Food Microbiology 46 (2015) 368–382

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Physico-chemical characterization of natural fermentation process of Conservolea and Kalamàta table olives and developement of a protocol for the pre-selection of fermentation starters

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ARTICLE INFO

Article history:
Received 13 May 2014
Received in revised form
21 July 2014
Accepted 26 August 2014
Available online 6 September 2014

Keywords:
Table olives
Fermentation
Microorganism selection
GC–MS analysis

ABSTRACT

Table olives are one of the most important traditional fermented vegetables in Europe and their world consumption is constantly increasing. Conservolea and Kalamàta are the most important table olives Greek varieties. In the Greek system, the final product is obtained by spontaneous fermentations, without any chemical debittering treatment. This natural fermentation process is not predictable and strongly influenced by the physical-chemical conditions and by the presence of microorganisms contaminating the olives. Natural fermentations of Conservolea and Kalamàta cultivars black olives were studied in order to determine microbiological, biochemical and chemical evolution during the process. Following the process conditions generally used by producers, in both cultivars, yeasts were detected throughout the fermentation, whereas lactic acid bacteria (LAB) appeared in the last staged of the process. A new optimized specific protocol was developed to select autochthonous yeast and LAB isolates that can be good candidates as starters. These microorganisms were pre-selected for their ability to adapt to model brines, to have beta-glucosidase activity, not to produce biogenic amines. Chemical compounds deriving by microbiological activities and associated to the three different phases (30, 90 and 180 days) of the fermentation process were identified and were proposed as chemical descriptors to follow the fermentation progress.

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1. Introduction

Table olives are one of the most important and popular traditional fermented vegetables in Western world and in particular in Southern European countries. This product, together with olive oil, represents an important food in the Mediterranean diet. World production of table olives is estimated (2013–2014 season) in 2,574,500 tons and the 27% of this production (698,000 tons) is located in the European Union (EU) (IOOC, 2013). Spain has a leading position in table olive production with 513,100 tons, followed by Greece with 94,000 tons, by Italy with 74,000 tons and Portugal with 11,900 tons. Table olives consumption is constantly increasing throughout both EU and the world and producer countries are also the most important consumers. According to their relevance on the international markets, Kalamàta and Conservolea are the most important table olive varieties together with Manzanilla, Sevillana and Hojiblanca and to a lesser extent Bella di Cerignola and Ascolana Tenera (Anon., 2003). Conservolea represents the most economically important cultivar in Greece, corresponding to at least 80–85% of Greek olive production, whereas Kalamàta is the second most important cultivar used in the production of Greek table olives for domestic and foreign market (Garrido-Fernandez et al., 1997). The most important industrial preparations of table olives are the green olives by Spanish style, the black oxidized olives by Californian method and the naturally black olives by Greek style (Garrido-Fernandez et al., 1997).

In the Greek system, the final product is obtained placing directly olives into brine, without any debittering pre-treatment and it is characterized by a fruity aroma and a slightly bitter taste. Today, this debittering process is carried out by spontaneous

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http://dx.doi.org/10.1016/j.fm.2014.08.021
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fermentations, not predictable and strongly influenced by the physical-chemical conditions (salt content, pH, aerobic/anaerobic conditions and temperature), the availability of fermentable substrates and by the presence of microorganisms contaminating the drupes (De Castro et al., 2002; Tassou et al., 2002; Alvarez et al., 2003; Abriouel et al., 2011). To standardize the fermentation process and consequently improve the quality of the final products, the identification of chemical descriptors for monitoring the fermentation is highly requested. The primary purpose of table olive fermentation is to achieve a preservation effect and enhance the organoleptic attributes of the processed product. Natural fermentation is mainly promoted by yeasts and lactic acid bacteria (LAB), naturally associated with drupes (Brenes et al., 2004; Romero et al., 2004).

Homo and hetero-fermentative LAB, which can produce lactic acid and other organic acids, are the most important group of bacteria in olives. Homo fermentative LAB such as *Lactobacillus*, *Streptococcus* and *Pediococcus* and hetero fermentative LAB such as *Leuconostoc* and some members of *Lactobacillus* are commonly detected in fermented olive preparations (Abriouel et al., 2012; Randazzo et al., 2012).

LAB are able to improve the aroma and flavor characteristics of the product (Panagou et al., 2008), to enhance the olive preservation due to a progressive acidification of the fermenting brine and the production of antimicrobial compounds and bacteriocins (Marsilio et al., 2005). *Lactobacillus plantarum* and *Lactobacillus pentosus* were used in different studies as starters to control fermentation processes, demonstrating that these microorganisms have the potential to allow the microbiological control of the process, increase the lactic acid yield and improve the quality of the final product (Lamzira et al., 2005; Marsilio et al., 2005; Sabatini et al., 2008; Panagou et al., 2008; Servili et al., 2006).

Yeasts can play both a positive and a negative role in table olive processing (Arroyo-López et al., 2008). In fact, yeasts are able to produce desirable volatile compounds and metabolites that improve the organoleptic properties (Garrido-Fernández et al., 1995), to enhance the growth of LAB (Tsapatsaris and Kotzekidou, 2004; Segovia Bravo et al., 2007) and to biodegrade phenolic compounds (Ettayebi et al., 2003). On the other hand, yeasts may cause gas pocket formation because of CO_2 production at the early stage of fermentation (Lamzira et al., 2005) and softening of the olive tissue (Hernández et al., 2007).

The aim of the present work was (i) to study the microbiological, biochemical and chemical profiles associated with natural fermentation of Conservolea and Kalamàta cultivars black olives, (ii) to identify chemical descriptors associated with the fermentation progress and (iii) to pre-select, by optimized specific protocols, autochthonous yeast and LAB isolates that can be good candidates as starters. For the first time in this study yeast and LAB strains suitable to be used as candidate mixed autochthonous starter cultures were isolated and chemical compounds associated to the three different phases of the fermentation process were identified in Conservolea and Kalamàta black olives.

2. Materials and methods

2.1. Olive samples and fermentation method

The pilot-scale fermentations were performed in triplicate on olive samples of Conservolea and Kalamàta cultivars in an industrial plant (Agricola Nuova Generazione, Martano, Lecce, Italy). Healthy black olives (90 kg) were collected at the black stage of ripening and washed with tap water to eliminate plant materials and superficial contaminants. The olives were then selected (caliber above 10–12 mm), washed and placed in plastic vessels of 30 kg capacity filled with 20 L of 8% NaCl (wt/vol). The olives were allowed to ferment at ambient temperature (8–30 °C).

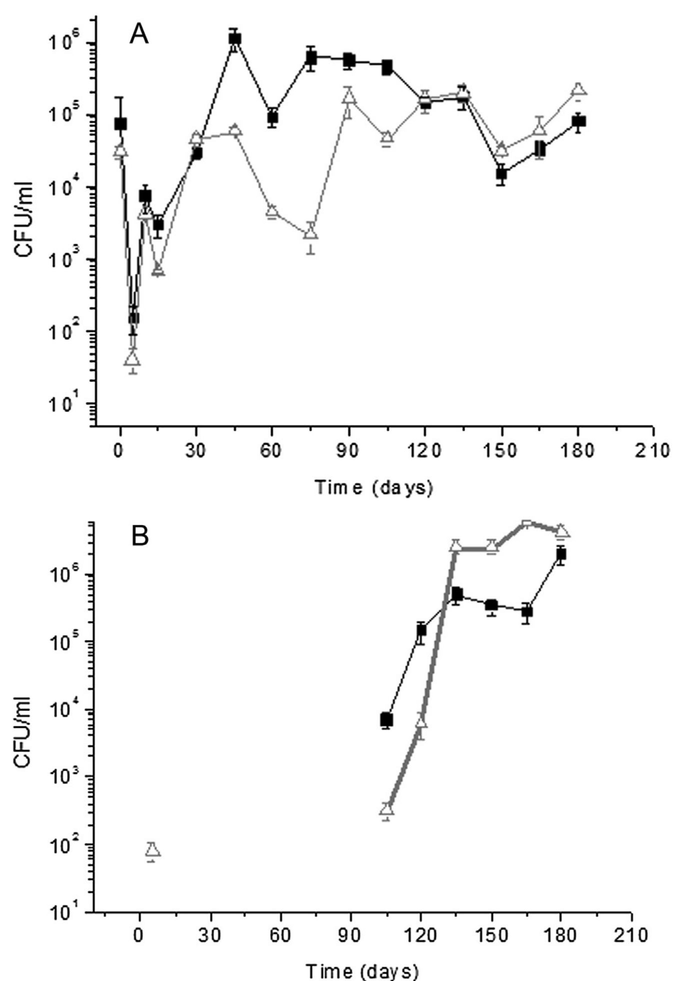

Fig. 1. Yeast and LAB evolution during Conservolea —■— and Kalamàta —△— fermentation. Yeast (A) and LAB (B) total counts (Log CFU/ml) of Conservolea and Kalamàta naturally fermented table olives.

2.2. Isolation of microbial population

To isolate epiphytic yeasts, *Enterobacteriaceae* and Lactic Acid Bacteria (LAB) from the olives, 10 drupes per sample were washed in 50 ml of sterile water on a rotary shaker at 200 rpm for 30 min. The sediment was recovered after centrifugation at 5000 × g for 10 min at room temperature and suspended in 0.5 ml of 0.1% (wt/vol) peptone water. The suspension was added with one volume of sterile glycerol and stored at −80 °C until microbiological analysis.

Salinity, pH and temperature were evaluated during fermentation at the following time points: 0, 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180 days (Fig. 2). At each different fermentation time, 7.5 ml aliquots of brines were collected diluted with one volume of sterile 100% glycerol and stored at −80 °C for further analysis.

Microbiological analyses on epiphytic microorganisms from olives and brines were performed by diluting samples serially with 0.1% (wt/vol) peptone water and applying them to agar slants containing the following media: Man, Rogosa and Sharpe Agar (MRS, LABM, UK) added with 0.05 g/l of nystatin for LAB identification and incubated at 30 °C under anaerobic conditions for

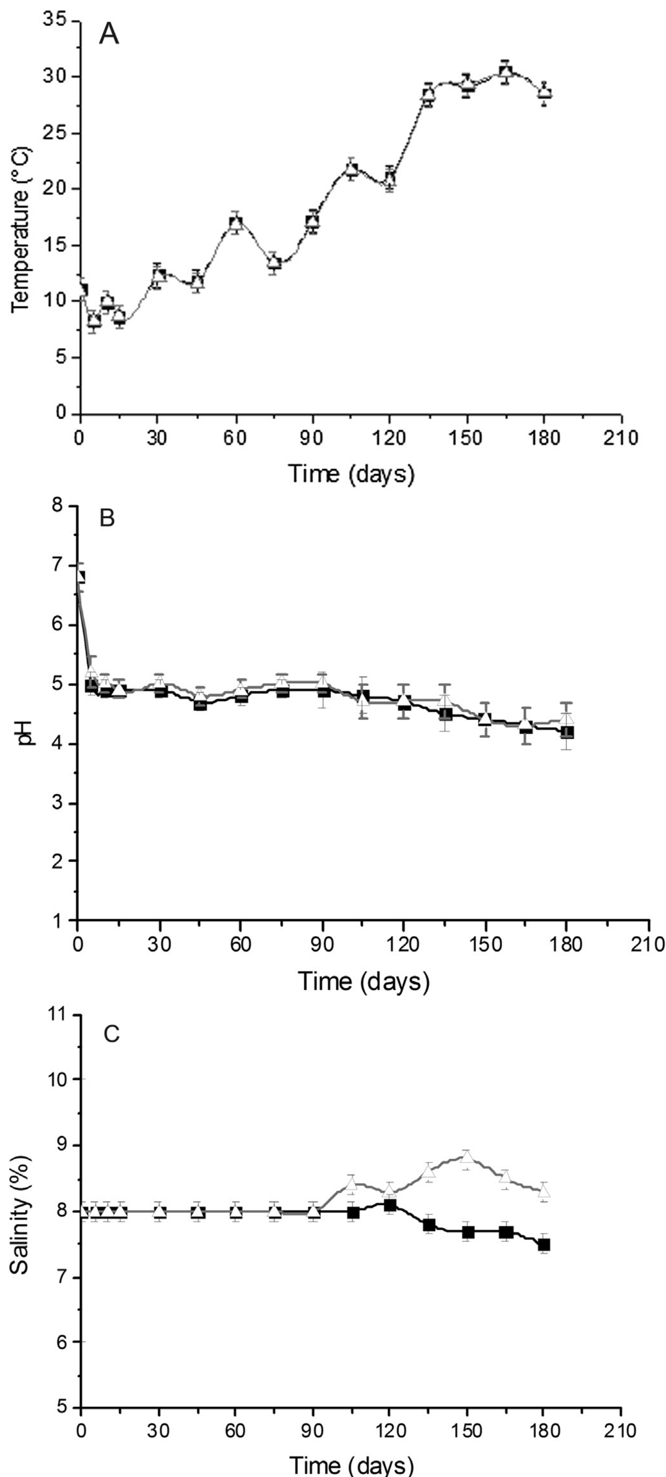

Fig. 2. Temperature, pH and salinity evolution during Conservolea and Kalamàta fermentation. Temperature (A), pH (B) and Salinity % (wt/vol) (C) values of Conservolea —■— and Kalamàta —△— naturally fermented table olives.

48–72 h; Violet Red Bile Glucose Agar (VRBGA, LABM, UK) for *Enterobacteriaceae* identification by incubation at 37 °C for 18–24 h; Sabouraud Dextrose Agar (LABM, UK) for yeasts identification added with 0.1 g/l of ampicillin and 0.05 g/l of kanamycin by incubation at 25 °C for 2–4 days. Isolates were counted in order to quantify the LAB, *Enterobacteriaceae* and yeasts in each sample. Thirty five colonies were randomly selected from the agar plates specific for LAB and yeasts corresponding for all the above indicated sampling time.

2.3. Detection, quantification and isolation of phenolic compounds

Brine samples were filtered through a 0.45 μm filter and then directly injection in the HPLC apparatus Agilent 1100 equipped with a photodiode array detector. The wavelengths used for quantification of phenol compounds were 280, 295 and 320 nm. Separation was achieved by using a Phenomenex-Luna 5 μ C18 (2) 100 Å column (250 × 4.6 mm) with the temperature of the column set to 30 °C. A gradient elution program was utilized with a mobile phase consisting of acetonitrile (solution A) and 1% (v/v) phosphoric acid in water (solution B) as follows: isocratic elution, 5% B, 0–30 min; linear gradient from 5% B to 15% B, 30–50 min; linear gradient from 5% B to 50% B, 50–55 min; linear gradient from 50% B to 100% B, 55–65 min; post time, 10 min before the next injection. The flow rate during the mobile phase was 1.0 ml/min, and the injection volume was 20 μl. All phenol compounds were quantified using calibration curves of authentic phenolic standards.

2.4. Detection and quantification of sugars, organic acids and alcohols

HPLC analyses of sugar, organic acid and alcohols composition of the brine were achieved by directly injecting the brine (filtered as stated above) in the chromatographic system (Agilent 1100) equipped with a RID-10A refractive index detector (for sugars and alcohols analysis) and with a photodiode array detector set to 210 nm (for the analysis of organic acids). Sugars, organic acids and alcohols were simultaneously analyzed, according to De Benedictis et al. (2011), using an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad) and kept at 55 °C. The analytical conditions used were as follows: flow 0.3 ml/min, eluent 0.045 NH_2SO_4 with 6% acetonitrile (v/v).

2.5. GC–MS analysis of volatile compound from olive fruit and brines

Volatile compounds of fermentations were identified by Solid Phase Micro-Extraction technique in Head Space followed by Gas Chromatography/Mass Spectrometry (HS-SPMEGC/MS) (Pawliszyn, 1997). Samples were prepared by homogenizing 5 g of stoned drupes and then transferring the slurry into 20-ml vials covered with a polytetrafluoroethylene (PTFE)/silicone rubber septum. Eight microliters of 4-methyl-2-pentanol in methanol (final concentration 50 μg/ml) were used as an internal standard. The 65 μm DVB/CAR/PDMS coated fiber (Supelco, Spain) was used according to Malheiro et al. (2011). The vials were heated to a controlled temperature (40 ± 0.5 °C) in order to reach equilibrium. On the basis of preliminary tests, 30 min exposure time proved suitable for fiber saturation and for reproducibility of the extraction procedure.

The analysis of volatile compounds in olive brines was performed using the fiber CAR/PDMS − 75 μm (needle length 1 cm, needle size 24 ga) (Supelco, Spain). The headspace SPME sampling conditions used were as follows: 10 ml of brine and 0.5 g NaCl were transferred in a 20 ml glass vial and spiked with 20 μL of a 4-methyl-2-pentanol (final concentration 50 μg/ml). The vial was tightly capped with a PTFE-faced silicone septum. The sample was equilibrated for 15 min at 35 °C and then the fiber was exposed to the headspace for 30 min, under the same conditions. Desorption of volatiles took place in the injector of the GC/MS for 5 min.

Before the first daily analysis, the fiber was conditioned in the injector for 10 min at 250 °C to remove any volatile contaminants. All samples were analyzed in triplicate.

HS-SPME analyses were performed using an AGILENT 6890N gas chromatograph coupled to an AGILENT 5973 mass spectrometer (Agilent, USA). Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The injection port was equipped with an SPME liner (0.75 mm × 6.35 mm × 78.5 mm). Separation of compounds was performed on a DB-WAX column (60 m, 0.25 mm i.d., 0.25 mm film thickness, Agilent). The injections were performed in split less mode. Oven temperature was maintained at 40 °C for 5 min, programmed at 3 °C/min to 150 °C for 20 min. The mass spectrometer was operated in electron impact mode with the electron energy set to 70 eV and a scan range of 30–350 *m/z*. The temperature of MS source and quadrupole were set at 230 and 150 °C. Analyses were performed in full-scan mode. Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic standards analyzed under the same conditions and by comparison of the retention indices (as Kovats indices) with literature data. MS fragmentation patterns were compared with those of pure compounds, and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 98 spectral database. Semi-quantitative determination was carried out by the internal standard method (IS, 4-methyl-2-pentanol). The volatile compounds were quantified by comparison of peak areas to those of internal standards.

2.6. Statistical analysis

Chemical data comparisons were subjected to one-way factor analysis of variance (ANOVA). Significant differences were separated using the Duncan test. The level of significance was set at $P < 0.05$. The comparison of volatile classes of compounds during fermentations was achieved by principal component analysis (PCA). All statistical analyses were carried out using the STATISTICA 7.0 software (StatSoft software package, Tulsa, OK, USA).

2.7. LAB and yeast selection

A specific protocol for the selection of autochthonous starter for table olive production was applied. Briefly, two different synthetic brines were formulated either for yeast and LAB selection. For yeast selection, brine samples collected at days 15th, 30th and 60th of fermentation and for LAB selection, brine samples collected every two weeks starting from day 105th until 180th from each olive cultivar, were analyzed by reversed-phase HPLC-DAD for the determination of the qualitative and quantitative profile of phenolic compounds. A model brine (MB) for yeasts was formulated by the following composition: 100 mg/l Tyrosol, 30 mg/l Caffeic acid, 1000 mg/l Oleuropein, 500 mg/l Verbascoside, 8–10% NaCl, 3 g/l Glucose, 0.5 g/l Yeast extract, pH 4–4.5, Agar 20 g/l. Yeast isolates were grown at 14 °C. The selection of bacterial isolates was carried out on MB formulated *ad hoc*: 100 mg/l Tyrosol, 15 mg/l Caffeic acid, 300 mg/l Oleuropein, 100 mg/l Verbascoside, 8% NaCl, 3 g/l Glucose, 0.5 g/l Yeast extract, 20 g/l Agar, pH 4.2. Microbial isolates were grown at 12 °C (Bleve et al., submitted).

Yeasts and LAB isolates able to grow in specific MBs were subjected to selection for their specific technological and safety traits: beta-glucosidase activity and absence of production of biogenic amines. Beta-glucosidase activity was determined by replica plating the yeast onto selective medium, for yeast (SC = 0.67% yeast nitrogen base (YNB), 0.5% arbutin, 2% agar, pH 5.0) and for bacteria (MRS medium, 0.5% arbutin, 2% agar, pH 5.0), each added with. Two milliliters of a filter sterilized 1% ammonium ferric citrate solution were added to 100 ml medium. Biogenic amines formation was determined using the method described by Nikolaou et al. (2006). Yeast and bacteria strains were inoculated on YPD or MRS agar plates, respectively, supplemented with bromocresol purple 0.006% and amino acid 1% (w/v). The amino acids histidine, tyrosine, phenylalanine, tryptophan, lysine, leucine and arginine were used. Selected yeasts and LAB were tested also for: the absence of extracellular protease production by replica plating yeast colonies onto YPD plates containing 2% casein and bacteria colonies onto MRS plates containing 2% casein; for the presence of lipase activity by replica plating colonies onto agar plates containing 5% peptone, 0.5% glucose, 0.1% $NaNO_2$, 0.1% KH_2PO_4, 0.1% $MgSO_4$, 2% Tween 80 and 0.01% Rhodamine B (Bleve et al., submitted).

2.8. LAB and yeast molecular identification

Total genomic DNA from the yeast strains was prepared according to the method used by Bleve et al. (2005) and diluted to 50 ng/μl. ITS1-5,8S-ITS2 region was amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTG ATATGC-3′) (White et al., 1990). The polymerase chain reaction (PCR) conditions were as described by Esteve-Zarzoso et al. (1999) with the following modifications: initial denaturation at 94 °C for 5 min, followed by 40 cycles consisting of 30 s at 94 °C, 30 s at 52 °C and 1 min at 72 °C, followed by a final extension at 72 °C for 10 min and subsequently cooled to 8 °C. The amplified DNA products were visualized by agarose gel electrophoresis.

Bacterial cells were grown for 3–5 days at 25 °C under vacuum in liquid culture media and total DNA was extracted as described by Wilson (2001). The nearly full-length 16S rRNA gene was amplified for all isolates by using the Universal 16S forward primer (5′-G GAGAGTTAGATCTT GGCTCAG-3′) and Universal 16S reverse primer (5′-AGAAAGGAGGTGATCCAGCC-3′). A reaction mixture (50 μl) containing 1 μl (50 ng/μl) genomic DNA, 10 × PCR buffer (Euroclone), 2 mM $MgCl_2$, 200 μM each dATP, dTTP, dCTP and dGTP, primers Universal 16S forward and reverse, 0.5 μM each, and 1 U DNA polymerase (Euroclone, Italy) was prepared. Genomic DNA was amplified with a 2-min denaturation step at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 55 °C and 1 min DNA chain extension at 72 °C. The PCR was completed by 5 min DNA chain extension at 72 °C. DNA sequencing was performed as previously described Bleve et al. (2011). Sequences were analyzed by Chromas program version 1.45 and BLAST program for sequence alignment and compared with the sequences in the GenBank database.

3. Results

3.1. Pilot-scale fermentations of black table olives

The analysis of microflora associated with each of the two varieties indicated a similar degree of yeast contamination (3–7.4 × 10^4 CFU/ml; Fig. 1A), whereas no LAB and *Enterobacteriaceae* were obtained from the olive surface of both the analyzed table olive cultivars (Fig. 1B). Olive samples were soaked in brines and samples of olives and brine were collected after specific time intervals (0, 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180 days) till the 180th day of fermentation.

Concerning LAB detection, the following results were obtained: i) LAB remained undetectable in Conservolea brine samples until the 105th day of fermentation, then they increased from 7 × 10^3 CFU/ml to 2 × 10^6 CFU/ml (180th day of fermentation); ii) LAB were isolated in Kalamàta brine samples only at the 5th day of fermentation (8 × 10 CFU/ml); they then became undetectable until the 105th day (3.25 × 10^2 CFU/ml) and finally they increased to 4.2 × 10^6 CFU/ml after 180 days (Fig. 1B).

Yeasts were present throughout the fermentative process. In both fermentations, yeast population showed a decrease during the first 15 days of fermentation and then increased to 2.9–4.4 $\times$ 10^4 CFU/ml, in Conservolea and Kalamàta brines, respectively (Fig. 1A). However, from the 15th to the 105th of fermentation the count of the yeast associated with the brines of the olives Conservolea was higher than that of the yeast associated with the brines of the olives Kalamàta. Finally, Conservolea and Kalamàta fermentations showed a similar behavior as far as yeast presence from the 120th day to the end of fermentation (Fig. 1A).

3.2. Physico-chemical changes during fermentation

The evolution of brine temperatures followed the seasonal trend ranging from 8 to 30 °C without sensitive difference between the two fermentations (Fig. 2A). The pH value decrease was noticeable in the two fermentations within the first 5–10 days. From the 15th to the 120th day of fermentation, no significant pH variations were recorded (4.6–5) and then pH declined slowly, reaching minimum values (4.2–4.3) (Fig. 2B). Salinity value was stable until the 90th day of fermentation and then it increased for the cultivar Kalamàta, whereas it slightly decreased for the Conservolea cultivar (Fig. 2C).

3.3. Chemical analyses of brines during fermentation

In both Conservolea and Kalamàta brines, glucose and fructose levels showed a continuous increase during fermentation until 90–120 days and then they were completely consumed (Fig. 3A, B). Contemporarily, an increase in glycerol concentration was observed starting from the day 150 in Conservolea brines to obtain a final concentration of 0.71 g/l. Ethanol appeared at day 60, after which it remained almost stable and finally it increased to day 180 up to 1.21 g/l (Fig. 3A). In Kalamàta brines, ethanol was detectable in the last part of fermentation, from day 150–180 (1.90 g/l), whereas glycerol increase was recorded in the period between 150 and 180 days to a final concentration of 1.24 g/l (Fig. 3B).

Organic acids were quantitatively more represented in Kalamàta brines (36.41 g/l) than in Conservolea brines (14.11 g/l). In particular, citric acid showed a similar behavior of increase during fermentation in the brines of both cultivars, lactic acid started to appear after 60 days of fermentation in Conservolea brines and then it increased in concentration until end of fermentation, whereas in Kalamàta samples it was observed from the initial stages and then it increased after 60 days of fermentation, it remained stable until the 120 day and finally it increased until the end of the process. A continuous increase of acetic acid was reported, during the process, for Conservolea brines up to 5.53 g/l. In Kalamàta samples, this compound was absent until day 120 and then it was produced during the time interval between 150 and 180 days (9.27 g/l). Although the trend of increasing concentration was similar in both brines, Kalamàta samples showed a higher content of tartaric acid (Fig. 3B).

The Fig. 4 shows the evolution of phenolic compounds associated with Conservolea (A) and Kalamàta (B) during the natural

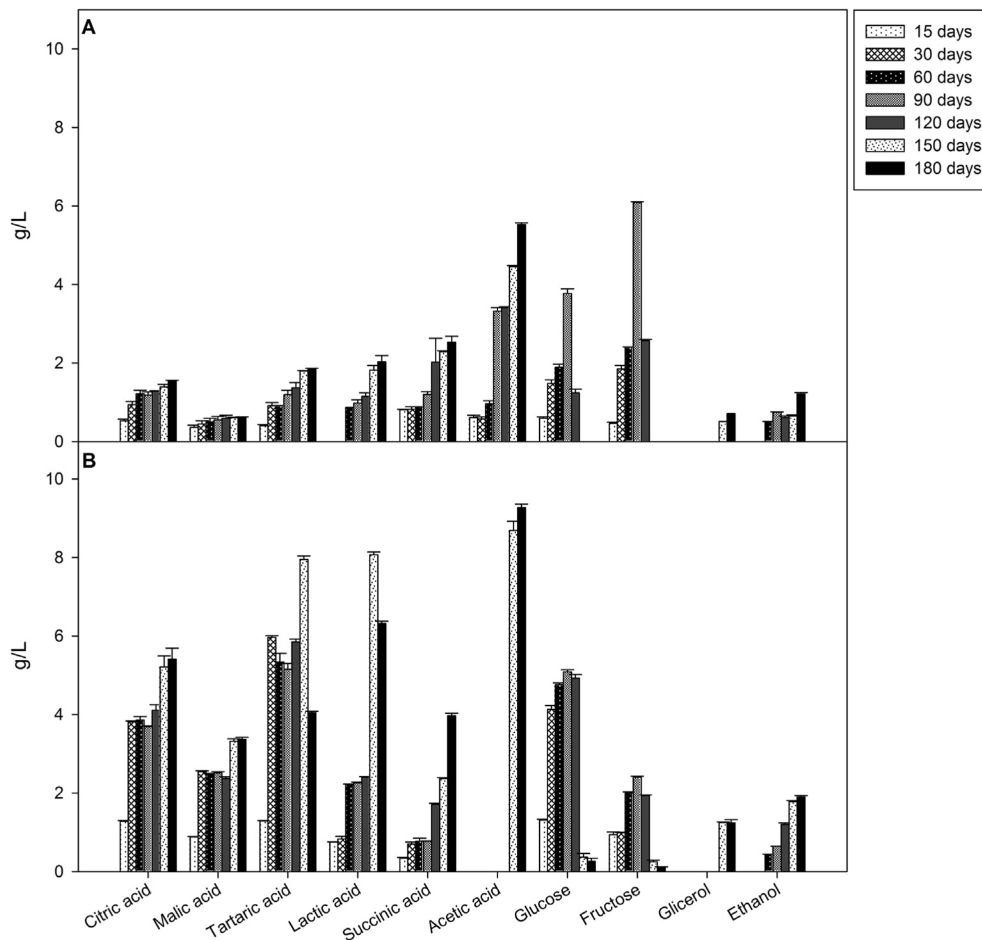

Fig. 3. Sugars, organic acid and alcohol evolution during Conservolea and Kalamàta fermentation. Conservolea (A) and Kalamàta (B) brines during natural fermentation process.

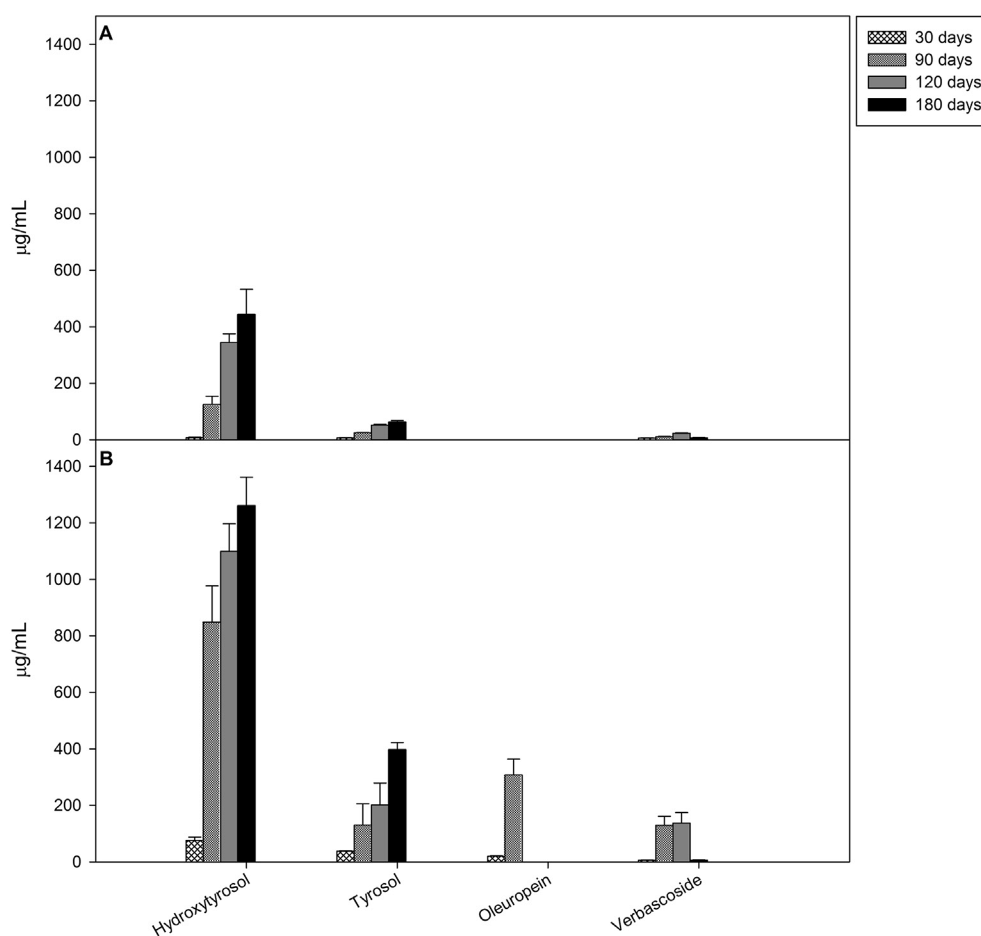

Fig. 4. Mono and polyphenol compound concentrations evolution during Conservolea and Kalamàta fermentation. Conservolea (A) and Kalamàta (B) brines during natural fermentation process.

fermentation process. All samples exhibited a similar profile of constituents: the main phenol compounds were hydroxytyrosol, tyrosol, verbascoside and oleuropein. Moreover Kalamàta brines showed a greater amount of phenol compounds than Conservolea. After 30 days of fermentation, the amount of hydroxytyrosol in Conservolea and Kalamàta brines was about 7.37 and 75.85 μg/ml, respectively. In Conservolea and Kalamàta brine samples hydroxytyrosol content increased throughout the process up to a level of 443.98 and 1261.30 μg/ml respectively at the end of the fermentation (Fig. 4A, B). In brine samples of both cultivars, tyrosol increased (up to 4 and 10-fold, respectively) in the period ranging from the 30th to the 90th day of fermentation then, it decreased to about 52 and 129 μg/ml at the end of the process, respectively in Conservolea and Kalamàta samples (Fig. 4). Oleuropein was undetectable in Conservolea brines, whereas the amount of oleuropein in Kalamàta brines was about 20 μg/ml at 30th day of fermentation, then it showed an increase to 19-fold after 90 days of fermentation and finally it became undetectable at the end of fermentation (Fig. 4A, B). Finally, in brines of both cultivar, verbascoside amount increased during the first 90 days of fermentation, then it remained almost stable at 120th day then it gradually decreased until the end of fermentation (Fig. 4A, B).

3.4. Analysis of volatile compounds in olives and brines

An analytical method based on the use of SPME/GC–MS was optimized and it was employed to correctly identify and quantify 46 different volatile compounds in Kalamàta and Conservolea table olives and brines. The identified volatile compounds were grouped into different classes, such as aldehydes–ketons, esters, alcohols, hydrocarbons, volatile phenols, fatty acids, monoterpenes and lactones. Figs. 5–7 report the average values (mean ± standard deviation) of volatile compounds concentration expressed in $\mu g_{(compound)}/Kg_{(olive\ fruit)}$ and $\mu g_{(compound)}/L_{(brines)}$ determined by analysis in triplicate using the solid-phase micro-extractions for Kalamàta and Conservolea table olives and brines. The evolution of aroma profile composition of table olives and brines is described in Fig. 5. These data were obtained by the analysis of each volatile chemical class at the time point corresponding to 30, 90 and 180 days of fermentation.

3.5. Volatile compounds in olives

Aldehydes and ketones. In Kalamàta and Conservolea olives, aldehydes were present in high amount at the initial stages of fermentation (30 days) and then their content showed a decreasing trend, whereas the ketone species acetoin appeared only in Kalamàta olives at the final stage of fermentation (180 days) (Figs. 5 and 6). Although the initial content of aldehydes was similar in the two cultivars, a more pronounced decrease in Conservolea olives than in Kalamàta olives was observed. Values ranged from 125.30 μg/kg to 23.54 μg/kg in Conservolea and from 135.72 μg/kg to 81.28 μg/kg in Kalamàta olives at 180 days of fermentation.

Alcohols. Differences in the alcohol content were observed during fermentations of Conservolea and Kalamàta olives. There was a slight increase of alcohols during fermentation in

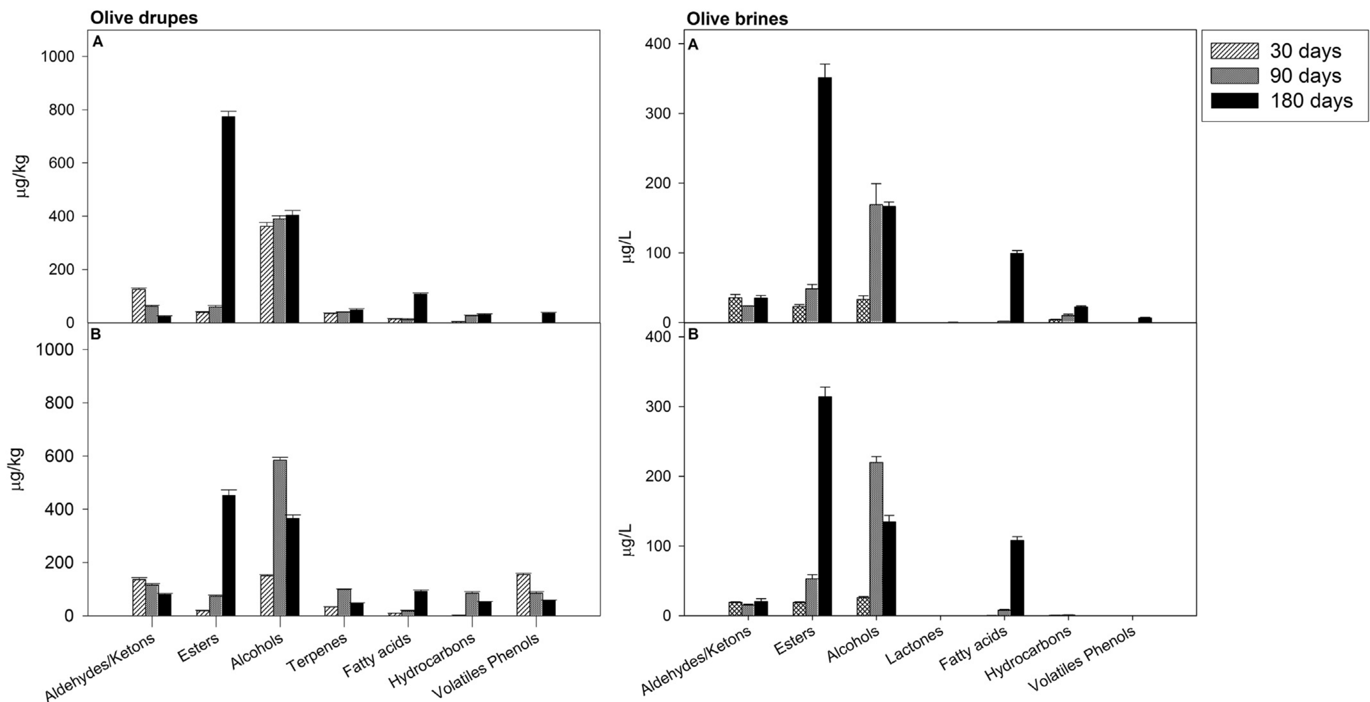

Fig. 5. Evolution of volatile compound classes associated to Conservolea and Kalamàta olives and brines. Conservolea (A) and Kalamàta (B) olive drupes (left panels) and brines (right panels) during natural fermentation process.

Conservolea olives from 362.41 μg/kg to 404.45 μg/kg, whereas a completely different behavior was observed in Kalamàta olives. In this cultivar, total alcohol content started from a concentration corresponding to 150.85 μg/kg after 30 days of fermentation, it then increased to a concentration peak of 548.26 μg/kg after 90 days of fermentation and finally it decreased to 366.30 μg/kg after 180 days of fermentation (Figs. 5 and 6). After ethanol, 2+3-methyl-1-butanol (isoamylalcohols), produced by yeast fermentation metabolic pathways, are the most representative alcohol species in both analyzed olive cultivars.

Esters. There was a slow production of esters during the 90 days of fermentation, whereas a rapid and significant increase was reported at the final stage of olive processing (180 days) from 58.22 μg/kg and 73.53 μg/kg (90 days) to 774.07 μg/kg and to 452.54 μg/kg in Conservolea and Kalamàta olives, respectively. Conservolea olive samples contained higher concentrations in these compounds than Kalamàta olive samples. The acetates of higher alcohols were more abundant than the ethyl esters of fatty acids. Among acetate esters, ethyl acetate is the most representative compound, followed by isoamyl acetate and methyl acetate, their concentrations increased during fermentation in the two table olive cultivars (Fig. 6). Isoamyl acetate (banana aroma), showed the highest concentration in all olive samples followed by ethyl octanoate (sweet aroma), ethyl hexanoate (green apple aroma) and phenyl acetate (fruity aroma) (Fig. 6). Ethyl hexanoate (fruity, apple, banana, strawberry aroma) was present only in Kalamàta olives, whereas methyl hexanoate was reported only in Conservolea olives. Several acetate esters (*n*-propyl acetate, isobutyl acetate, phenyl acetate), propionic esters (ethyl propanoate, ethyl-2-methyl propanoate), and ethyl esters (ethyl butanoate, ethyl propanoate, ethyl lactate), some of them responsible of green, fruity and floral aromas were detected only in the final stage of fermentations (180 days) in olives of both cultivars (Fig. 6).

Short chain fatty acids. Volatile short chain fatty acids increased during fermentations. The only two species identified in Conservolea and Kalamàta olives were acetic and propionic acids. Acetic acid content showed a slight decrease in Conservolea (from 13.65 μg/kg after 30 days to 11.36 μg/kg after 90 days) or a limited increase in Kalamàta (from 9.46 μg/kg after 30 days to 18.13 μg/kg after 90 days) and then a significant increase (to 101.52 μg/kg in Conservolea and to 88.52 μg/kg in Kalamàta olives) at the final stage of fermentations (Figs. 5 and 6). In both Conservolea and Kalamàta olives it was observed an increase in styrene concentration In Conservolea olives styrene showed a slightly continuous increase during the fermentation, whereas in Kalamàta olives, after an increase at the middle stage of fermentation (90 days) its content reduced from 84.81 μg/kg to 52.92 μg/kg.

Volatile phenols. Guaiacol is the only volatile phenol detected in Conservolea and Kalamàta olives. This compound, responsible of spices-curry aroma, was not detectable during the process in both olive cultivars until the end stage (180 day), where its concentration reached 36.00 μg/kg in Conservolea and to 154.72 μg/kg in Kalamàta olives.

Terpenes. Among terpene class, α-farnesene, α-cubebene, 3,7-dimethyl-1,3,7-octatriene were identified by NIST 05 data base. The most abundant sesquiterpene was 3,7-dimethyl-1,3,7-octatriene, followed by α-farnesene and α-cubebene. In Conservolea olives terpenes showed a slightly continuous increase during the fermentation from 34.70 μg/kg to 47.92 μg/kg, whereas in Kalamàta olives, after an increase at the middle stage of fermentation (90 days) its content reduced from 99.42 μg/kg to 47.06 μg/kg.

Volatile compounds in brines. Volatile compounds present in the free fraction of brines deriving from spontaneous fermentation of Conservolea and Kalamàta table olives were identified and characterized (Figs. 7 and 5). The differences observed in the volatile composition of the two cultivar brines seemed to be quantitative rather than qualitative, except for hydrocarbons class that was in higher concentration in Conservolea samples. In both Conservolea and Kalamàta brines collected after 90 and 180 days of

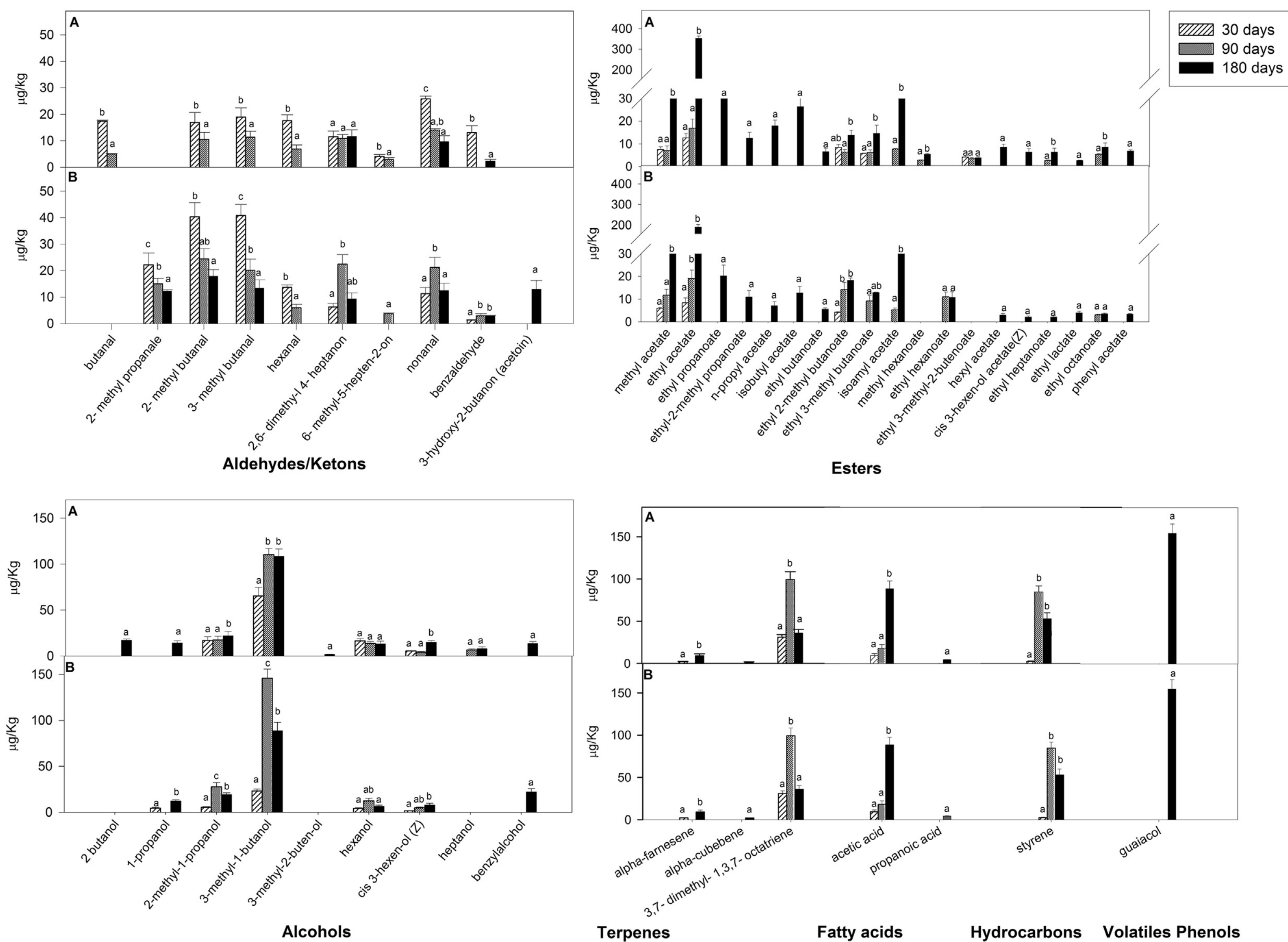

Fig. 6. SPME/GC–MS analysis of Conservolea and Kalamàta olives during fermentation. SPME/GC–MS quantitative data, including concentration (μg/Kg) with standard deviation (SD) of the volatile compounds identified in Conservolea (A) and Kalamàta (B) table olives during natural fermentation. Results expressed as means 3 replicates. For each compound, according to the result of the one-way ANOVA test, concentration values that do not share a common superscript are significantly different ($P < 0.05$).

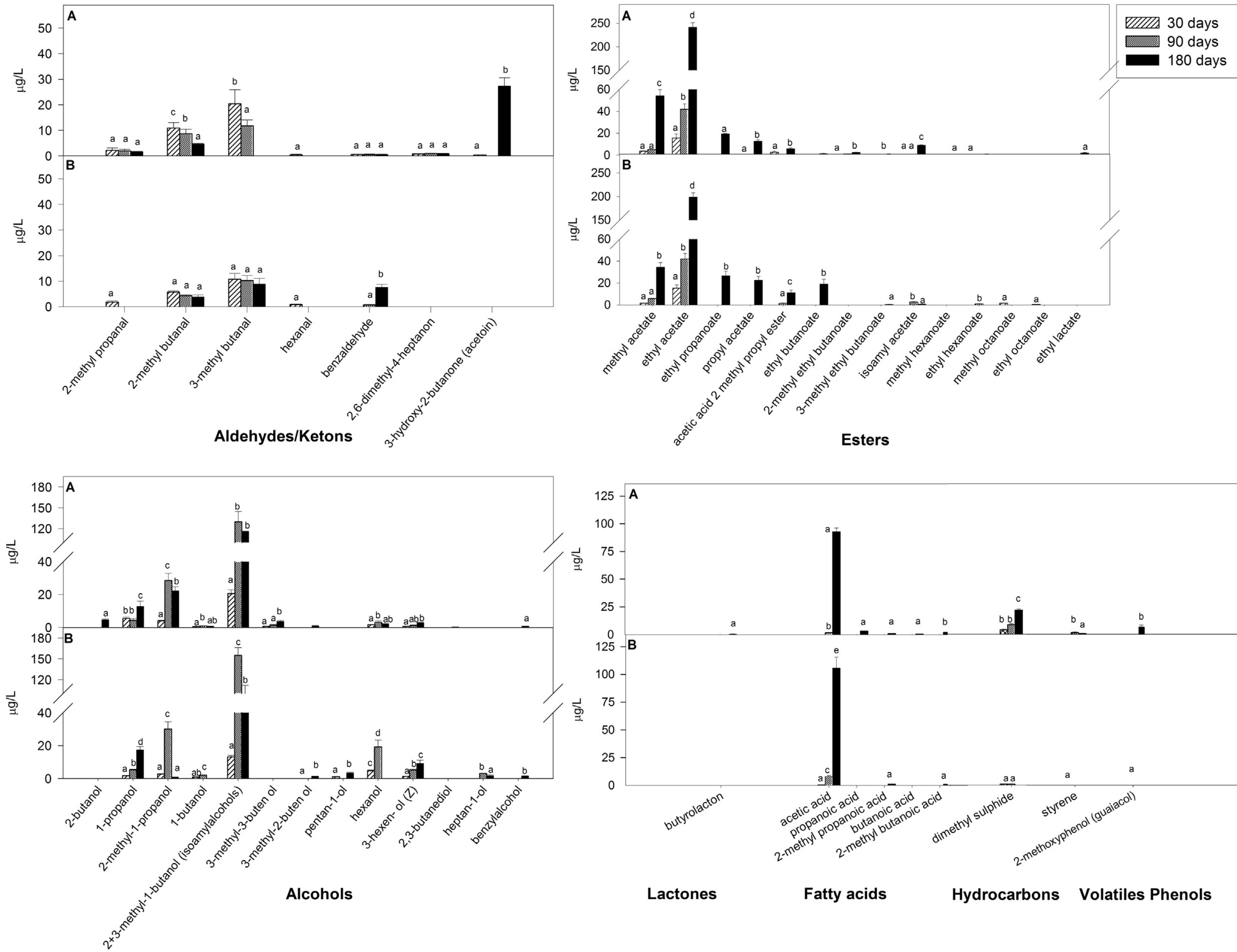

Fig. 7. SPME/GC–MS analysis of Conservolea and Kalamàta brines during fermentation. SPME/GC–MS quantitative data, including concentration (µg/L) with standard deviation (SD) of the volatile compounds identified in Conservolea (A) and Kalamàta (B) brines during natural fermentation. Results expressed as means 3 replicates. For each compound, according to the result of the one-way ANOVA test, concentration values that do not share a common superscript are significantly different ($P < 0.05$).

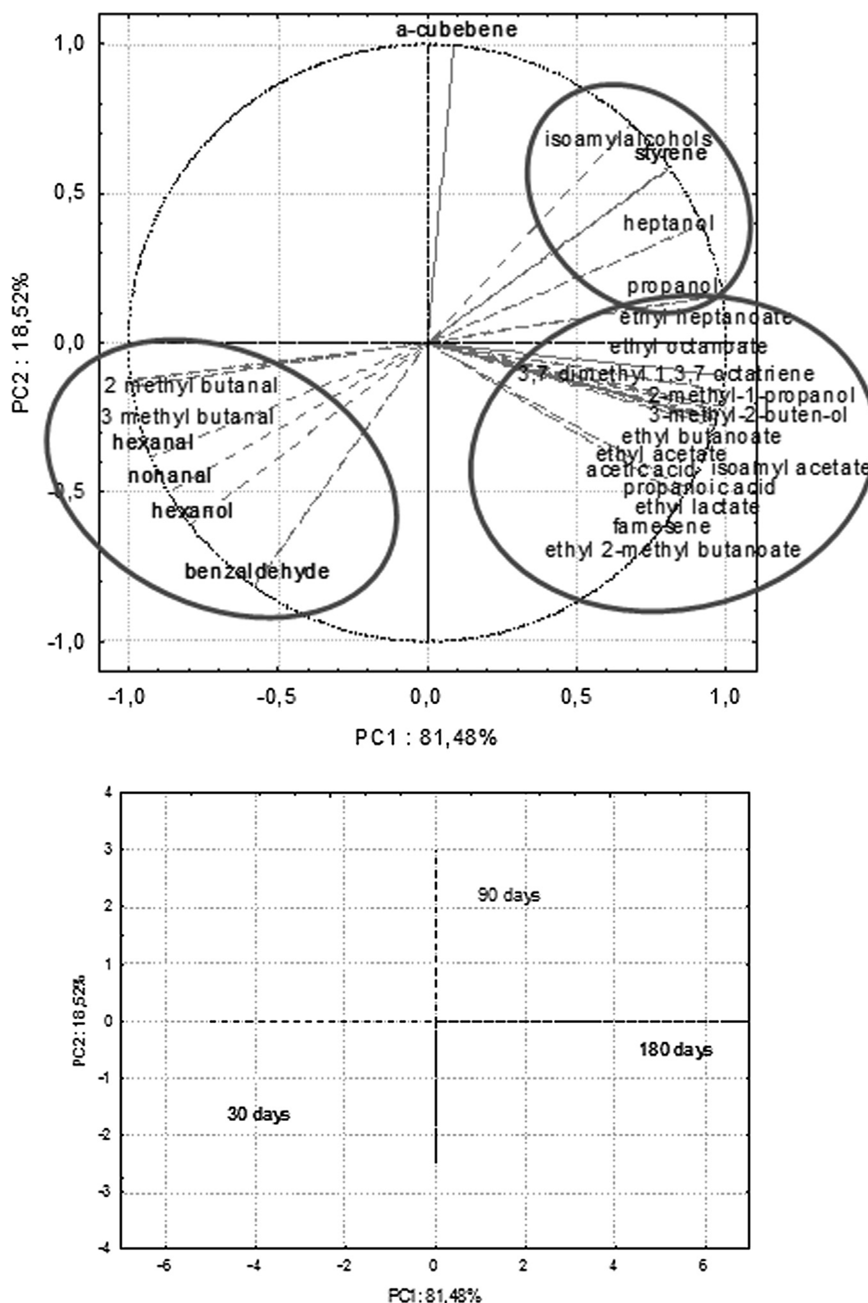

Fig. 8. PCA of volatile compounds associated with Conservolea fermented table olives. PCA variables were the data obtained from the analysis of concentration and presence of volatile compounds at three different fermentation times. The figure is bi-plot, displaying the sample scores and variable loadings in the planes formed by PC1–PC2.

fermentation it was observed a lesser concentration of esters, alcohols and short chain fatty acids than in the corresponding olives. Analogously to what observed in olive samples, the most representative ester species were ethyl acetate and methyl acetate. Moreover, propionic esters (ethyl propanoate, propyl acetate, acetic acid-2-methyl propyl ester) were detected in relatively high concentration in the last stage fermentation in both the Conservolea and Kalamàta brines. Alcohols detected in higher concentrations were 3-methyl-1-butanol, followed by 2-methyl propanol and propanol in both the two olive cultivars, analogously to what observed in olives. In Kalamàta brines there was a higher amount of fatty acids than in Conservolea brines. In particular, acetic acid was the most abundant compound, followed by propanoic acid and 2-methylpropanoic acids. Terpens were not detectable in brines, since they are lipophilic compounds.

3.6. Principal component analysis (PCA)

PCA analysis of the data obtained by the GC–MS analysis was carried out in order to identify different compounds to be used as descriptor for monitoring yeast and bacteria activities during the fermentation process.

PCA was applied to the mean quantity value of volatile compounds identified in each of the two table olive fermentation at three different fermentation times (30, 90 and 180 days).

A two-dimensional model was produced to explain the 100% variance in the data, and two bi-plots displayed PC1 vs PC2 are reported in Fig. 8 and Fig. 9, which show the projection of the variables on the plane defined by the first and second principal components. In Conservolea and Kalamàta planes, the first principal component (PC1) explained 81.48% and 64.92%, respectively,

of the total variability, between volatile compounds produced during fermentation and the second principal component (PC2) accounted for an additional 18.52% (Conservolea) and 35.08% (Kalamàta). PCA analysis (Figs. 8 and 9) revealed in both the analyzed table olive samples, one group, consisting mainly of aldehydes, that proved to be closely related to the first stage of fermentation (30 days); a second group represented by higher alcohols (isoamylalcohols) and styrene associated with the middle stage of fermentation (90 days), characterized by the presence of yeasts and the third one that consisted of acetate esters and acetic acid linked to the final step in olive fermentation (180 days) characterized by the presence of the bacteria.

3.7. Microbiological characterization of yeasts and bacteria isolates

The protocol described by Bleve et al. (submitted) was applied for the isolation and characterization of yeasts and bacteria from fermented Conservolea and Kalamàta table olives and for the preparation of authochthonous starter cultures suitable for industrial production. The protocol consisted of three steps:

1. selection of isolated yeasts and bacteria on model brines;
2. selection of yeast and bacteria deriving from the first step of selection against specific physiological and technological

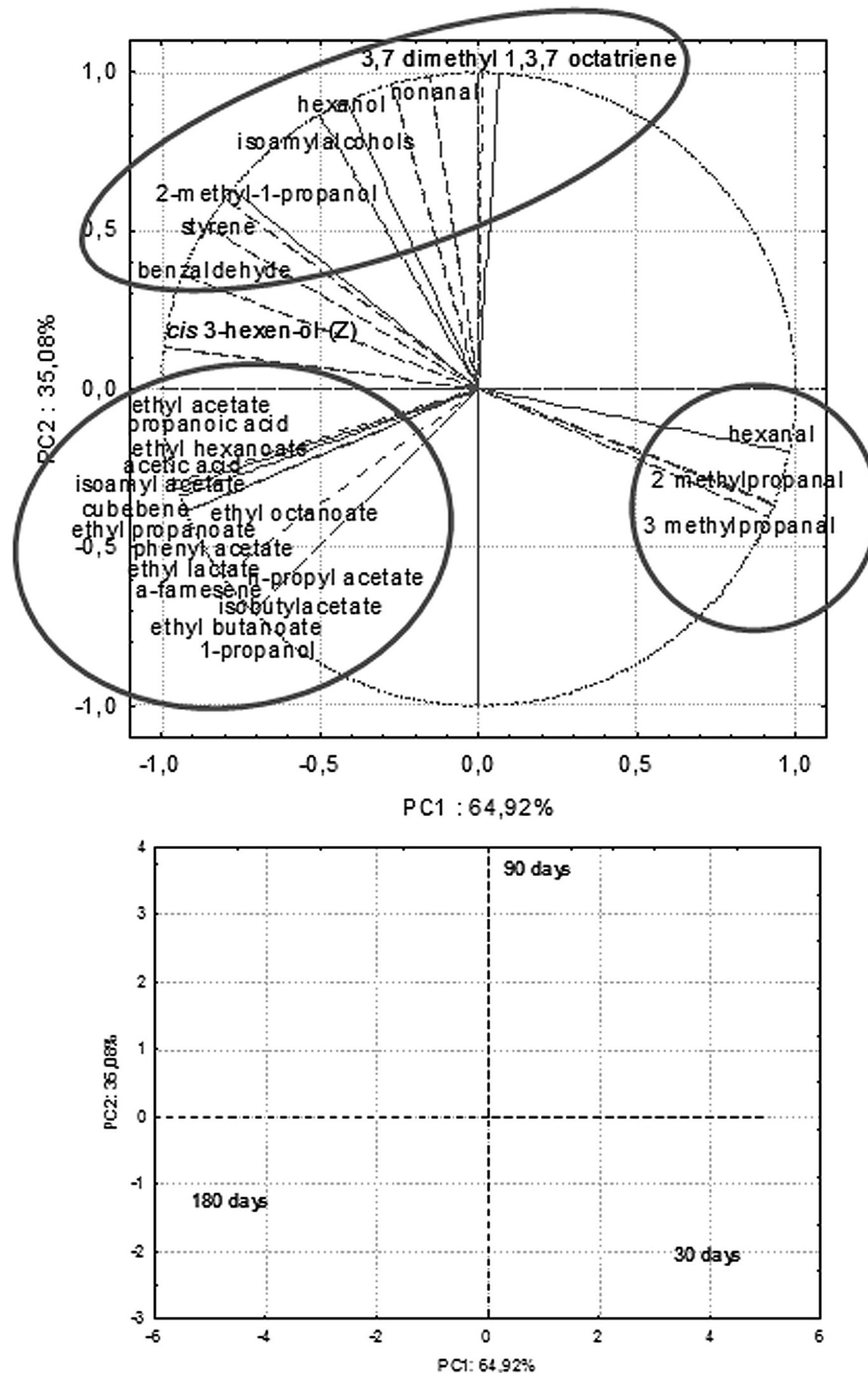

Fig. 9. PCA of volatile compounds associated with Kalamàta fermented table olives. Score plot of variables (concentration of volatile molecules) and three different fermentation times in the plane formed by the first two principal components (PC1 against PC2).

properties (the presence of beta-glucosidase activity and the inability to produce biogenic amines);

3. identification at the molecular level of the isolates characterized by the selected physiological and technological properties;

Five hundred yeast isolates for each of the two olive cultivars were grown on MB for 15 days at 14 °C. After this first selection step, the yeast population able to survive and showing the best score in a pattern ranging from 3 (highest growth) to 0 (lowest growth) to the above constraints was isolated and it consisted of 128 isolates from Conservolea and 362 isolates from Kalamàta fermentations.

As second selection step, the yeast population able to produce beta-glucosidase activity and unable to produce biogenic amines was isolated. The score assigned for beta-glucosidase activity was 3 (intense brown), 2 (light brown), 1 (yellow-milky), 0 (white), whereas, concerning amino acids decarboxylation activities, the value 0 was assigned for isolates that remained white, the value 1 for isolates producing slight blue halo, 2 for isolates which produced intense blue halo and 3 for isolates surrounded by an intense purple halo. The yeast population characterized by intense beta-glucosidase activity (score 3) and absence of amino acids decarboxylation activities (score 0) consisted of 37 Conservolea isolates (out of 128 original isolates grown on MB) and 41 Kalamàta isolates (out of 362 original isolates grown on MB).

These isolates were evaluated for the presence of protease and lipase activities: 9 isolates from Conservolea and 8 isolates from Kalamàta showed protease activity on plate against casein, whereas none of the isolates deriving from the second selection step demonstrated lipase activity.

In the third step the yeast isolates characterized by the above described enzymatic activities were identified at the molecular level.

Yeast species identified as Generally Recognized As Safe (GRAS) species in Conservolea olives were *Debaryomyces hansenii* (56%), *Pichia anomala* (44%). From Kalamàta fermentations, yeast isolates identified belonged to the GRAS species were *Saccharomyces cerevisiae* (40%), *Pichia anomala* (23%), *D. hansenii* (19%) and *P. membranifaciens* (11%) and to the non-GRAS species *Guehomyces pullulans* (7%).

Five hundred bacterial isolates for each of the two olive cultivars were applied on MB and incubated for 15 days at 14 °C. After this selection step, a bacterial population being resistant to the above constraint was isolated and it consisted of 166 isolates from Conservolea and 250 isolates from Kalamàta. The microbial isolates were then tested by plate assays for the presence of beta-glucosidase activity and the inability to produce biogenic amines.

In order to characterize the presence of beta-glucosidase and amino acids decarboxylation activities on bacterial isolates, the same chromatic scales on the plate used for yeasts was applied.

After this second selection step, the LAB population which satisfied the above parameters (intense beta-glucosidase activity, score 3, and absence of amino acids decarboxylation activities, score 0) consisted of 10 Conservolea isolates (out of 166 original isolates grown on MB) and 20 Kalamàta isolates (out of the 250 original isolates grown on MB). These isolates were then evaluated for the presence of protease and lipase activities. None of the isolates from Conservolea and Kalamàta showed protease and/or lipase activity on plate.

By the third step, molecular identification of the selected strains revealed that bacterial isolates obtained from Conservolea fermentations belonged to species *L. plantarum* (86%), and *Acetobacter tropicalis* (4%). Bacterial isolates selected from Kalamàta fermentation belonged to *Leuconostoc mesenteroides* (62%) and the *L. plantarum* (38%) species.

4. Discussion

In the traditional Greek method for table olive production, the anaerobic fermentation of black olives is a spontaneous process carried out by coexisting communities of yeasts and lactic acid bacteria (LAB).

In general, the fermentation is carried out by homo- and hetero-fermentative LAB and/or yeasts and depends on the cultivars itself and on industrial and agricultural practices. The table olive initial processing and the subsequent changes occurring during fermentation leads to the dominance of LAB and/or yeasts able to participate in conferring the required product characteristics.

Yeasts are associated with the surface of Conservolea and Kalamàta olive fruits and they play a substantial role throughout natural fermentations occurring during table olive production (Deiana et al., 1992; Arroyo-López, 2007). Analogously to what observed by Alves et al. (2012), counts of the yeast population increased all along the Conservolea and Kalamàta fermentation process from 6×10–10^2 CFU/ml (beginning) to 10^5 CFU/ml (end). However, the role of yeasts during olive fermentation is under discussion, since they can produce compounds important for quality and flavor of the final product (Garrido-Fernandez et al., 1997; Segovia Bravo et al., 2007), but they can also act as spoilage microorganisms, causing gas pockets, swollen containers, cloudy brines and off-flavors and off-odors (Lamzira et al., 2005; Hernández et al., 2007; Turantas et al., 1999).

Since the use of a high level of NaCl during fermentation (>8% in the equilibrium) could favor the growth of yeasts against LAB, table olive industries have reduced salt level to 6–8% favoring a mixed fermentation by LAB and yeasts that coexist until the end of fermentation resulting in a product with better characteristics (pH, acidity) compared to the traditional process (Tassou et al., 2002).

In fresh prepared brines of both green and black olives, the growth of LAB is partially inhibited due to the presence of phenolic compounds. In fact, in both Conservolea and Kalamàta brines, LAB remained undetectable until 105 days of fermentation. In agreement with Doulgeraki et al. (2013), the microbiological analyses of olive samples revealed a final LAB populations of about 10^6 CFU/ml in the brines of Conservolea and Kalamàta.

It has been reported that both olive variety and differences in the spontaneous fermentation process influenced the phenol content (Morello et al., 2004). The evolution of phenolic compounds found in the brines of the two cultivars can be attributed to the ability of yeast and LAB species to hydrolyze compounds such as oleuropein by means of beta-glucosidase (Servili et al., 2006). As expected, after fermentation process, the concentration of the bitter glucoside oleuropein decreased in the brine.

At the same time, the concentrations of mono-phenolic compounds, such as hydroxytyrosol and tyrosol increased during the process in both fermentations. Our results are in agreement with those reported by Ben Othman et al. (2009) and Pistarino et al. (2013) who identified hydroxytyrosol as the main simple phenolic compound in the brine of Tunisian black and in Taggiasca olives, respectively. The appearance of this compound in the brine can be related to the hydrolysis of oleuropein (Parinos et al., 2007) or hydroxytyrosol-4-beta-glucoside (Romero et al., 2004). Furthermore, the increase of tyrosol concentration during the olive fermentation was observed by Romero et al. (2004) and Ben Othman et al. (2009).

Ethanol concentration varied greatly between the two table olive cultivars, reflecting different yeasts activity and hetero-fermentative LAB. Ethanol is of great importance with respect to the organoleptic properties of naturally fermented black olives (Flemin et al., 1969).

The trends of sugars consumption and the corresponding synthesis of glycerol ad ethanol confirmed the fermentation evolution. In agreement to other studies performed on green and black olive fermentations, lactic, citric, tartaric and acetic acids were the major metabolic products with a significant presence in the brines (Nychas et al., 2002; Chorianopoulos et al., 2005; Panagou et al., 2008). In accordance to Panagou et al. (2008), during the increase of the organic acid content a decrease in pH value in both fermentation was observed and the final value was maintained at about 4.0, which is satisfactory for naturally black olive fermentation. The high level of acetic acid in the final stages of fermentations can be attributed to hetero-fermentative metabolism of bacteria under conditions of environmental stress such as oxygen and nutrient limitation, salt concentration, low pH value (Bobillo and Marshall, 1991).

The evolution of higher alcohols (isoamylalcohols) concentration in olive and brine samples of Conservolea and Kalamàta cultivars could be considered important markers identifying the yeasts as responsible of the of the first part of the fermentation (90 days) (Romano et al., 2003). It could be hypothesized that the differences in C6 alcohols concentration observed between Conservolea and Kalamàta fermented olives can be linked to the different yeast population associated with the olive fermentations. In fact, according to Torrens et al. (2008), C6 alcohols such as 1-hexanol and cis-3-hexen-1-ol, characterized by a “vegetal” and “herbaceous” aroma, seem to be linked to the different yeast strain used for wine production.

Ethanol is a precursor of ethyl esters, with ethyl acetate being the major representative, derived from the ethanolysis of acetyl-CoA (Roza et al., 2003). In a similar manner to the effect that it can produce in wines, the relevant presence of the ethyl-acetate ester at the end of fermentation in both Conservolea and Kalamàta fermentations suggests that this compound adds complexity to the aroma of the final product (Mallouchos et al., 2002), noticeably, it did not exceed 200 mg/l, a quantity threshold that could confer disagreeable odor (Roza et al., 2003).

Other esters identified were isoamyl acetate and methyl acetate, the former was in higher quantity in Conservolea olives and the latter in Kalamàta olives. Isoamyl acetate belongs to the acetic acid ester group, which is mainly responsible for the sweet-fruity-banana aroma in wine, whereas methyl acetate may give a specific contribution to olive aroma since it does not present the typical fruity aroma, characteristic of other acetic esters (Capone et al., 2013).

In both cultivars, hexanal (Z)-hex-3-enol, hexanol (Z)-hex-3-enol acetate and hexyl acetate were found at various concentrations, various levels of hexanal, hexanol and (Z)-hex-3-enol and this data were consistent with the data reported by Sabatini and Marsilio (2008) who hypothesized that microbial enzymes affect the metabolism of polyunsaturated fatty acids during brining.

Fatty acids have been described as giving rise to fruity, cheesy, fatty and rancid notes. As reported in wines, also in table olives they could be very important for aromatic equilibrium because they balance the hydrolysis of the corresponding esters, thus enhancing the aroma complexity (Boidron, 1988; Gil et al., 2006). The different concentrations of fatty acids (acetic acid and propionic acid) in the two cultivar of table olives depends on the bacteria population responsible of the second part of fermentation. Terpenes production is closely linked to cultivars, geographical area, climatic conditions and proliferation of specific pests and micro-organisms characteristic of a given production area (Damascelli and Palmisano, 2013). In fact, the two sesquiterpenes farnesene and cubebene and monoterpene cimene are associated with Conservolea and Kalamàta olives, whereas they are not detectable in the two Italian table olive cultivars Leccino and Cellina di Nardò (Bleve et al., submitted).

Styrene content shows an increase during fermentation in both cultivars. This compound could be produced by L-phenylalanine deamination (Ashurts, 1999; Tressler et al., 1977) and decarboxylation of trans-cinnamic acid (Shimada et al., 1992) or by the dehydration of 2-phenylethanol, which is produced in yeasts by L-phenylalanine catabolism (Patterson et al., 1992).

PCA analysis was used to reveal the volatile compounds that can be linked to the metabolic activities of yeasts and bacteria, mainly LAB, during the process. In Fig. 8 (Conservolea table olives) negative loadings for PC1 and PC2 and in Fig. 9 positive loading for PC1 (Kalamàta table olives) were releated to aldehydes (2-methylbutanal, 3-methylbutanal, hexanal, hexanol, nonanal, 2-methyl propanal, 3-methylpropanal) linked to first stage of fermentation (30 days). The presence of C5-C6 aldehydes and alcohols suggests that the lipoxygenase pathway may be activated during black-ripe table olive processing. The middle stage of fermentation (30–90 days) was dominated by the presence of yeasts and it can be described by the presence of isoamylalcohols and styrene. In fact, in both PCA graphics, positive loading for PC2 were characterized by the presence of these compounds.

Esters, acetate and fatty acids were located in a quadrant of PCA plot closely associated with the final step of processing (180 days) characterized by the presence of bacteria (Sabatini et al., 2008).

These considerations suggest that, volatile composition of table olives is the results of the interaction of two principal metabolism, yeast and bacteria, and their sequential activity influence chemical composition significantly and produce a more complex aroma.

By means of the PCA analysis, it was possible to identify during the fermentation of Conservolea and Kalamàta olives three main temporary steps characterized by the presence of the following chemical descriptors: aldehydes at the beginning, isoamylalcohols and styrene in the middle and ethyl esters and fatty acids at the end of fermentation. The above evidences are not likely to be dependent from the Conservolea and Kalamàta drupes, but they might be extended also to other black olive cultivar and they could help in monitoring the fermentation process.

Table olive fermentations occur spontaneously in many cases without adding any starter culture (Ruiz-Barba and Jiménez-Díaz, 2012). However, interest in the development and use of starter cultures for table olive production is increasing (Randazzo et al., 2012). LAB and yeasts play an important role in the production of treated and natural table olives (Arroyo-López et al., 2012a). LAB have been widely studied due to their abilities to produce lactic acid and bacteriocins and to degrade oleuropein, producing the biological debittering of the fruits, whereas there is little information available about the real effects of yeasts on the organoleptic properties of the fruits and their interactions with the other microorganisms present during olive fermentation (Arroyo-López et al., 2012a).

The data reported in this study demonstrated that the spontaneous fermentation of Conservolea and Kalamàta black olives is driven by yeasts during the first half of the process (at least 90 days) and then in cooperation with LAB for the remaining period (90–180 days). On the basis of these evidences, we decided to study the microbiota associated with these spontaneous fermentation in order to formulate mixed autochthonous starters for the industrial table olive production in a method that mimics the natural succession of these microorganisms during the process.

In the first selection step, two model brines, one specific for yeasts and another useful for bacteria were used, in order to combine in a single test the conditions usually encountered in olive

fermentation. By these model brines, microorganisms isolated from different time points during Conservolea and Kalamàta olives fermentations were subjected at the same time to several constraints, normally encountered in industrial plant of table olive production, such as low pH, high NaCl concentration, low temperature, mono- and polyphenol compounds. In the second step, yeasts and LAB were selected according to two key criteria: i) beta-glucosidase activity, which make the strains able to hydrolyze oleuropein and to enhance olive aroma (Restuccia et al., 2011); ii) no production of biogenic amines, which represent an emerging problem in table olives, wine and other fermented products (Garcia et al., 2004; Spano et al., 2010; Tristezza et al., 2013). The promising yeasts and bacteria were analyzed also for other important enzymatic traits with important effects on olive quality, such as proteolytic and lipolytic activities (Arroyo-López et al., 2012b). In fact, the presence of lipase activity is highly requested, since it could improve the aromatic profile of fermented olives by increasing their free fatty acid content (Rodriguez-Gomez et al., 2012), whereas LAB and yeast isolates would be selected for the absence of proteolyitc activity, which could have a negative impact on olive quality because it is related to olive softening (Arroyo-López e al., 2008).

Moreover, among several possible enzymatic activities that could decrease the quality of the product, the absence of pectolytic activities would be also interesting as criterium for the selection of yeasts with good technological properties (Bevilacqua et al., 2013). In fact, although polygalacturonase (PGases), pectate lyase and pectin methyl esterase (PME) are applied in wine industries to facilitate juice extraction, viscosity reduction and clarification, releasing more color and flavor compounds (Van Rensburg and Pretorius, 2000), they are undesirable properties in table olives production since they that could produce fruit softening (Hernández et al., 2007).

After the assessment of the enzymatic features, promising yeast and LAB isolates candidate as starters were identified by molecular approaches and GRAS strains were identified. The yeast species identified in Conservolea olive fermentation were *D. hansenii* and *P. anomala*, as previously reported by Nisiotou et al. (2010), whereas in Kalamàta fermentation the isolates belonged to *S. cerevisiae*, *P. anomala*, *D. hansenii* and *P. membranifaciens*. Analogously to the data reported by Doulgeraki et al. (2013), molecular identification of the selected strains revealed that GRAS bacterial isolates obtained from Conservolea and Kalamàta fermentations belonged to species *L. plantarum* and *L. mesenteroides*.

5. Conclusions

The chemical descriptors identified in this study could be used by producers to monitor the fermentation of black table olives produced by Greek method, making the process more predictable and standardized.

For the first time, the characterization of yeast and LAB isolates associated to Conservolea and Kalamàta olive fermentations represents the first stage of a selection of new microorganisms that can be candidate as autochthonous starters. Then, a new a approach in controlling the fermentation process is proposed by the use of yeasts together with LAB to shorten the time of fermentation, to standardize the process and to improve organoleptic and nutritional properties of olives.

New experiments are now under the way for the validation of GRAS yeasts (*D. hansenii* and *S. cerevisiae* isolates for Conservolea and Kalamàta olives, respectively) and LAB (*L. mesenteroides* for Kalamàta and *L. plantarum* for Conservolea olives) as candidate starter in pilot-scale fermentations of Conservolea and Kalamàta olives performing co-inoculation or sequential inoculation strategies for the optimization of mixed starters.

Acknowledgments

This work was supported by BIOLEA I1.11.04 (subsidy contract number) project (Interreg Greece–Italy) and by Regione Puglia.

Conservolea and Kalamàta black olives were kindly provided by Prof. Tasioula-Margari from Ioannina (Greece). The authors wish to thank Dr. Vittorio Falco for his high specific technical assistance for the DNA sequencing. The authors wish to thank also Mr. Pantaleo Piccinno and Mr. Benedetto De Serio (Coldiretti Lecce) for encouraging research and for helpful and critical discussion.

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