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LWT - Food Science and Technology 41 (2008) 2017–2022
LWT
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# Volatile compounds in uninoculated and inoculated table olives with *Lactobacillus plantarum* (*Olea europaea* L., cv. Moresca and Kalamata)

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Received 5 July 2007; received in revised form 11 December 2007; accepted 13 December 2007

**Abstract**

Table olives' flavour plays an important role in consumer's acceptability and it depends on various factors such as varieties, intrinsic characteristics, ripening of fruit and processing technologies. Flavour biogenesis is also influenced by addition in brine medium of lactic acid bacteria as inoculants, which reduce spoilage risks and improve sensory characteristics. In this work, flavour profiles of uninoculated and inoculated table olives with *Lactobacillus plantarum* (cv. Moresca and Kalamata) have been analytically characterised and compared. Twenty-one volatile compounds comprise alcohols, aldehydes, ketones, esters as well as acids formed during Greek-style olive fermentation (3 months brining after) have been characterised by gas chromatography and GC/mass spectrometry. Very high contents of ethanol and appreciable amounts of ethyl acetate, isobutanol, 2-butanone, 1-propanol and 1-hexanol were revealed in all samples with a significant increase in inoculated samples with respect to uninoculated ones. Also 1-butanol, 3-pentanol, 3-hydroxy-2-butanone, *cis*-3-hexen-1-ol and 2-butanol which were present in lower amounts, disclosed a meaningful increase in inoculated samples of both varieties, especially in Moresca inoculated sample. Acetic acid, isopentanol, 2-pentanol, propyl acetate, ethyl propanoate and 4-penten-1-ol showed a significant increase in inoculated Kalamata sample. These results showed that inoculation of brine medium with lactic acid bacteria starters significantly influenced aroma profiles of both varieties, in particular an increase in concentration of various flavour compounds has been revealed in inoculated table olives.


*Keywords:* *Olea europaea* L.; Table olives; Volatile compounds; *Lactobacillus plantarum*

## 1. Introduction

Table olives are currently the most important fermented vegetable products in the developed world. Worldwide production is around 1.5 million tons, of which nearly half is produced in European Union, predominantly in Spain, Greece, Italy and Portugal (IOOC, 2005). For table olive consumption, the fruits are opportunely processed and served as an appetizer or as a complement to salads, pasta, pizza and other foods (Marsilio, Russi, Iannucci, & Sabatini, 2008). The main purposes of table olives fermentation are to improve the preservation and the organoleptic properties of the final product. An important criterion to determine the effectiveness of fermentation is the concentration of lactic acid. However, the production of other end products of microbial metabolism, such as volatile compounds present in large or trace concentrations, may affect the sensory properties of table olives, especially flavour and aroma (Panagou & Tassou, 2006). Table olives' well-odours test a good fermentation and quality of the end product. The production of volatile flavour components tends to be the first mechanism considered for the development of a specific flavour to a particular fermented food (McFeeters, 2004). Flavour is tight connected with the quali-quantitative composition of volatile compounds playing an important role in consumer's acceptability (Koprivnjak, Conte, & Totis, 2002; Sabatini & Marsilio, 2008). Volatile compounds are not produced in significant amounts during fruit growth but increase during the climacteric stage of ripening and during fermentation process (Kalua et al., 2007). Fermenting olives are typically very complex ecosystems with active

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doi:10.1016/j.lwt.2007.12.002

enzyme systems from the raw materials interacting with the metabolic activities of microorganisms (McFeeters, 2004). Fermented foods are popular throughout the world and the production of fermented food products is important in many countries in providing income and employment. Fermentation is a technique that has been employed for generations to preserve food for consumption at a later date and to improve food security. The lowering of the pH operated from lactic acid bacteria inhibits the growth of food spoiling or poisoning bacteria and destroys certain pathogens (Hammes & Tichaczek, 1994).

Furthermore, fermentation can improve the flavour and appearance of food. In food fermentations, the by-products play a beneficial role in preserving and changing the texture and flavour of the food substrate. For example, acetic acid is the by-product of the fermentations of some fruits. This acid not only affects the flavour of the final product, but more importantly has a preservative effect on the food. Several alcohol-fermented foods are preceded by an acid fermentation and in the presence of oxygen and *Acetobacter*, alcohol can be fermented to produce acetic acid. *Lactobacillus plantarum* has been known to play a preponderant role in olive fermentation. This species has been extensively studied also with the aim of its use as *starter cultures*. Lactic acid bacteria are employed in olive fermentation to enhance the olive preservation due to a progressive acidification of the fermenting brine with a consequent pH decrease and the production of antimicrobial substances and bacteriocins (Marsilio et al., 2005; Ruiz et al., 2005; Ruiz-Barba, Cathcart, Warner, & Jiménez-Díaz, 1994). Also, the use of lactic acid bacteria as inoculants during olive fermentation is a practice that improves the current processing technologies. The inhibitory effect of lactic acid bacteria is due to the accumulation of main primary metabolites (lactic and acetic acids, ethanol and carbon dioxide) as well as to the production of other antimicrobial compounds, such as formic and benzoic acids, hydrogen peroxide, diacetyl, acetoin and bacteriocins (Delgado, Brito, Fevereiro, Peres, & Figueiredo-Marques, 2001). Starter cultures also prevent contamination of secondary microflora responsible for the gas pocket in the olives (Leal-Sánchez, Jiménez-Díaz, Maldonado-Barragán, Garrido-Fernández, & Ruiz-Barba, 2002). They also improve the aroma and flavour characteristics of the product (Borcakli, Özay, & Alperden, 1995), so it is of a great interest to study the effect of starter cultures on the quali-quantitative composition of volatile compounds in most important industrial varieties of table olives. Until today, the determination of volatile compounds in treated green olives fermentation has attracted little attention, mainly by Spanish researches to detects spoilage incidents (García-García, Romero-Barranco, Durán-Quintana, & Garrido-Fernández, 2004; Montaño, De Castro, & Rejano, 1992; Montaño, Sánchez, & De Castro, 1993; Montaño, Sánchez, & Rejano, 1990), whereas no data are available in literature for Greek table olives (Panagou & Tassou, 2006). In this work, *Greek-style* table olives' flavour profiles of two different cultivars (Moresca and Kalamata) have been compared. In addition, the effects of a selected lactic acid bacteria starter culture on the olive fermentation and volatile compounds biogenesis have been assessed.

## 2. Materials and methods

### 2.1. Plant material and processing

Olive fruits of Moresca and Kalamata cultivar from Brindisi area (Italy) were used in this study. Olives, hand harvested at the black ripening stage, were processed by Greek method, according to the Unified Qualitative Standard applying to Table Olives in International Trade (International Olive Oil Council, 2004). Olives were washed twice with water and directly put into a brine solution made up of 7% w/v NaCl and fermented at room temperature (Greek method).

A selected oleuropeinolytic *L. plantarum* bacterial strain (LAB B1-2001) was used as inoculant. The bacterial strain was propagated in MRS broth (Oxoid) for 18 h at 30 °C, then the culture was inoculated into MRS broth and incubated until the exponential phase of growth was reached. Cells were pelleted by centrifugation at 10,000×*g* for 15 min at 4 °C, washed twice with sterile water, and suspended at a concentration of ca. $10^{10}$ CFU/mL. The culture was then inoculated into the fermenting brine in a ratio of 40 ml/L. All the fermentation processes were carried out at ambient temperatures. The experimental trials were carried out in triplicate. Fig. 1 shows the experimental set up.

### 2.2. Volatile compounds extraction

Dynamic headspace method (Solinas, Marsilio, & Angerosa, 1987), largely used to analyze quality and quantity flavour molecules of olive oil has been updated and used in this work to extract volatile compounds. Chemical–physical characteristics of table olives are different from that of olive oil. Fermented

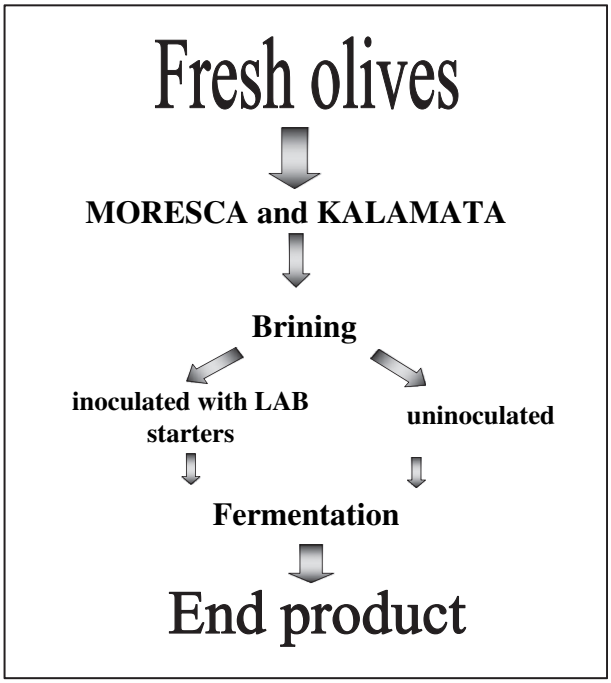



Fig. 1. Experimental set up.

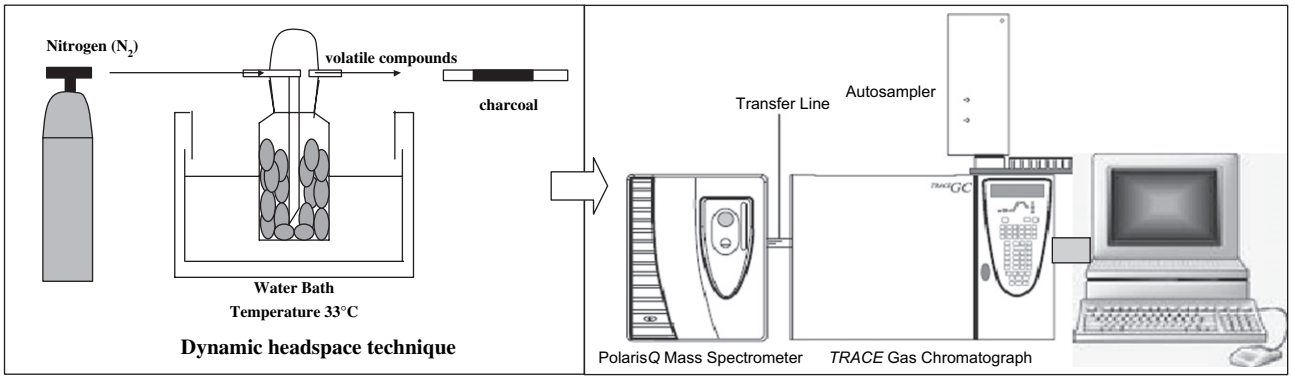



Fig. 2. Description of dynamic headspace technique for olive fruits.

olive is a solid phase made up by hydrophilic and hydrophobic portions, while olive oil is only a hydrophobic liquid phase. For this reason, it has been necessary to change some parameters linked with the technique described for olive oil.

Temperature of extraction was diminished to 30–33 °C (37 °C for olive oil) to avoid excessive water evaporation, which could inactivate charcoal (table olives contain high concentration of water); charcoal concentration was increased to 100 mg (30 mg for olive oil); volume of diethyl ether (elution solvent) was diminished to 1 mL (1.5 mL for olive oil).

So, 60 g of stoned olive fruits were put into a 120 mL Drechsel gas washing bottle. Volatiles were stripped with $N_2$ (1.0 $dm^3$/min) at 33 °C for 2 h, trapped on 100 mg of activated charcoal and then eluted with 1 mL of diethyl ether (Fig. 2).

### 2.3. Reagents

Ethyl acetate, 2-butanone, ethanol, ethyl propanoate, propyl acetate, 2-butanol, 1-propanol, *n*-propyl propanoate, isobutanol, 3-pentanol, 2-pentanol, 1-butanol, isopentanol, 1-pentanol, 1-hexanol, *cis*-3-esen-1-ol, acetic acid, propionic acid and 1-nonanol were purchased from Sigma–Aldrich (St Louis, MO, USA). Activated charcoal (0.5–0.85 mm; 20–35 mesh ASTM) was from Merck (Stuttgart, Germany).

### 2.4. GC analysis

Gas chromatography was carried out with a Carlo Erba (Milan, Italy) 5160 Mega series instrument equipped with a flame ionization detector (FID) and a Supelcowax-10 capillary column (Supelco, Sigma–Aldrich) 60 m × 0.3 mm (id), 0.1 μm film thickness was used, with hydrogen as carrier gas at 40 kPa. The column temperature was programmed as follows: at 35 °C for 10 min, from 35 to 45 °C at 0.8 °C/min, from 45 to 200 °C at 5.5 °C/min and then held there for 20 min. The temperature of detector was 240 °C. The sample (1 μL) was injected "on-column" mode. Quantitative analysis was obtained by peak area integration with a Carlo Erba Mega series integrator.

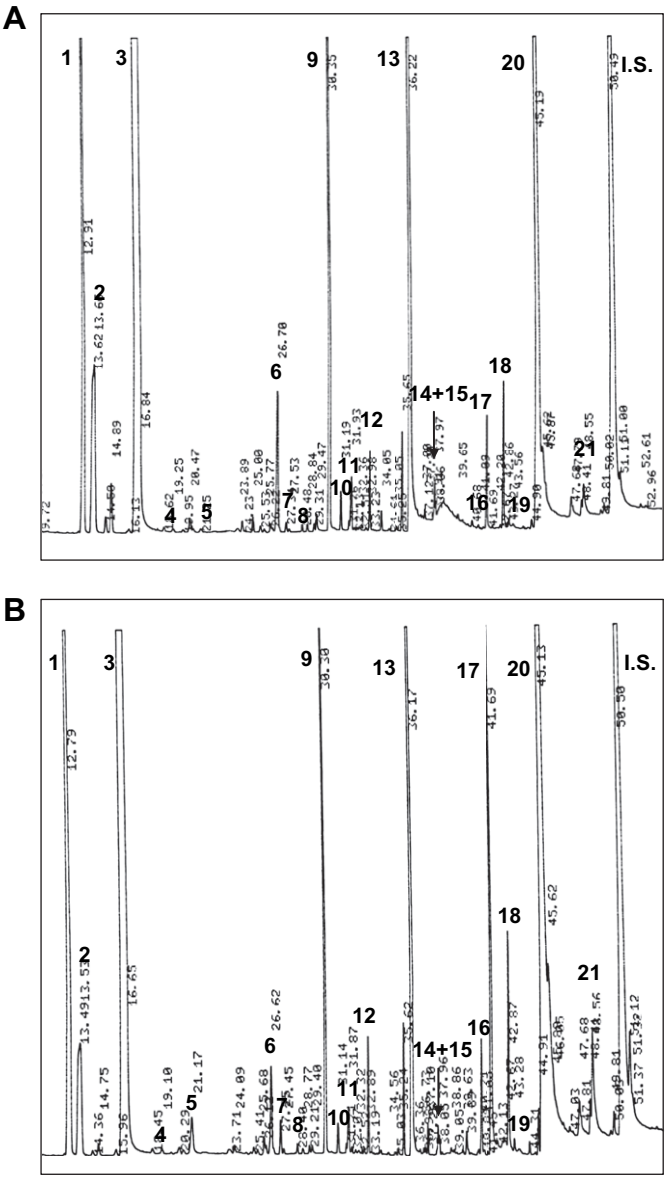



Fig. 3. Gas chromatograms of volatile compounds of inoculated Kalamata (A) and Moresca (B) varieties after 3 months brining.

### 2.5. GC/mass spectrometry analysis

GC/mass spectrometry analysis was carried out with a Thermo Finnigan (San Jose, CA, USA) gas chromatograph coupled to a Polaris Q quadrupole mass-selective spectrometer.

Sample aliquots of 2 μL were injected. Analysis was provided with split-less injection port.

The GC injector temperature was 200 °C with 50 mL/min split flow and the transfer line temperature was 210 °C. Helium at 100 kPa was employed as the carrier gas. The oven temperature program was run at 39 °C for 10 min, then raised at 5 °C/min to 200 °C, and held there for 30 min. The ion source temperature of Polaris Q was 250 °C and electron impact mass spectra recorded at 70 eV.

### 2.6. Qualitative and quantitative analysis

Compounds were identified by comparison of their mass spectra and retention time with those of standard compounds. 1-Nonanol has been used as standard. Each value is the mean of triplicate analyses expressed in $\mu g_{(compound)}/Kg_{(olive\ fruit)} \pm SD$. Statistical analysis have been carried out by Student's *t*-test at $p < 0.05$.

### 2.7. Microbiological assays

At given fermentation times, brine samples were withdrawn from the containers and serial dilutions in sterile distilled water for microbiological counts by the standard plate method from each dilution were prepared; 0.1 mL was spread on the media plates. Lactic acid bacteria were enumerated on MRS agar (Oxoid) at 30 °C for 72 h and yeasts on Malt Extract agar (Oxoid) at 28 °C for 72 h. Microorganisms' enumeration in each solution was made in duplicate. Colony forming units/mL of brine (CFU/mL brine) were calculated.

## 3. Results

Gas chromatograms of volatile compounds of inoculated Moresca and Kalamata varieties after 3 months brining are shown in Fig. 3. Details on peak identities and volatile compounds amounts expressed in $\mu g_{(compound)}/Kg_{(olive\ fruit)}$ are shown in Table 1. Among identified organic compounds, there are alcohols, aldehydes, ketones, esters as well as acids. Very high contents of ethanol and appreciable amounts of ethyl acetate, isobutanol, 2-butanone and 1-propanol were revealed in all samples with a significant increase in inoculated samples with the respect to uninoculated ones. Also 1-hexanol, 1-butanol, 3-pentanol, 3-hydroxy-2-butanone, *cis*-3-hexen-1-ol and 2-butanol which were present in lower amounts, disclosed a meaningful increase in inoculated samples of both varieties, especially in Moresca inoculated sample. Acetic acid, isopentanol, 2-pentanol, propyl acetate, ethyl propanoate and 4-penten-1-ol showed a significant increase in inoculated Kalamata sample, but on the other hand, they disclosed an appreciable decrease in inoculated Moresca sample. Nonanal and propionic acid amounts diminished in incolutated samples of both varieties with respect to uninoculated ones. Little amounts of propyl propanoate were detected only in Moresca inoculated sample, while 1-pentanol was revealed only in Kalamata commodities and above all in inoculated sample. Although Kalamata inoculated sample disclosed a major

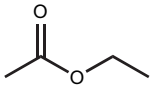
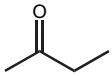
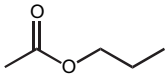
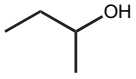
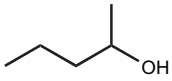
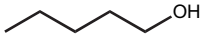
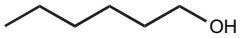
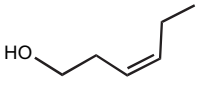
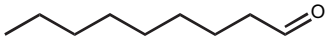
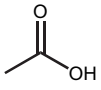
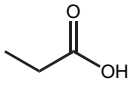
Table 1
Volatile compounds amounts of uninoculated and inoculated Kalamata and Moresca table olives, after 3 months brining expressed in $\mu g_{(compound)}/kg_{(olive\ fruit)} \pm SD$

| Peak number | Compound | Kalamata (uninoculated) | Kalamata (inoculated) | Moresca (uninoculated) | Moresca (inoculated) |
|---|---|---|---|---|---|
| 1 | Ethyl acetate | 102.6 ± 9.0* | 262.5 ± 10.6* | 956.7 ± 24.0* | 1.300 ± 14.0* |
| 2 | 2-butanone | 53.9 ± 5.5* | 147.8 ± 11.0* | 55.2 ± 4.5* | 200.5 ± 3.5* |
| 3 | Ethanol | 8.208.0 ± 342.0* | 12.973.0 ± 386.0* | 6.884.0 ± 260.0* | 19.660 ± 848.5* |
| 4 | Propyl acetate | 0.9 ± 0.03 | 4.9 ± 0.2 | 6.8 ± 0.3 | 5.3 ± 0.4 |
| 5 | Ethyl propanoate | 0.7 ± 0.02 | 3.2 ± 0.1 | 15.8 ± 0.7 | 10.8 ± 0.7 |
| 6 | 2-butanol | 0.5 ± 0.01* | 2.0 ± 0.1* | n.d. | 2.7 ± 0.1* |
| 7 | 1-propanol | 20.9 ± 1.8* | 60.2 ± 5.6* | 28.8 ± 1.1* | 52.0 ± 2.8* |
| 8 | Propyl propanoate | n.d. | n.d. | n.d. | 8.0 ± 0.4 |
| 9 | Isobutanol | 385.2 ± 24* | 698.4 ± 49.5* | 257.3 ± 16.9* | 410 ± 28.3* |
| 10 | 3-pentanol | 3.4 ± 0.1* | 7.6 ± 0.6* | 3.0 ± 0.1* | 4.7 ± 0.1* |
| 11 | 2-pentanol | 5.0 ± 0.2 | 20.6 ± 0.08 | 51.5 ± 4 | 24 ± 1.0 |
| 12 | 1-butanol | 4.2 ± 0.3* | 14.4 ± 1.1* | 10.1 ± 0.3* | 17.2 ± 0.5* |
| 13 | Isopentanol | 1.603.0 ± 70.0 | 3.272.0 ± 167.0 | 1.722.0 ± 100.0 | 1.506.0 ± 100.0 |
| 14 | 1-pentanol | 2.6 ± 0.1 | 5.5 ± 0.3 | n.d. | n.d. |
| 15 | 4-penten-1-ol | 1.1 ± 0.07 | 3.3 ± 0.1 | 4.5 ± 0.3 | 3.3 ± 0.2 |
| 16 | 3-hydroxy-2-butanone | 4.1 ± 0.3* | 5.5 ± 0.5* | 22.6 ± 3.5* | 38.5 ± 3.5* |
| 17 | 1-hexanol | 8.2 ± 0.5* | 17.2 ± 1.6* | 55.0 ± 4.2* | 155.5 ± 9.0* |
| 18 | *cis*-3-Hexen-1-ol | 10.0 ± 1.0* | 22.3 ± 2.4* | 22.0 ± 0.7* | 34.5 ± 2.1* |
| 19 | Nonanal | 5.6 ± 0.2 | 3.8 ± 0.2 | 11.4 ± 0.9 | 7.1 ± 0.4 |
| 20 | Acetic acid | 247.0 ± 12.0 | 598.0 ± 30.0 | 3.435.0 ± 200.0 | 1.259.0 ± 70.0 |
| 21 | Propionic acid | 10.4 ± 0.5 | 5.0 ± 0.3 | 6.0 ± 0.4 | 6.0 ± 0.5 |

Each value is the mean of triplicate analyses expressed in $\mu g_{(compound)}/kg_{(olive\ fruit)} \pm SD$. (*$p < 0.05$) statistical significance of the increase of volatile compound contents (inoculated versus uninoculated).
n.d. = not determined.

number of molecules which increased their concentrations, Moresca inoculated sample showed a most significant increment in the contents of various volatile molecules with respect to Kalamata sample (Table 1). Table 2 reports the odorous information of some volatile compounds (detected in Moresca and Kalamata table olives) obtained from the literature isolated from other foods and fruits such as olive oil, cidrus, cucumber, honey, etc.

Table 2
Odour information of *some volatile molecules* (detected in Moresca and Kalamata table olives) obtained from the literature isolated from other foods and fruits such as: olive oil, cidrus, cucumber, honey, etc.

| Compound | Structures | Odour information |
|---|---|---|
| Ethyl acetate |  | Sweet, aromatic[a] |
| 2-butanone |  | Fragrant, pleasent,[a] ethereal, fruity[b] |
| Ethanol |  | Alcohol[b] |
| Ethyl propanoate |  | Sweet, strawberry, apple[a] |
| 2-butanol |  | Winey[b] |
| 2-pentanol |  | Pungent[a] |
| 1-butanol |  | Sweet, fusel oil[d] |
| 1-pentanol |  | Fruity,[e] strong, sticky, balsamic[b] |
| 1-hexanol |  | Fruity, aromatic, soft,[a] green,[c] fresh grass[d] |
| *cis*-3-Hexen-1-ol |  | Banana,[a] fresh green grass[d] |
| Nonanal |  | Green floral,[c] faint elder flower,[d] citrus like[f] |
| Acetic acid |  | Sour, vinegary[b] |
| Propionic acid |  | Pungent, sour, aromatic[b] |

[a] Kiritsakis (1998).
[b] Morales, Luna, and Aparicio (2005).
[c] Selli, Rannou, Prost, Robin, and Serot (2006).
[d] Jorgensen, Hansen, Christensen, Jensen, and KaacK (2000).
[e] Aparicio and Luna (2002).
[f] Reiners and Grosch (1998).

## 4. Discussion

Most food spoilage organisms cannot survive either alcoholic or acidic environments. The changes that occur during fermentation of foods are the result of enzymatic activity. The lactic acid bacteria are a group of Gram positive bacteria, non-respiring non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. The lactic acid bacteria belong to two main groups: the homofermenters and the heterofermenters. The pathways of lactic acid production differ for the two. Homofermenters produce mainly lactic acid, via the glycolytic (Embden–Meyerhof) pathway. Heterofermenters produce lactic acid plus appreciable amounts of ethanol, acetate and carbon dioxide, via the 6-phosphoglucanate/phosphoketolase pathway (*FAO corporate document repository*). Our results suggest that during olive processing all four samples have undergone alcoholic and heterolactic fermentation which lead to the production of high amounts of ethanol, acetic acid and other alcohols and esters. Moresca variety was more sensitive to lactic acid bacteria starters inoculation since there was a more significant increase in the contents of a greater number of aroma molecules. In fact, Moresca inoculated sample disclosed concentrations of $10^8$ CFU/mL brine of lactic acid bacteria and $10^5$ CFU/mL brine of yeasts, higher than Kalamata inoculated sample, where lactic acid bacteria and yeasts reached $10^5$ and $10^4$ CFU/mL brine, respectively. This was probably due to a greater total phenol contents in Kalamata fresh olives (5200 mg/kg) with respect to Moresca fresh olives (3200 mg/kg) (Cavallo, 2007). It is well known, in fact, that phenols inhibit microorganism growth during olives fermentation (Sousa et al., 2006).

Although there are surely different biogenesis pathways of volatile compounds between table olives and olive oil, it is indeed important to consider some enzymatic pathways which occurs in olive oil flavour compounds biosynthesis. $C_5$ and $C_6$ aldehydes and alcohols and their corresponding esters are the most volatile compounds present in olive oils and are produced enzymatically by polyunsaturated fatty acids through the "lipoxygenase pathway" (Feussner & Wasternack, 2002). Lipoxygenases (LOX) are enzymes that are ubiquitous in the plant and animal kingdoms. They catalyze the oxygenation of polyunsaturated fatty acids containing a 1,4-*Z*,*Z*-pentadiene moiety using molecular oxygen. The LOX pathway is critical in olive fruit for the formation of various flavours or scent components of virgin olive oil (Gardner, 1991; Williams & Harwood, 2000). It is known that lipoxigenases, after their release owing to the disruption of fruit cells during the milling in olive oil produce 9- and 13-hydroperoxides of linolenic and linoleic acids (Angerosa, 2002; Angerosa, Mostallino, Basti, & Vito, 2000; Ridolfi, Terenziani, Patumi, & Fonatanazza, 2002). Thus, it is a conceivable lipoxigenases pathway for our samples as we found out some compounds which occur in it, as showed by the presence of 1-hexanol, and *cis*-3-hexen-1-ol. But nevertheless, we could also consider a lipoxigenases-like metabolism of polyunsaturated fatty acids affected by enzymes produced in brine medium by lactic

acid bacteria and yeasts together with other different microorganisms. This research showed that the inoculation of brine medium of Greek-style Kalamata and Moresca olives with a selected lactic acid bacteria starter culture caused a significant change in aroma profile of both varieties. In particular, an increase in concentration of a greater number of flavour compounds has been revealed in inoculated samples, especially in Moresca table olives.

In our samples, only well-flavours have been found because olives have undergone a good fermentation process (no coliforms have been revealed in brine medium). Thus, in the future, it will be very useful to identify also off-flavours formed by anomalous fermentations, in order to reveal them, just in small traces, in premature times, so to obtain the recovery of the fermentation process.

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