution. The result was expressed in meq O2 / 1000 g of sample.

The total polyphenols were determined by colorimetric method with Folin-Ciocalteau reagent. An extraction of the water-alcohol mixture with multiple mixture of hexane and methanol / water (80:20) was carried out, then the samples were transferred into

a flask with the addition of Folin and sodium carbonate at 20% , they were left to react overnight. The measurement was carried out through spectrophotometric reading at 765 nm index of the quantity of phenol present, their value increases with the coloration of the mixture. The concentration of total polyphenols was expressed in mg / kg of gallic acid.

The volatile compound content of the oils after two months of storage was performed as previously described (Paragraph 3.3.2) by PromoFirenze - Laboratorio Chimico Merceologico, Special Agency of the Florence Chamber of Commerce, Florence, Italy.

Fatty acid composition of oil

The fatty acids were extracted and methylated by the BF_3-methanol reagent (Supelco, Bellefonte, PA). One drop (~50 µl) of oil was taken in 6 ml test tube and 2 ml of the BF_3-methanol reagent was added under a nitrogen stream. After 5 minutes at 70°C, it was allowed to cool to room temperature and 1 ml of a saturated NaCl solution was added. The fatty acid methyl esters were extracted three times with 2 ml of petroleum ether. The combined petroleum ether extracts were reduced to dryness with a nitrogen stream and dissolved with 150 µl of n-hexane prior to injection. The fatty acid methyl esters were analyzed with a gas chromatograph (GC8380, Fisons Instruments, Milano, Italy) equipped with a flame ionization detector and a capillary column (SP 2380, 30m x 0.25 mm i.d.). The operating conditions were the following: injector temperature 250°C, split ratio 70:1, detector temperature 250°C, helium as carrier gas at a flow rate of 1.5 ml / min, injector volume of 1 µl, oven temperature from 90 to 220°C at 2.5 °C per min. Identification of fatty acid methyl esters was performed by comparing their relative retention times with standard mixtures (Supelco and Sigma).

Statistical analysis

Microbiological and chemical data were processed by analysis of variance (ANOVA, $p < 0.05$).

3.4.3 RESULTS

3.4.3.1. Zymogram screening for specific enzymes

117 yeast isolates were screened for cellulase, polygalacturonase, ß-glucosidase, lipase and peroxidase activity. Results of the strains studied are shown in table 22. As reported in other studies (Fernandez at al., 2000; Arévalo Villena et al., 2005) enzymatic activities were strain-dependent. The standardization of the inoculum had allowed to evaluate the intensity of the enzymatic activities (no activity, weak activity, moderate activity and strong activity) based on the spread of clear halo for polygalactunorase, the amount of white precipitation areas for lipase or growth for cellulase and ß-glucosidase.

All of the isolates assayed were cellulase negative, while only 2% of the assayed yeasts, belonging to *S. cerevisiae* species, were polygalacturonase positive (Tab. 22). Usually, these enzymatic activities are able to influence olive oil quality increasing antioxidant phenol compound levels, with a protective effect and a prolonging oil shelf life, and also hydrolyze olive cell-wall polysaccharides, with a yield increase (De Faveri et al., 2008).

More than half of the strains tested (52%) showed strong ß-glucosidase activity, 15% moderate activity, 17% weak activity and 16% no activity (Tab 22). All the strains belonging to *C. molendinolei* and *C.norvegica* displayed strong activity, while *C. diddensiae, C. adriatica, C. wickerhamii* and *P. kluiveri* showed respectively the 82%, 70%, 62% and 50% of isolates with strong activity. On the contrary, no strain of *Z. mrakii* showed ß-glucosidase activity (Tab.22). This enzymatic activity has an important role in the degradation of the main phenol compound in olives, oleuropeine, into simpler and no longer bitter compounds characterized by a high antioxidant activity (Ciafardini et al., 1994; Ciafardini and Zullo, 2002b).

The 65% of the strains tested displayed no lipase activity and in particular 11% a weak activity, 9% a moderate activity and 15% a strong activity (Tab.22). As reported by Ciafardini et al. (2006b), Zullo et al., (2010), and Ciafardini and Zullo (2015), a large number of the strains belonging to *Y. terventina, C.diddensiae, C. adriatica* and

S.cerevisiae displayed lipase activity; indeed, *Y. terventina* and *C. diddensiae* showed respectively the 53% and 64% of strains with strong lipase activity, while *C. adriatica* and *S.cerevisiae* only respectively the 7% and 9% (Tab.22). Yeasts with lipolytic activity could modify the nutritional composition of the oil through triglycerides hydrolysis, with the increase of diglyceride and the acidity levels (Cardenas et al., 2001; Zullo and Ciafardini, 2008).

All 117 strains displayed peroxydase activity (Tab.22). As shown by Gomez-Rico et al. (2008), this enzyme has a negative influence on olive oil quality due to oxidative degradation of the phenol compound present.

Table 22: Cellulase, polygalacturonase, ß-glucosidase, lipase and peroxidase activity of the 117 yeast isolates tested. The signs indicate the intensity of each enzymatic activity. -: no activity, +: weak activity, ++: moderate activity, +++: strong activity.

Yeast species	N. of isolates	Enzymatic activity																	
		Cellulase				Polygalacturonase				ß-glucosidase				Lipase				Peroxidase	
		-	+	++	+++	.-	+	++	+++	.-	+	++	+++	.-	+	++	+++	-	+
C.molendinolei	23	23				23							23	23					23
C. wickerhamii	21	21				21				4		4	13	20			1		21
Y. terventina	15	15				15				6	5	3	1		6	1	8		15
C. diddensiae	11	11				11					1	1	9	2	1	1	7		11
C. adriatica	13	13				13					1	3	9		4	8	1		13
S. cerevisiae	11	11				9			2	5	6			8	2		1		11
Z. fermentati	6	6				6					5	1		6					6
C. railanensis	4	4				4					2	2		4					4
P. kluyveri	6	6				6						3	3	6					6
C.norvegica	3	3				3							3	3					3
Z. mrakii	4	4				4				4				4					4
Total	117	117				115			2	19	20	17	61	76	13	10	18		117

3.4.3.2 Effects of yeast inoculation into olive pastes and commercial OEVO

Inoculation into olive pastes

The choice of the species to inoculate was performed taking into account the abundance of the individual species in the extraction process (Pharagraph: 3.2.2.4) Distribution of yeast species in the different extractive phases), which were: *Candida molendinolei* and *Candida wickeramii* into pastes and *Yamadazima terventina* into oil from decanter. The three yeast strains were chosen for their high enzymatic activity: *Candida molendinolei* PG194-2013 with strong peroxidase and glucosidase activities and no lipase activity; *Candida wickeramii* DM15-2012 and *Yamadazima terventina* DFX3-2011 displaying moderate β-glucosidase activity and strong peroxidase and lipase activities.

The three yeast strains were singularly inoculated (axenic cultures) into crushed pastes and two negative controls (pastes without any inoculums) were prepared: one before and one after the inoculation of the three strains. Acidity, number of peroxides and determination of total polyphenols performed on the inoculated or not inoculated oils obtained by centrifugation after 1 hour of incubation are reported in Figure 26.

As reported in Figure 26-A, the strains *C. wickeramii* DM15-2012 and *Y. terventina* DFX3-2011 were able to increase of about 25% the acidity of the oils compared to the samples without inoculation and the samples inoculated with *C. molendinolei* PG194-2013 (Fig. 26-A). These results confirmed the results obtained with the enzymatic screening. In fact, *C. wickeramii* DM15-2012 and *Y. terventina* DFX3-2011 showed lipase activity, while in *C. molendinolei* PG194-2013 this enzymatic capability was absent, as in all isolates of this species analyzed.

As regards the number of peroxides, no statistically significant difference was found between oils from inoculated and not inoculated pastes (Fig. 26-B).

On the contrary, all the three strains inoculated were able to significantly decrease the number of total polyphenols compared to the oils from not inoculated pastes (Fig. 26-C). *C. wickerhamii* DM15-2012 and *Y. terventina* DFX3-2011 showed a significant decreases in the content of total polyphenols (from 13000 to about 11000 mg / kg of

gallic acid) even if the most significant decrease was observed when *C. molendinolei* PG194-2013 was inoculated (from 13000 to less than 10000 mg / kg of gallic acid) (Fig. 26-C). These results seems to confirm that the enzymatic capability of yeasts could modify the quality of the olive oil. In fact, all the strains inoculated displayed ß-glucosidase activity, which directly affects the content of polyphenols and in particular, the reduction of oleuropein and ligstroside. Moreover, oils from pastes inoculated with the strain *C. molendinolei* PG194-2013, characterized by a high ß-glucosidase activity, showed a lower content of polyphenols than the other two strains assayed characterized instead by a moderate ß-glucosidase activity.

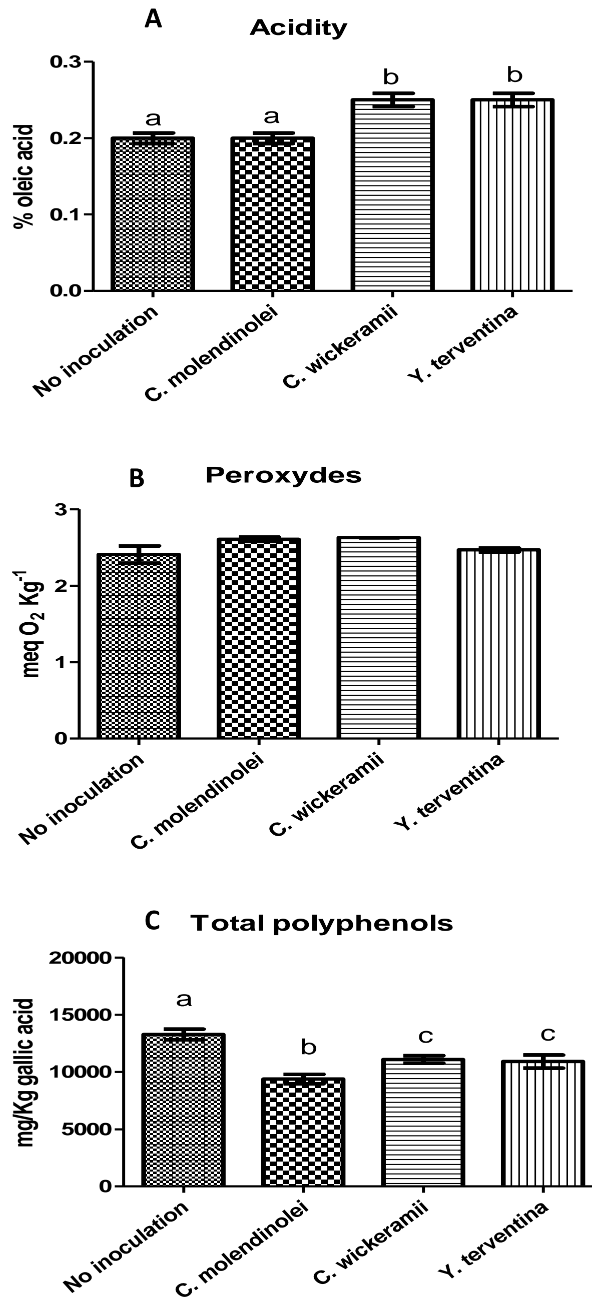


Figure 26: Chemical analysis of the oils obtained from olive pastes inoculated or not: A) oil acidity, B) peroxide value and C) total polyphenols. Different letters indicate significant differences (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found.

Inoculation into commercial OEVO

The three yeast strains previously described were singularly inoculated (axenic cultures) into commercial OEVO and a negative controls (oil without any inoculums) was prepared. Acidity, number of peroxides, total polyphenols and yeast viability, performed after 3 hours, 30 and 60 days of dark storage, are reported in Figure 27.

As shown in Figure 27 D, all isolates showed a mortality of 15% after the first three hours of incubation; after 30 days the yeast concentrations ranged between values of about 10^4 CFU/mL (*C. molendinolei* PG194-2013 and *C. wickerhamii* DM15-2012) and 10^5 CFU/mL (*Y. terventina* DFX3-2011). Finally, after 60 days of incubation the strain *C. molendinolei* PG194-2013 showed values of about 10^2 CFU/mL while *C. wickerhamii* DM15-2012 and *Y. terventina* DFX3-2011 of about 10^3 CFU/mL.

As regard the acidity (Fig. 27 A), *C. molendinolei* PG194-2013 and the control (sample without inoculum) did not shown any changes over time, on the contrary, the other two strains showed an increase during the first month of storage and then remained at the same levels of acidity in the following month.

No change was found between the control and *C. molendinolei* PG194-2013 and *C. wickerhamii* DM15-2012 strains as regards the number of peroxides. On the contrary, *Y. terventina* DFX3-2011, remaining for 30 days to higher concentrations (10^5 UFC/mL), showed an increase of about 17 meq O2 / 1000 g of sample during the first month of storage (Fig. 27 B).

All the assayed strains showed a decrease in polyphenol concentrations (expressed as mg / Kg of gallic acid) compared to the control (Fig. 27 C).

Statistical analysis (ANOVA, $p < 0.05$) conduced on the content of polyphenols, peroxides and acidity after 60 days of incubation between the uninoculated (control) and inoculated oils are shown in figure 28.

As reported for the inoculated pastes, both *C. wickerhamii* DM15-2012 and *Y. terventina* DFX3-2011 were able to increase the oil acidity (20%), while no significant difference occurred between *C. molendinolei* PG194-2013 and the control (Fig. 28 A).

Peroxides increased significantly (24%) only in the oil inoculated with *Y. terventina* DFX3-2011. This was the only strain that survived in a significant concentration (10^5 CFU/mL) after 30 days of incubation in oil (Fig. 28 B).
In agreement with the results obtained for the inoculation into pastes, total polyphenols decreased by 12% in all inoculated samples compared to control (Fig. 28 C).
Volatile compounds contents of olive oil samples, inoculated or not, after 60 days of storage were subdivided into chemical classes, as reported in Tables 23. Underlined volatile compounds are intermediate of LOX pathway and they are considered to be responsible for olive oil “fruity”, “grassy” and other positive attribute (Di Giacinto et al., 2010; Kotti et al., 2011; Aparicio et al., 2012). All the oils inoculated showed values of cis-3-hexenyl acetate, hexenal, trans-2-hexenal and trans-2-hexenol significantly lower than the control. On the contrary, the oil inoculated with *C. molendinolei* PG194-2013 showed value of trans-2-hexenyl acetate significantly higher than the other oils, including the control. Finally, cis-3-hexenol was detected in significantly lower concentration in the oils inoculated with *C. wickerhamii* DM15-2012 and *Y. terventina* DFX3-2011 than the oil inoculated with *C. molendinolei* PG194-2013 and the control.
With regard to the compounds related to the oil defects, as reported by Morales et al., 2005, (Paragraph 3.3.3.1, Tab. 15), all the inoculated oils showed a content of trans-2-heptenal, associated with "musty" and "rancid" defect, well above the odour threshold and significantly higher than the control. For some compounds different trends were noted. These depend on the strain inoculated:

1. the content of ethyl acetate, associated with "winey–vinegary" defect, was significantly higher then the control and detected above the odour threshold in the oil inoculated with *C. wickerhamii* DM15-2012, while the oils inoculated with *C. molendinolei* PG194-2013 and *Y. terventina* DFX3-2011 showed value significantly lower than the control;

2. propionic acid, associated with “fusty” defect, were significantly higher than the control and above the odour threshold in oils inoculated with *C. wickerhamii* DM15-2012 and *C. molendinolei* PG194-2013, while oil inoculated with *Y. terventina* DFX3-2011 showed value significantly lower than the control;
3. the oils inoculated with *C. wickerhamii* DM15-2012 and *Y. terventina* DFX3-2011 showed value of heptanoic acid significantly higher and above the odour threshold than the control and the oil inoculated with *C. molendinolei* PG194-2013;
4. two volatile compounds associated to "rancid" defect, butyric acid and trans-2-decenal, were above the odour threshold in oil inoculated respectively with *Y. terventina* DFX3-2011 and *C. wickerhamii* DM15-2012; in both cases the values were significantly higher than the other oils (including the control);
5. values of 1-octen-3-one, associated to “musty” defect, were significantly lower in the oil inoculated with *C. molendinolei* PG194-2013 than the other oils.

Finally, the composition of fatty acids, unsaturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids resulted not significantly different between non inoculated and inoculated oils after 60 days of storage (T-test, $p<0.05$).

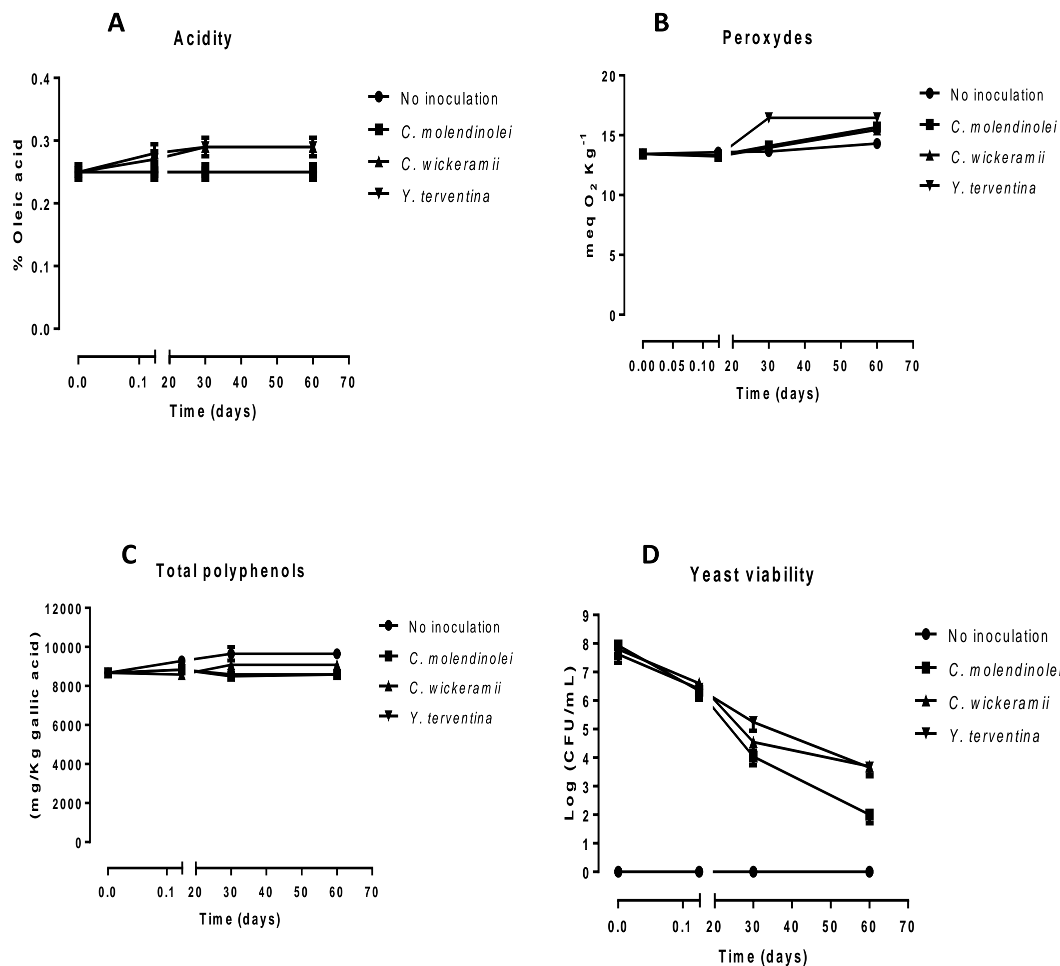


Figure 27: A) Oil acidity, B) number of peroxides, C) total polyphenol and D) vitality of yeast strains inoculated in the commercial OEVO during dark storage at 15° C. Analyses were performed after 3 hours, 30 and 60 days after the yeasts inoculation.

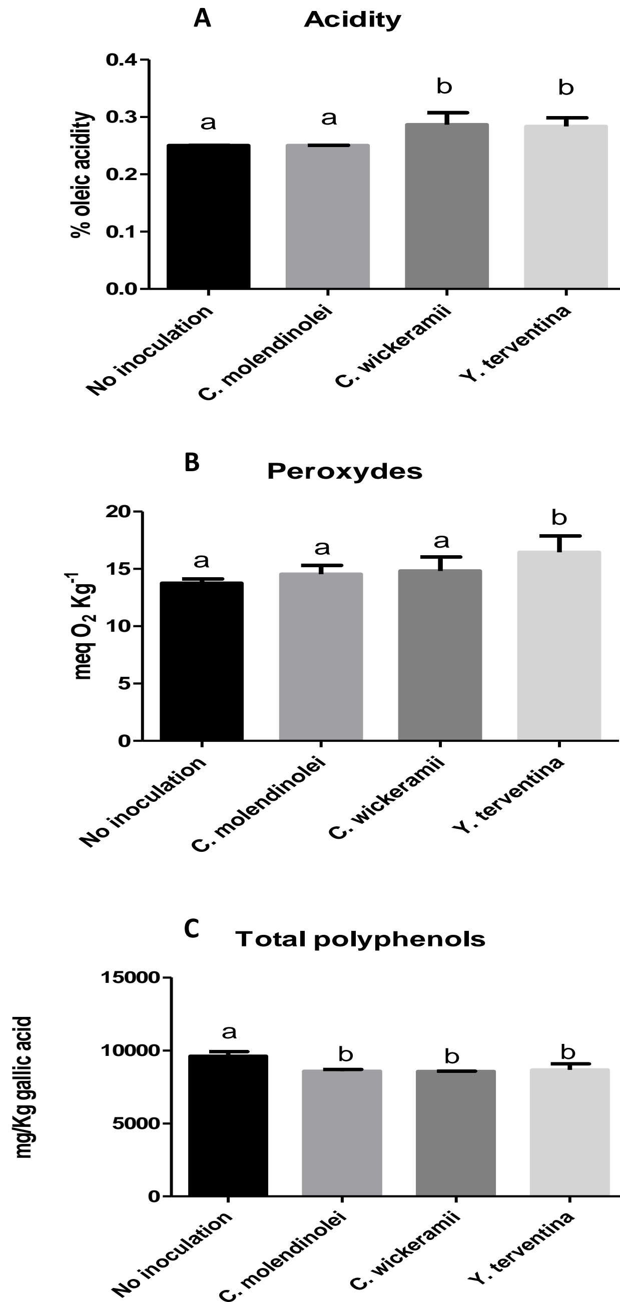


Figure 28: Chemical analysis of the commercial OEVO inoculated or not with different yeast strains: A) oil acidity, B) peroxide value and C) total polyphenols. Different letters indicate statistically significant differences (ANOVA; $p < 0.05$).

Table 23: Concentration of volatile compounds in extra virgin olive oil inoculated with the strains: *Yamadazima terventina* DFX3-2011, *Candida wickeramii* DM15-2012, *Candida molendinolei* PG194-2013, and the control (not inoculated) after 60 days of storage. Samples are encoded in relation to the chemical classes of the compounds analyzed. Different letters indicate significant differences between different extractive processes (ANOVA; $p < 0.05$); when no letter is reported, no significant difference was found. Underlined volatile compounds are intermediate of LOX pathway and they are considered to be responsible for olive oil positive attribute.

A. Class of esters, acids and hydrocarbons

	***Y. terventina* DFX3-2011** (mg/Kg)		***C. wikerhamii* DM15-2012** (mg/Kg)		***C. molendinolei* PG194-2013** (mg/Kg)		**Control** (mg/Kg)	
Component Name	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Methyl acetate	0.412[b]	0.012	0.574[c]	0.017	0.013[a]	0.0004	0.431[b]	0.013
Ethyl acetate	0.695[b]	0.055	1.039[d]	0.083	0.026[a]	0.002	0.906[c]	0.072
Hexyl_acetate	0.061	0.003	0.072	0.004	0.088	0.004	0.09	0.004
Cis-3-hexenyl_acetate	0.320[a]	0.013	0.353[a]	0.014	0.347[a]	0.14	0.397[b]	0.016
Trans-2-hexenyl_acetate	0[a]	0	0[a]	0	0.059[b]	0.002	0[a]	0
Ethyl-butirrate	0.019	0.0007	0.021	0.0008	0.016	0.0008	0.026	0.001
Ethyl-propionate	0.014	0.0005	0.011	0.0004	0.005	0.0002	0.01	0.0004
Methyl-propionate	0.019	0.007	0.025	0.001	0.038	0.001	0.017	0.0006
Butyric acid	0.226[b]	0.009	0.114[a]	0.004	0.108[a]	0.004	0.072[a]	0.003
Octanoic acid	0.811[c]	0.032	0.837[c]	0.033	0.248[b]	0.01	0.062[a]	0.002
Heptanoic acid	1.021[c]	0.04	0.430[b]	0.017	0[a]	0	0.017[a]	0.0006
Pentanoic acid	0.022	0.0008	0.038	0.001	0.022	0.0009	N/A	
Propionic acid	0.064[a]	0.002	0.884[d]	0.035	0.715[c]	0.028	0.112[b]	0.005
Heptan	0.013	0.0005	0.011	0.0004	0.01	0.0004	0.01	0.00004
Octan	0.650[c]	0.032	0.579[b]	0.029	0.519[a]	0.023	0.524[a]	0.026

B. Class of aldehydes

	Y. terventina **DFX3-2011** (mg/Kg)		***C. wikerhamii*** **DM15-2012** (mg/Kg)		***C. molendinolei*** **PG194-2013** (mg/Kg)		**Control** (mg/Kg)	
Component Name	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Valeraldeide	0.032^{a}	0.001	0.046^{a}	0.002	0.071^{a}	0.003	0.201^{b}	0.01
Isovaleraldeide	0^{a}	0	0^{a}	0	0^{a}	0	0.116^{b}	0.005
Hexanal	0.419^{a}	0.02	0.429^{a}	0.021	0.453^{a}	0.022	0.847^{b}	0.042
2-methyl-butanal	0.021^{a}	0.002	0.052^{a}	0.005	0.043^{a}	0.004	0.209^{b}	0.02
Trans-2-pentenal	0.021	0.0002	0.018	0.0001	0.017	0.0001	0.043	0.0004
Cis-3-hexenal	0.055	0.001	0.053	0.001	0.052	0.001	0.064	0.001
Heptanal	0^{a}	0	0^{a}	0	0^{a}	0	0.190^{b}	0.007
Trans-2-hexenal	0^{a}	0	0^{a}	0	0^{a}	0	0.744^{b}	0.007
Octanal	0.299^{a}	0.012	0.402^{c}	0.016	0.352^{ab}	0.014	0.310^{a}	0.012
Trans-2-heptenal	0.861^{c}	0.034	1.052^{d}	0.042	0.647^{b}	0.026	0.543^{a}	0.022
Trans-2-decenal	0^{a}	0	0.470^{b}	0.019	0a	0	0^{a}	0
2,4-decadienal	0.039	0.001	0.073	0.003	0.059	0.002	0.024	0.0009
2,4-heptadienal	0.027	0.001	0.035	0.001	0.022	0.001	0	0
2,4-nonadienal	0.085^{b}	0.003	0.151^{c}	0.006	0.036^{a}	0.001	0^{a}	0

C. Class of alcohols

Component Name	*Y. terventina* DFX3-2011 (mg/Kg)		*C. wikerhamii* DM15-2012 (mg/Kg)		*C. molendinolei* PG194-2013 (mg/Kg)		Control (mg/Kg)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1_penten-3-ol	0.353[c]	0.01	0.145[a]	0.004	0.262[b]	0.007	0.233[b]	0.007
Trans-2-hexenol	0.459[a]	0.006	0.432[a]	0.006	0.473[a]	0.007	0.633[b]	0.009
Pentanol	0.037[ab]	0.003	0.044[bc]	0.004	0.083[c]	0.008	0[a]	0
Hexanol	0.578[a]	0.011	0.684[b]	0.013	0.830[c]	0.016	0.687[b]	0.014
Octanol	0.024[a]	0.001	0.058[a]	0.003	0.129[b]	0.006	0.056[a]	0.002
Trans-3-hexenol	0.061	0.002	0.052	0.002	0.056	0.002	0.038	0.001
Cis-3-hexenol	0.806[a]	0.016	0.904[b]	0.018	1.045[c]	0.021	1.025[c]	0.02
Cis-2-pentenol	0.167	0.003	0.147	0.002	0.137	0.002	0.126	0.002
Trans-2-pentenol	0.022	0.0003	0	0	0	0	0.044	0.0006
2-e-3-methyl-1-butanol	0.079	0.003	0.07	0.003	0.067	0.002	0.073	0.003
Phenyl_ethanol	0.359[b]	0.014	0.294[a]	0.011	0.274[a]	0.011	0.290[a]	0.011
Heptanol	0.018	0.0007	0.022	0.0008	0.024	0.0009	0.02	0.0008
Iso-butanol	0.015	0.0006	0	0	0	0	0	0
Nonanol	0[a]	0	0.156[b]	0.006	0.156[b]	0.006	0[a]	0
Propanol	0		0		0.003	0.0001	0.032	0.001

D. Class of ketones and phenols

	Y. terventina **DFX3-2011** (mg/Kg)		***C. wikerhamii*** **DM15-2012** (mg/Kg)		***C. molendinolei*** **PG194-2013** (mg/Kg)		**Control** (mg/Kg)	
Component Name	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2-butanone	0.025	0.0005	0.032	0.0006	0.019	0.0004	0.008	0.0001
2_octanone	0.156[d]	0.006	0.070[b]	0.003	0.102[bc]	0.004	0[a]	0
1-octen-3-one	0.08[b]	0.003	0.097[b]	0.004	0.029[a]	0.001	0.073[b]	0.003
3-pentanone	0.393[b]	0.011	0.596[d]	0.017	0.463[c]	0.014	0.299[a]	0.008
Ethyl_vinil_ketone	0.033[a]	0.0003	0.028[a]	0.0002	0.032[a]	0.0003	0.115[b]	0.001
6-methyl-5-hepten-2-one	0.114[bc]	0.004	0.151[c]	0.006	0.087[b]	0.003	0[a]	0
Guaiacol	0	0	0	0	0	0	0.005	0.0002
Phenol	0.149[c]	0.004	0.085[b]	0.002	0.013[a]	0.0004	0.014[a]	0.0004
Ethyl_guaiacole	0.074	0.003	0.065	0.002	0.067	0.002	0.066	0.002
4-ethyl-phenol	0.111	0.004	0.129	0.005	0.087	0.003	0.131	0.005

Table 24: Fatty acid composition (mean±standard deviation of Area %) and some calculated parameters of extra-virgin olive oil (UFAs: unsaturated fatty acids; MUFAs: mono-unsaturated fatty acids; PUFAs: poly-unsaturated fatty acids) in extra virgin olive oil inoculated with the strains: *Yamadazima terventina* DFX3-2011, *Candida wickeramii* DM15-2012, *Candida molendinolei* PG194-2013, and the control (not inoculated) after 60 days of storage.

Parameters	**Control**	***Y. terventina* DFX3 2011**	**t-test**	***C. wikerhamii* DM15 2012**	**t-test**	***C. molendinolei* PG194 2013**	**t-test**
C16:0	11.5±0.5	11.0±0.0	ns[a]	11.2±0.5	ns	11.4±1.3	ns
C16:1Δ9	0.8±0.0	0.7±0.1	ns	0.8±0.1	ns	0.7±0.0	ns
C16:2n4	0.1±0.1	0.2±0.0	ns	0.2±0.1	ns	0.1±0.0	ns
C18:0	3.0±0.1	3.1±0.2	ns	3.0±0.0	ns	2.9±0.1	ns
C18:1 Δ9	76.4±0.2	77.3±0.2	ns	77.0±0.4	s	77.4±1.2	ns
C18:1 Δ11	2.5±0.3	2.1±0.3	ns	2.2±0.1	ns	1.8±0.0	ns
C18:2cis	4.6±0.1	4.7±0.0	ns	4.8±0.0	ns	4.7±0.0	ns
C18:3αn3	0.6±0.0	0.7±0.0	ns	0.7±0.0	ns	0.7±0.0	ns
C20:0	0.5±0.2	0.3±0.0	ns	0.3±0.1	ns	0.3±0.0	ns
C18:1/C18:2	17.0±0.2	16.9±0.0	ns	16.5±0.0	ns	16.7±0.2	ns
UFAs	85.0±0.6	85.6±0.2	ns	85.6±0.4	ns	85.4±1.2	ns
MUFAs/PUFAs	14.7±0.1	14.5±0.1	ns	14.2±0.1	ns	14.4±0.3	ns

[a]ns: not significantly different for t-test ($p<0.05$) between non inoculated and inoculated oils.

3.4.4 Conclusion

Some olive oil yeast strains are considered useful, as they are able to hydrolyze the bitter tasting secoiridoid compound of the oil, whereas others are considered harmful, as they can damage the quality of the oil (Zullo et al., 2013). In this study:

1. All the 117 assayed isolates resulted peroxidase positive. As reported by Gomez-Rico et al. (2008), this activity has a negative influence on olive oil quality because it is responsible of the oxidative degradation of the phenol compounds.
2. The 66% of the assayed isolates were β-glucosidase positive. The β-glucosidase activity has an important and useful role in the degradation of the main phenol compound in olives, oleuropeine, into simpler and no longer bitter compounds characterized by a high antioxidant activity (Ciafardini et al., 1994; Ciafardini and Zullo, 2002b).
3. The 22% of the isolates were lipase positive. Lipolytic activity could modify the nutritional composition of the oil through triglycerides hydrolysis, with the increase of diglyceride and the acidity levels (Cardenas et al., 2001; Zullo and Ciafardini, 2008).
4. All the 117 assayed isolates were cellulose negative and only the 2% of the isolates were polygalacturonase positive. These two enzymes are able to influence olive oil quality increasing antioxidant phenol compound levels, with a protective effect and a prolonging oil shelf life, and also hydrolyze olive cell-wall polysaccharides, with a yield increase (De Faveri et al., 2008).

Therefore, most of the chemical modifications of the oil caused by yeasts are to be considered negative for oil quality.

Three strains, *Candida molendinolei* PG194 with high peroxidase and glucosidase activities; *Candida wickeramii* DM15 and *Yamadazima terventina* DFX3 displaying high β-glucosidase, peroxidase and lipase activities, were separately inoculated in crushed pastes and filtered extra virgin olive oil to investigate their influence on the oil quality. After 1 hour of incubation, crushed pastes were centrifuged and the oils obtained were analyzed (acidity level, peroxide value, total polyphenols, yeast concentrations)

and statistically compared with the control (oil incubated without yeast inoculation). The strains *C. wickeramii* DM15-2012 and *Y. terventina* DFX3-2011 were able to increase of about 25% the acidity of the oils compared to the sample without inoculation and the sample inoculated with *C. molendinolei* PG194-2013. As regards the number of peroxides, no statistically significant difference was found between oils obtained from inoculated and not inoculated pastes. On the contrary, all the three strains inoculated were able to significantly decrease (about 15%) the number of total polyphenols compared to the oils from not inoculated pastes. After two months of storage, the filtered extra virgin olive oils were analyzed (acidity level, peroxide value, total polyphenols, yeast concentrations) and statistically compared with the control (oil incubated without yeast inoculation). The acidity level of the oil was about 20% higher when *C. wickeramii* DM15 and *Y. terventina* DFX3 were present. Peroxide values increased (20%) only in the presence of *Y. terventina* DFX3, while total polyphenols decreased (about 10%) independently of the inoculated yeast strain.

Therefore, the presence of yeasts both in the pastes and in the oil have had negative effects on the oil quality.

In order to confirm the significant correlations between yeast concentrations of oil from decanter and the aromatic compounds of oil (paragraph 3.3.3.3), a comparison between the results obtained from the yeasts inoculated oils has been made.

The inoculated oils showed values of aromatic compounds related to olive oil positive attribute (cis-3-hexenyl acetate, hexenal, trans-2-hexenal and trans-2-hexenol), which were significantly lower than the control. These results confirmed the correlation studies (table 19, paragraph 3.3.3.3). As a matter of fact, negative correlations between yeast concentrations in oil from decanter and the same volatile compounds were found. These findings confirmed the results of Zullo et al. (2013), which reported that olive oil samples inoculated with yeasts showed lower concentration of total C6 volatile carbonyl compounds and hexanal, responsible for positive olive oil sensory attributes, if compared to the uninoculated sample.

Concentrations of trans-2-heptenal, associated with "musty" and "rancid" defects, well above the odour threshold and significantly higher than the control were found in all inoculated oils. On the contrary, no correlation was found between this compound and yeast concentrations in oil from decanter (table 19, paragraph 3.3.3.3).

In the experimental inoculated oil, compounds related to olive oil defects, such as ethyl acetate, propionic acid, heptanoic acid, butyric acid, trans-2-decenal and 1-octen-3-one, showed significantly lower or higher values than the control. This was depending from the inoculated species. However, the aromatic composition of oil from decanter taken from a real extraction process was influenced by the combination of various yeast species. Consequently, the results obtained from the two studies, real oil extraction processes (paragraph 3.3.3.3) and inoculated oil (paragraph 3.4.3.2), did not agree because the biodiversity of the yeasts was higher in the real processes than in the experimental ones.

However, Zullo et al. (2013) found in olive oil samples treated with *Candida adriatica, C. wickerhamii and C. diddensiae* specific strains, muddy-sediment, rancid or both defects.

These findings demonstrate that the presence of yeasts with specified enzymatic activities, may negatively affect the chemical composition of olive oil during the storage.

REFERENCES

Arévalo Villena M., Úbeda J.F., Cordero Otero R.R., Briones A.I. 2005. Optimisation of a rapid method for studying the cellular location of b-glucosidase activity in wine yeasts. Journal of Applied Microbiology, 99, 558-566.

Cardenas F., Alvarez E., de Castro-Alvarez M.S., Sánchez-Montero J.M., Valmaseda M., Élson S.W., Sinisterra J.V. 2001. Screening and catalytic activity in organic synthesis of novel fungal and yeast lipases. Journal of Molecular Catalysis B*:* Enzymatic, 14, 111-123.

Ciafardini G., Marsilio V., Lanza B., Pozzi N. 1994. Hydrolysis of oleuropein by Lactobacillus plantarum strains associated with olive fermentation. Applied and Environmental Microbiology, 60(11), 4142-4147.

Ciafardini, G., Zullo, B.A., 2002a. Survival of microorganisms in extra virgin olive oil during storage. Food Microbiology, 19, 105-109.

Ciafardini G., Zullo B.A. 2002b. Microbiological activity in stored olive oil. International Journal of Food Microbiology, 75, 111–118.

Ciafardini G., Zullo B.A., Iride A. 2006a. Lipase production by yeasts from extra virgin olive oil. Food Microbiology, 23, 60–67.

Ciafardini G., Zullo B.A., Cioccia G., Iride A. 2006b. Lipolytic activity of Williopsis californica and Saccharomyces cerevisiae in extra virgin olive oil. International .Journal of Food Microbiology, 107, 27–32.

Ciafardini G., Zullo B.A. 2015 Effect of lipolytic activity of *Candida adriatica, Candida diddensiae* and *Yamadazyma terventina* on the acidity of extra-virgin olive oil with a different polyphenol and water content. Food Microbiology, 47, 12-20.

De Faveri D., Aliakbarian B., Avogadro M., Perego P., Converti A. 2008. Improvement of olive oil phenolics content by means of enzyme formulations: effect of different enzyme activities and levels. Biochemical Engineering Journal, 41, 149-156.

Fernández, M., Úbeda Iranzo, J.F., Briones Pérez A.I. 2000. Typing of non-Saccharomyces yeasts with enzymatic activities of interest in winemaking. International Journal of Food Microbiology, 59, 29-36.

Fernández González M., Úbeda J., Vasudevan T.G., Cordero Otero R., Briones, A. 2004. Evaluation of polygalacturonase activity in Saccharomyces wine strains. FEMS Microbiology Letters, 237, 261-266.

Gomez-Rico A., Fregapane G., Salvador M.D. 2008. Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils. Food Research International, 41, 433-440.

Migliorini M., Cecchi L., Cherubini C., Trapani S., Cini E. and Zanoni B. 2012. Understanding degradation of phenolic compounds during olive oil processing by inhibitor addition. European Journal of Lipid Science *and* Technology*,* 114, 942-950.

Nuero O.M., Garcia-Lepe R., Lahoz M.C., Santamaria F., Reyes F., 1994. Detection of lipase activity on ultrathin-layer isoelectric focusing gels. Analytical Biochemistry, 222, 503-505.

Palomares O., Villalba M.M. and Rodriguez R. 2003. The C-terminal segment of the 1,3-β-glucanase Ole e 9 from olive (*Olea europea*) pollen is an indipendent domain with allergenic activity: expression in *Pichia pastoris* and characterization. Biochemical Journal, 369, 593.

Romo-Sánchez S., Alves-Baffi M., Arévalo-Villenaa M., Úbeda-Iranzoa J., Briones-Pérez A. 2010. Yeast biodiversity from oleic ecosystems: Study of their biotechnological properties. Food Microbiology 27, 487-492.

Vichi S., Romero A., Tous J. and Caixach J. 2011. The activity of healthy olive microbiota during virgin olive oil extraction influences oil chemical composition. Journal of Agricultural and Food Chemistry, 59, 4705-4714.

Zullo B.A. and Ciafardini G. 2008. Lypolitic yeast distribution in commercial extra virgin olive oil. Food Microbiology, 25, 970-977.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2010. Distribution of dimorphic yeast species in commercial extra virgin olive oil. Food Microbiology. 27, 1035-1042.

Zullo, B.A., Cioccia, G., Ciafardini, G., 2013. Effects of some oil-born yeasts on the sensory characteristics of Italian virgin olive oil during its storage. Food Microbiol. 36, 70-78.

Session 2A: Yeasts in food biotechnology: biodiversity and ecology in foods and beverages

Enzymatic capabilities of oil-born yeasts and their impact on olive oil quality during its storage

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The olive oil microbiota is mainly composed of yeasts. Some olive oil yeasts are considered useful, as they are able to hydrolyze the bitter tasting secoiridoid compound of the oil, whereas others are considered harmful, as they can damage the quality of the oil (Zullo et al 2013). To assess the incidence of these abilities in oil-born yeasts, 117 yeast isolates coming from pastes, centrifuged oil and pomaces, collected during 35 olive oil extraction processes carried out in the same oil mill during three different harvest years, were taken into consideration. The yeasts were at first identified by using PCR-RFLP of rITS and sequencing rRNA genes (11 species in total) and then assayed for β-glucosidase, cellulase, polygalacturonase, peroxidase and lipase activities. All of the isolates were peroxidase positive and cellulase negative, while β-glucosidase, lipase and polygalacturonase activities were found in 66, 22 and 2% of the assayed yeasts, respectively. Three strains, *Candida molendinolei* PG194 with high peroxidase and glucosidase activities; *Candida wickeramii* DM15 and *Yamadazima terventina* DFX3 displaying high β-glucosidase, peroxidase and lipase activities, were separately inoculated in filtered olive oil to investigate their influence on the oil quality. After two months, the oils were analyzed (acidity level, peroxide value, total polyphenols, yeast concentrations) and statistically compared with the control (oil incubated without yeast inoculation). The acidity level of the oil was about 20% higher when *C. wickeramii* DM15 and *Y. terventina* DFX3 were present. Peroxide values increased (20%) only in the presence of *Y. terventina* DFX3, while total polyphenols decreased (about 10%) independently of the inoculated yeast strain. These findings show that enzymatic activities of oil-born yeasts may negatively affect the chemical composition of olive oil during the storage.

KEYWORDS: Olive oil quality, Yeasts, Enzymatic activities

REFERENCES:

Zullo BA, Cioccia G, Ciafardini G(2013). Effects of some oil-born yeasts on the sensory characteristics of Italian virgin olive oil during its storage. Food Microbiology 36: 70-78

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INTRODUCTION

The microbiota of the olive oil extraction process is mainly composed of yeasts[1]. Some oil-born yeasts are considered useful, as they are able to hydrolyze the bitter tasting secoiridoid compound of the oil, whereas others are considered harmful, as they can damage the quality of the oil[2].

AIM

To assess the incidence of oil-born yeast abilities to modify the chemical composition of the olive oil.

MATERIALS AND METHODS

1. 117 yeast isolates coming from pastes (a: crushed pastes, b: kneaded pastes), centrifuged oil (c) and pomaces (d) (Fig. 1), collected during 35 olive oil extraction processes carried out in the same oil mill during three different harvest years, were assayed.
2. The yeasts were identified by using PCR-RFLP of rITS (data not shown) and sequencing rRNA genes (Tab. 1).
3. The yeasts were assayed for β-glucosidase, cellulase, polygalacturonase, peroxidase and lipase activities as reported by Sanchez et al. (2010); acidity level, peroxide value, total polyphenols were measured according to EU official method (EC Reg. 1989/2003); yeast concentrations were quantified on MYPG agar after three days of incubation at 30°C.

Fig. 1

Tab.1

Species	Sequence of D1/D2 region of isolates and type strains (Matching nucleotides; identity %)
Candida molendinoiei	508/508 (100)
Candida wickerhamii	552/553 (100)
Yamadazyma terventina	508/511 (99.4)
Candida diddensiae	511/512 (99.8)
Candida adriatica	550/550 (100)
Saccharomyces cerevisiae	558/558 (100)
Lachancea fermentati	552/554 (99.6)
Candida oleiensis	540/541 (99.8)
Pichia kluyveri	556/556 (100)
Candida norvegica	556/558 (99.6)
Zygotorulaspora [illegible]	565/565 (100)

Tab. 2

		Level of enzymatic activity														
		Cellulase	Polygalacturonase				β-glucosidase				Lipase				Peroxidase	
Yeast species	N° of isolates	-	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+
C. molendinoiei	33	33	33						2	31	33					33
C. wickerhamii	21	21	21				8	2	11		20			1		21
Y. terventina	16	16	16				13	2	1		8	1	1	6		16
C. diddensiae	11	11	11				1	1	4	5	3		5	3		11
C. adriatica	13	13	13				1	3	4	5	4	8		1		13
S. cerevisiae	11	11	9		1	1	11				10	1				11
L. fermentati	8	8	8				7	1			8					8
C. oleiensis	4	4	4				2	2			4					4
P. kluyveri	6	6	6						3	3	6					6
C. norvegica	3	3	3						3		3					3
Z. [illegible]	4	4	4				4				4					4
Total	117	117	115		1	1	40	22	28	26	91	8	7	10		117

RESULTS

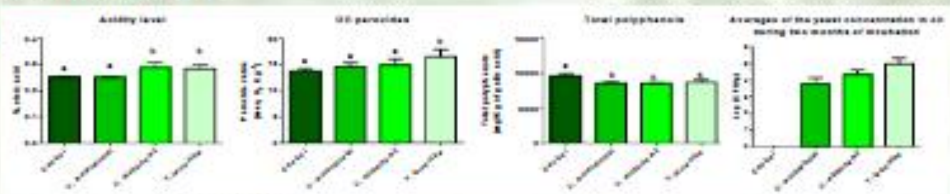
1. All of the isolates were peroxidase positive and cellulase negative, while β-glucosidase, lipase and polygalacturonase activities were found in 66, 22 and 2% of the assayed yeasts, respectively (Tab. 2).
2. Three strains (Tab. 3: *C. molendinoiei* PG194 with high peroxidase and glucosidase activities; *C. wickerhamii* DM15 and *Y. terventina* DFX3 displaying high β-glucosidase, peroxidase and lipase activities) were separately inoculated (concentration of 10^5 UFC/g^{-1}) in filtered extra-virgin olive oil (N_2 atmosphere, dark bottles) to investigate their influence on the oil quality.

Tab. 3

Species	Candida molendinoiei	Candida wickerhamii	Yamadazyma terventina
Isolate	PG194	DM15	DFX3
Origin	Pastes	Oil from decanter	Oil from decanter
Enzymatic activity			
Peroxidase	+	+	+
Lipase	-	+++	+++
β-glucosidase	+++	++	+++

3. After two months, the oils were analyzed (acidity level, peroxide value, total polyphenols, yeast concentration) and statistically compared (ANOVA, p<0.05) with the control (oil incubated without yeast inoculation). The acidity level of the oil was about 20% higher when *C. wickerhamii* DM15 and *Y. terventina* DFX3 were present. Peroxide values increased (20%) only in the presence of *Y. terventina* DFX3, while total polyphenols decreased (about 10%) independently of the inoculated yeast strain (Fig. 2).

Fig. 2



CONCLUSIONS

The enzymatic activities of oil-born yeasts may negatively affect the chemical composition of olive oil during the storage.

4. FINAL CONCLUSION

The aim of the thesis was to investigate on the yeast microbiota occurring in olive oil extraction process and in particular the impact of the yeast population on the olive oil quality.

The thesis is articulated in four parts:

Investigation on the microbiota of the olive oil extraction process (Chapter 3.1)

In this part 35 olive oil extraction processes were considered to investigate the yeast microbiota and its impact on olive oil quality. The samples have been carried out in the same manufacture located in Tuscany during different days of the harvest time in three consecutive crop seasons (2011, 2012 and 2013).

The microbiota occurring in the extra virgin olive oil extraction process was composed mainly by yeasts and the microbial concentrations in the samples, analyzed in the three years, ranged between values below 10 and above 10^5 CFU/g and the filtered olive oil showed microbial cell counts lower than 10^2 CFU/100 mL. Correlation studies demonstrated that yeast cell densities in olive paste and in oil from decanter were not statistically related, suggesting that yeast growth could be encouraged by malaxation and/or "decanting" steps. Indeed, in the three consecutive years considered, the yeast concentration in the pomaces resulted statistically higher than in pastes and oil from decanter suggesting a possible accumulation of yeasts during the subsequent centrifugations of the kneaded pastes in the two-phase decanter.

Yeast species biodiversity of olive oil extraction process (Chapter 3.2)

In this second part yeast isolates occurring in the different phases of the extraction process were isolated and identified. A reproducible molecular method for differentiating the yeast species from olive oil environment was provided.

The dominant yeast species identified on the process were seventeen: *C. norvegica*, *C. adriatica*, *C. diddensiae, C. railenensis*, *C. molendinolei, C. wickerhamii*, *C. oleophila*, *L. fermentati, M. fructicola, P. kluyveri, P. manshurica, R. glutinis, R. mucilaginosa, R. sloffiae*, *S. cerevisiae*, *Y. terventina* and *Z. mrakii*. The dominant yeast species detected

on the washed olives were eleven but only three of them, were also found in oil samples at significant isolation frequencies (*C. adriatica*, *C. molendinolei*, and *C. wickerhamii)*. On the contrary, same yeast species showed significant isolation frequencies only in oil samples, such as *Y. terventina*, or in kneaded pastes and pomaces, such as *Z. mrakii*. These observations suggested a possible contamination of the plant for oil extraction (malaxation equipment and decanter in particular) that might select some yeast species at the expense of others. Finally, the application of RAPD method with primer M13 proved to be an effective, low cost and efficient tool to identify at level species the yeasts isolated from olives, olive oil and its by-products.

Chemical analysis of oils (Chapter 3.3)

In the third part the aromatic and polyphenolic compounds of oils from the studied extraction processes were analyzed. Correlations studies between these compounds and yeast concentrations in the different phases of the process were carried out.

Trans-2-hexenal, responsible with other compounds for olive oil “fruity”, “grassy” and other positive attribute (Aparicio *et al*., 2012), was the most abundant compound present in all three years of study. As regards the aromatic compounds related to olive oil defects (Morales et al., 2005), all the oils of the 2011 showed a content of trans-2-heptenal, trans-2-decenal and 1-octen-3-ol significantly above the odour threshold, while in the 2012 only 1-octen-3-ol was detected above the odor threshold in all the extracted oils. On the contrary, in the oils of the 2013, compounds associated with defect were not detected or detected below the odor threshold. Correlation studies showed that 24, 13 and 12 volatile compounds were significantly correlated with the yeast concentrations quantified in respectively one, two and all steps (crushed pastes, oil from decanter and pomaces) of the extraction process. Oleuropein and its derivatives were predominant compared to the other classes of phenolic compounds in all samples of oil. 3,4-DHPEA-EDA was the most abundant phenolic compound in 2011 and 2013 oils, while in 2012 oils

the most abundant phenolic compound was oleuropein. Correlation studies showed that a total of 10, 5 and 2 polyphenolic compounds were significantly correlated with the yeast concentrations quantified in respectively one, two and all steps (crushed pastes, oil from decanter and pomaces) of the extraction process.

Investigation on the metabolic behavior of the yeast (Chapter 3.4)

Finally, in the fourth part, the enzymatic capabilities of the yeast isolates belonging to species most frequently isolated from the different extractive processes were investigated and their abilities to modify the chemical composition of the olive oil was assessed.

From the screening of 117 yeast isolates :

1. All the 117 assayed isolates resulted peroxidase positive.
2. The 66% of the assayed isolates were β-glucosidase positive.
3. The 22% of the isolates were lipase positive.
4. All the 117 assayed isolates were cellulose negative and only the 2% of the isolates were polygalacturonase positive.

Three yeast strains with different enzymatic capability were inoculated into pastes and commercial extra virgin olive oil. The strains with lipase activity were able to increase the acidity of the oils of about 25% (in oils from pastes) and 20% (in inoculated oils) compared to the samples without inoculation and the samples inoculated with the strain without lipase activity. Furthermore, in both tests, all the assayed strains showed a decrease in polyphenols concentrations compared to the control. Finally, as regards the number of peroxides, no statistically significant difference was found between oils from inoculated and not inoculated pastes, while, in the inoculated oils, peroxide values increased only in the presence of the strain that survived in a significant concentration (10^5 CFU/mL) after 30 days of incubation in oil.

Regarding the aromatic compounds related to olive oil positive attribute (cis-3-hexenyl acetate, hexenal, trans-2-hexenal and trans-2-hexenol), the inoculated oils showed values significantly lower than the control. These results confirmed the correlation

studies between yeast concentrations of oil from decanter and the aromatic compounds of oil.

On the contrary, in the experimental inoculated oil, compounds related to olive oil defects, such as ethyl acetate, propionic acid, heptanoic acid, butyric acid, trans-2-decenal and 1-octen-3-one, showed significantly lower or higher values than the control. This was depending from the inoculated species. However, the aromatic composition of oil from decanter taken from a real extraction process was influenced by the combination of various yeast species. Consequently, the results obtained from the two studies, real oil extraction processes and inoculated oil, did not agree because the biodiversity of the yeasts was higher in the real processes than in the experimental ones.

To conclude, the thesis point out that the yeast populations occurring in olive oil extraction processes are numerically significant and originate not only from the yeasts contaminating the olives but also from the yeasts colonizing the oil extractive plants. Some aromatic and polyphenolic compounds of oils from the studied extraction processes are significantly correlated with the yeast concentrations quantified in one or more steps of the extraction process, suggesting a role of yeasts contamination in the modification of olive oil characteristics. Actually, most of the yeasts present into the extraction process of extra virgin olive oil have enzymatic activities that can change both positively but mostly negatively the quality of the oil. The inoculation of three strains with different enzymatic abilities in crushed pastes and extra virgin olive oil confirms their negative effect on the chemical and aromatic composition of olive oil.

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