Microbiota and metabolome of un-started and started Greek-type fermentation of Bella di Cerignola table olives

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Abstract

This study aimed to utilize an "omics" approach to evaluate the ability of selected lactobacilli and yeasts to improve the fermentation process of Bella di Cerignola table olives. Four types of fermentations were performed at the pilot-plant scale: un-started fermented olives used as a control (Ctrl); olives started with a commercial Lactobacillus plantarum strain (S); commercial L. plantarum strain and autochthonous yeast Wickeramomyces anomalus DiSSPA73 (SY); and L. plantarum, W. anomalus DiSSPA73, autochthonous L. plantarum DiSSPA1A7 and Lactobacillus pentosus DiSSPA7 (SYL). Compared to Ctrl, S, SY, SYL showed a higher acidification ($P < 0.05$) of the brine, which reached a pH value of 4.49 after one day of fermentation. The microbiota of unfermented olives and brine after one day of fermentation was primarily composed of Enterobacteria that belonged to Hafnia alvei and Methylobacterium. However, L. plantarum and L. pentosus dominated the total and metabolically active microbiota of the Ctrl brines and olives at the end of the fermentation. The use of lactobacilli and W. anomalus DiSSPA73 as a starter culture markedly affected the microbiota of the brines after one day of fermentation. The number of species (OTU) and the results of an alpha diversity analysis indicated that the microbial diversity of the brines was markedly simplified by the S, SY and, in particular, SYL fermentations. According to the lowest biodiversity, S, SY and SYL samples showed the lowest abundance of Proteobacteria, including Enterobacteriacea, Lactococcus lactis, Propionibacterium acidipropionici and Clostridium. The lactobacilli and W. anomalus DiSSPA73 used in this study markedly affected the amounts of free amino acids, phenolic and volatile organic compounds. Both a texture profile analysis and a sensory evaluation showed the highest appreciation for all of the started table olives. As shown through microbiological, biochemical, and sensory analyses, an accelerated fermentation of Bella di Cerignola table olives was achieved using the selected lactobacilli and yeast strains.

1. Introduction

For centuries, fermented foods (milk, meat, cereal and olive products) have been manufactured through spontaneous fermentations performed by the indigenous microbiota activity present in both raw food ingredients and the environment. The need to improve and standardize the food-making process as well as the sensory, nutritional and functional properties and shelf-life of foods has also led to the development of technologies to speed up the fermentation using selected microorganisms (Gobbetti et al., 2010). Overall, many efforts studies have investigated the selection and commercialization of starter preparations for dairy, cereal and meat products, but many vegetables, such as table olives, are still produced using the adventitious microbiota (Heperkan, 2013). Table olives are the fermented fruit of the Olea europaea L., which is traditionally cultivated in the Mediterranean countries (Spain, Italy, Greece and Turkey, mainly) and, more recently, in America, Australia and the Middle East (IOC, 2012). Table olives are an important component of the Mediterranean diet with potential beneficial effects on human health due to all of the above-described

antioxidant properties of phenolic compounds (Corsetti et al., 2012). Immediately after harvesting, olives are inedible due to their high amount of oleuropein, a phenolic compound with a bitter taste (Garcia et al., 2004). Several traditional protocols are used for the treatment of table olives, and these include the Spanish- and Greek-type protocols, which are universally adapted to the production of table olives. Based on the Spanish-type protocol, olives are treated with 2e3% NaOH aqueous solution to reduce their bitterness by the hydrolysis of polyphenol compounds. After washing in water, the olives are brined with an initial concentration of 8e12% NaCl and allowed to ferment for 30 e 60 days at room temperature (Garcia et al., 2004). Based on the Greek-type protocol, olives are directly brined and allowed to ferment for 8e12 months until they lose their bitterness. Overall, the fermentation period depends on the type of cultivar, NaCl content and temperature (Tassou et al., 2002; Arroyo-Lopez et al., 2008; Panagou et al., 2008). In general, the fermentation process of table olives is carried out by endogenous lactic acid bacteria and yeasts without the addition of starters (Heperkan, 2013; Corsetti et al., 2012). The microbial composition of some table olives during fermentation was previously characterized and reviewed (Botta and Cocolin, 2012; Heperkan, 2013). The microbiota of processed olives and/or brines is composed of a complex association of bacteria, such as lactic acid bacteria, Enterobacteriaceae, Clostridium, Staphylococcus, yeasts and, occasionally, molds. More than twenty lactic acid bacteria species belonging to the Leuconostoc, Pediococcus, Enterococcus, Streptococcus, Weissella, and, particularly, Lactobacillus genera have been isolated from olives and/or brines (Botta and Cocolin, 2012; Heperkan, 2013). The most commonly isolated genera of yeasts are Candida, Pichia, Debaryomyces, Saccharomyces, Issatchenkia, Rhodotorula and Wickerhamomyces. A single fermentation process of table olives can harbor simple (few species) to very complex microbial consortia. The final microbiota is a result of the complex interactions between the indigenous microbiota of the olives and the house microbiota (e.g., fermentation vessels and pipelines) (Panagou et al., 2003). For example, some Gram-negative bacteria, such as Enterobacteriaceae, are found at the beginning of the process but, due to acidification of the environment by lactic acid bacteria, exhibit a decrease after a few weeks of fermentation (Panagou et al., 2003; Abriouel et al., 2011). In addition to the above-mentioned acidification, lactic acid bacteria are also necessary for the hydrolysis of bitter compounds (Greek-type table olives), to improve the aroma and to stabilize the final product (Hurtado et al., 2012). Together with lactic acid bacteria, yeasts contribute to the organoleptic quality and shelf-life of table olives (Arroyo-Lopez et al., 2008). The final quality of table olives is also determined by the complex interactions between the indigenous olive microbiota, the house microbiota and, if added, the starter culture (Heperkan, 2013). Increased studies are investigating the selection of starter cultures for the fermentation of table olives to standardize the process (Bevilacqua et al., 2015). However, the diffusion of starter cultures remains limited. The selection process is complex and requires validation at the industrial level. A better knowledge of the microbial composition and the relative metabolomics of table olives may undoubtedly help the selection and use of starter cultures in table olive fermentation. As adapted for other fermented foods, “omics” approaches can aid in the design of autochthonous multiple strain cultures for table olive fermentation. This study aimed to evaluate the ability of selected lactobacilli and yeasts to accelerate the fermentation and improve the sensory properties of Bella di Cerignola table olives.

2. Materials and method

2.1. Microbial strains and culture conditions

Lactobacillus plantarum DiSSPA1A7, Lactobacillus pentosus DiSSPA7 and Wickeramomyces anomalus DiSSPA73 were previously isolated from cv. Bella di Cerignola table olives and genetically identified by partial sequencing of the 16S rRNA (lactobacilli) and D1/D2 domain of 26S rDNA (yeasts) genes. DiSSPA1A7, DiSSPA7, and DiSSPA73 were selected due to their ability to ferment table olives during the micro-fermentation activities funded by the Ministero dello Sviluppo Economico and Fondo Europeo di Sviluppo Regionale (PON02_00186_3417037, project PROINNO_BIT). All of the strains were able to grow in olive brine and to

hydrolyze oleuropein. In addition, L. plantarum DiSSPA1A7, L. pentosus DiSSPA7 and W. anomalus DiSSPA73 showed antimicrobial activity against some bacterial (Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Clostridium butyrricum, Propionibacterium sp., Cellulomonas flavigena, S. aureus and Bacillus cereus) and/or fungal (Penicillium roqueforti DPPMAF1; Penicillium sp., Aspergillus parasiticus CBS 971.97, Penicillium carneum CBS 112297, Penicillium bialowiezense CBS, Penicillium aethipicum, Debaryomyces sp., Candida sp. and Pichia sp.) strains. The well-diffusion assay (Schillinger and Lucke, 1989) was used to determine the inhibitory activity of L. plantarum DiSSPA1A7, L. pentosus DiSSPA7 and W. anomalus DiSSPA73 toward bacteria, whereas to determine their antifungal activity, a method based on the determination of the hyphal radial growth rate of fungi was used (Coda et al., 2011). In particular, the water/salt-soluble extracts obtained from dough inoculated with the selected strains were sterilized by filtration through 0.22-mm membrane filters (Millipore Corporation, Bedford, MA, USA) and added (30%, vol/vol, final concentration) to sterilized potato dextrose agar (PDA) medium (Oxoid). After mixing, aliquots of 15 ml were poured into Petri plates (90-mm diameter). The control plates contained PDA alone. The assay was conducted by placing a 3-mm-diameter plug of growing mycelia onto the center of Petri dishes containing the culture medium. The plates were incubated aerobically at 25°C. Three replicates were run simultaneously. The radial growth of mycelia (colony diameter, mm) in all of the plates was measured eight days after inoculation. Each datum point is the mean of at least four measurements of a growing colony. The percentage of growth inhibition was calculated from the mean values as follows: Percentage of inhibition ¼ [(mycelial growth under control conditions - mycelial growth in the presence of water/salt soluble extract)/mycelial growth under control conditions] x 100. L. plantarum DiSSPA1A7 and L. pentosus DiSSPA7 were propagated at 30 °C for 24 h in de Man, Rogosa and Sharpe broth (MRS, Oxoid, Basingstoke, Hampshire, England). W. anomalus DiSSPA73 was propagated at 30°C for 24 h in Sabouraud dextrose broth (Oxoid).

2.2. Olive samples, inoculation and pilot plant fermentation

Natural green olives cv. Bella di Cerignola from the 2013/2014 crop were kindly provided by a local farm (Puglia Conserve S.r.l.) sited in Modugno, southern Italy. Before processing, olives with mechanical or insect damage were discarded. The remaining olives were washed with tap water and fermented according to the Greektype protocol. Four different fermentation conditions were tested: (i) conventional fermentation by indigenous bacteria and yeasts, which was used as the control (Ctrl); (ii) fermentation started with an inoculum of a commercial L. plantarum strain (Sacco S.r.l., Como, Italy) as a starter (S); (iii) fermentation started with an inoculum of a commercial L. plantarum strain and the selected autochthonous yeast W. anomalus DiSSPA73 (SY); and (iv) fermentation started with an inoculum of a commercial L. plantarum strain, W. anomalus DiSSPA73, L. plantarum DiSSPA1A7, and L. pentosus DiSSPA7 (SYL). The cells used for the inoculation of the olives were cultured in MRS broth (lactobacilli) and Sabouraud dextrose broth (yeast) supplemented with 4.5% NaCl to allow the strains to adapt to the saline environment of the brine (De Castro et al., 2002). After 24 h at 30 °C, the cells were centrifuged at 10,000 rpm for 10 min, washed in sterile 20 mM potassium phosphate buffer at pH 7.0, harvested and resuspended in sterile brine (water containing 7% NaCl). Each strain was inoculated into the container of olives at a final cell density of approximately 7 log CFU/mL of brine. The fermentations were performed in a container with 25 kg of olives and 25 L of brine (7% of NaCl) at room temperature (18 and 25 °C). Each fermentation condition was performed in triplicate. The fermentations were monitored every 15 days for a total fermentation period of 90 days by determining the pH, temperature, and contents of lactic and acetic acids.

2.3. Acidification and synthesis of organic acids

The acidification was monitored by a pH meter (Model 507, Crison, Milan, Italy) after 1, 15, 75 and 90 days of fermentation. The concentration of organic acids in Ctrl, S, SY and SYL samples was determined by HPLC using an AKTA Puri € fier™ system (GE Healthcare, Uppsala, Sweden).

2.4. Enumeration of cultivable bacteria and yeasts

Olive and brines samples (25 g) after 1, 75 and 90 days of fermentation were mixed with 80 ml of sterilized physiological solution and homogenized. The lactic acid bacteria were enumerated using MRS agar supplemented with cycloheximide (0.1 g/l) (lactobacilli and enterococci). The plates were incubated under anaerobiosis (AnaeroGen and AnaeroJar, Oxoid Ltd., Basingstoke, UK) at 30 °C for 48 h. Presumptive coliforms were detected on VRBGA medium at 37 C for 24 h. The number of yeasts was estimated on Sabouraud dextrose agar (SDA) (Oxoid) supplemented with chloramphenicol (0.1 g/l). The colonies were counted after incubation at 30 °C for 48 h.

2.5. DNA extraction from olive and brine samples

The DNA from un-fermented olives and olive and brine samples after 1, 75 and 90 days of fermentation was extracted. After homogenization with RNA later (Sigma), the olive and brine samples were mixed 1:1 with distilled water in a sterile plastic pestle. The homogenate was subjected to mechanical disruption with the FastPrep® instrument (BIO 101), and the total DNA was extracted using the FastDNA® Pro Soil-Direct Kit (MP Biomedicals, Illkrich, France), according to the manufacturer's instructions. An aliquot of approximately 300 ml of each sample was diluted in 1 ml of PBSEDTA (phosphate buffer 0.01 M, pH 7.2, and 0.01 M EDTA). After centrifugation (14,000 g and 4 °C for 5 min), the pellet was washed twice to decrease the content of PCR inhibitors. The resulting pellet was re-suspended in 300 ml of PBS-EDTA and used for DNA extraction with a FastPrep kit. The product obtained consisted of 50e100 ml of application-ready DNA. The quality and concentration of the DNA extracts were determined using 1% agarose-0.5X TBE gels, which were stained with Gel Red™ 10,000X (Biotium, Inc.) and analyzed through spectrophotometric measurements at 260, 280 and 230 nm using a NanoDrop® ND-1000 Spectrophotometer (ThermoFisher Scientific Inc., Milan, Italy).

2.6. RNA extraction from olives and brines samples

The RNA from olive and brine samples after 90 days of fermentation was extracted. An aliquot of approximately 200 mg of the olive and brine samples was used for RNA extraction with the Stool total RNA purification kit (Norgen, Thorold, TO). The total RNA was treated with RNase-free DNase I (Roche, Almere, Netherlands; 10 U of DNase per 20 mg of RNA) for 20 min at room temperature. The quality and concentration of the RNA extracts were determined using 1% agarose-0.5X TBE gels and spectrophotometric measurements at 260, 280 and 230 nm obtained using a NanoDrop® ND1000 spectrophotometer. The total RNA extracted (approximately 2.5 mg) was retrotranscribed to cDNA using random hexamers and a Tetro cDNA synthesis kit from Bioline (Bioline, Freiburg, Germany), according to the manufacturer's instructions (Gowen and Fong, 2010).

2.7. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) and data analyses

For each sample, three DNA or cDNA samples were pooled and used for bTEFAP analysis. bTEFAP was performed by Research and Testing Laboratories (Lubbock, TX), according to standard laboratory procedures and using a 454 FLX Sequencer (454 Life Sciences, Branford, CT). The primers 28F (forward, GAGTTTGATCNTGGCTCAG) and 519R [reverse, GTNTTACNGCGGCKGCTG, based on the V1eV3 region (E. coli position 27e519) of the 16S rRNA gene] were used (Suchodolski et al., 2012). The bTEFAP procedures were performed according to RTL protocols (http://www.researchandtesting.com, Research and Testing Laboratories, Lubbock, TX). The raw sequence datawere screened, trimmed and filtered using default settings with the QIIME pipeline (version 1.4.0, http://qiime.sourceforge.net). Chimeras were excluded using the B2C2 (http://www. researchandtesting.com/B2C2.html) (Gontcharova et al., 2010). Sequences less than 250 bp were removed. The FASTA sequences for each sample, without chimeras, were evaluated using BLASTn against a database derived from GenBank (http://ncbi.nlm.nih.gov) (Dowd et al., 2005).

2.8. Taxonomic identification

The sequences were first clustered into Operational Taxonomic Unit (OTU) clusters with 97% identity (3% divergence) using USEARCH (Edgar, 2010). To determine the identities of bacteria, the sequences were first queried using a distributed BLASTn NET algorithm (Dowd et al., 2005) against a database of high-quality 16S bacterial sequences derived from NCBI. The database sequences were characterized as high quality based on criteria originally described by the Ribosomal Database Project (RDP, v10.28) (Cole et al., 2009). Using a NET and C# analysis pipeline, the resulting BLASTn outputs were compiled and validated using taxonomic distance methods, and a data reduction analysis was performed as previously described (Dowd et al., 2008). Based on the BLASTn derived sequence identity percentage, the sequences were resolved at the appropriate taxonomic levels as follows: >97% (<3% divergence), species level (OTUs); 95e97%, genus; 90e95%, family; 85e90%, order; 80e85%, class; and 77e80%, phyla. Any match below these identity levels was discarded. The percentage of each bacterial identification (ID) was individually analyzed for each olive or brine sample to obtain relative abundance information regarding the relative numbers of reads within a given sample. Divergences of 3 and 5% were indicative of sequences that differed at the species and genus levels, respectively. Alpha diversity (rarefaction, Good's coverage, Chao1 richness and Shannon diversity indexes) and beta diversity measures were calculated and plotted using QIIME (Suchodolski et al., 2012; Chao and Bunge, 2002; Shannon and Weaver, 1949). Final datasets at the species and other relevant taxonomy levels were compiled into separate worksheets for compositional analyses among the samples (Finegold et al., 2010). The differences in microbial communities between the olive and brine samples were also investigated using the phylogeny-based unweighted UniFrac distance metric (Suchodolski et al., 2012)

2.9. Determination of phenolic compounds

The phenolic compounds from the olive samples were recovered by liquideliquid extraction using methanol as the solvent and following the procedure reported by Montedoro et al. (1992), with some modifications. Approximately 1 g of homogenized sample was added to 1 ml of hexane and extracted using 5 ml of methanol/ water (70:30 v/v) for 10 min. The hydroalcoholic phase containing phenolics was separated from the oily phase by centrifugation (6000 rpm and 4 °C for 10 min). The hydroalcoholic phases were collected and subjected to another centrifugation (9000 rpm and room temperature for 4 min), and the hydroalcoholic extracts were recovered with a syringe and then filtered through nylon filters (pore size 0.45 mm, Sigma, Ireland) to obtain a clear supernatant liquid, which was stored at -20 °C. For HPLC analysis, the homogenized sample was preliminary added with 0.25 ml of a methanol/water (70:30, v/v) solution of gallic acid (internal standard, 100 mg kg-1). The HPLC analysis of the phenolic extracts was performed using an UHPLC binary system (Dionex Ultimate 3000 RSLC, Waltham, MA, USA) equipped with a 7725 Rheodyne injector, a 20 ml sample loop, a diode array detector (Dionex RS 3000, Waltham, MA, USA), and the Chromeleon software for data acquisition. The stationary phase was a Nova-Pack C18 analytical column (150 x 3 mm i.d.) with a particle size of 3 mm (Thermo Scientific, Waltham, MA, USA). The mobile phases for chromatographic analysis were (A) water/ acetic acid (98:2, v/v) and (B) methanol at a constant flow rate of 1 ml/min, and the column temperature was 30 °C. The gradient solver program was as follows: 0-16 min 95% A; 16-26 min 80% A; 26-36 min 60% A; 36-45 min 40% A; and 45-55 min 0% A. The detection of phenolic compounds was conducted at 280 nm. Spectra were recorded at wavelengths between 240 and 380 nm. The identification of phenolic compounds was performed by comparing the peak retention times with those obtained by the injection of pure standards. The phenolic compounds were quantified according using a gallic acid internal standard.

2.10. Determination of free amino acids and volatile organic compounds

Brine samples fermented for 75 and 90 days were centrifuged at 3000 g and 4 °C for 30 min. The supernatant was filtered through Whatman No. 2 paper, and the pH of the extract was adjusted to 4.6 using 1 N HCl. The suspension was centrifuged at 10,000 g for 10 min, and the supernatant was filtered through a Millex-HA 0.22- mm-pore-size filter (Millipore Co., Bedford, MA). The total and individual free amino acids (FAA) contained in the brine samples were analyzed using a Biochrom 30 series amino acid analyzer (Biochrom Ltd., Cambridge Science Park, England) with a sodium cation-exchange column (20 by 0.46 cm [inner diameter])

(Siragusa et al., 2007). The volatile organic compounds (VOC) of table olives after 90 days of fermentation were extracted by solid-phase micro-extraction (SPME) and analyzed using a gas-chromatographic system equipped with a mass spectrometer (GCeMS). In particular, an aliquot of the sample (1 g ± 0.05) was placed inside 12-mL glass vials, which were then closed with a butyl rubber septa and an aluminum seal. The olive sample was homogenized for 2 min using a laboratory vortex shaker. Before extraction, stabilization of the headspace in the vial was achieved by equilibration for 5 min at 40 °C. The extraction was performed by exposing a 50 x 30-mm polydimethylsiloxane/divinylbenzene/carboxen (PDMS/DVB/Carboxen) fiber (Supelco, Bellefonte, PA, USA) in the headspace of the sample at 40 °C for 15 min. Once the extraction process was completed, the fiber was removed from the vial and desorbed in the injection port of the GC in the splitless mode. The GCeMS instrumentation included an Agilent 6850 gas chromatograph (Milan, Italy) equipped with an Agilent 5975 mass spectrometer. The compounds were resolved on an HP-Innowax (30 m x 0.18 mm, 0.18 mm film thickness) polar capillary column (Agilent) under the following conditions: injector temperature, 220 °C; helium as the carrier gas at a flow rate of 15 mL/min for 7 min; oven temperature was initially set to 40 °C/0.70 ml/min (linear speed of 36 cm/s), increased at 18 °C/min to 180 °C, and then increased at 20 °C/min to 220 °C. The mass spectrometer was operated in the electron impact mode (electron energy ¼ 70 eV), and the ion source temperature was 250 °C. The mass range was m/z 20e250. The volatile compounds were identified by comparison with the mass spectra present in the NIST and Wiley libraries, quantified and expressed as a percentage of the integrated area. 2.11. Texture profile analysis Texture profile analysis (TPA) was performed with a Texture Analyzer (Instron, Zwick Roell Z1.0., USA). The testxpert II program was used for processing the data. The TPA was performed in the compression mode, and twenty uniform olives were used for each determination. A 50 kg load cell was used. The textures of the olive samples were determined after 90 days of fermentation. The samples were subjected to a two-cycle compression, and the measurements were obtained by pressing each olive with a flat rectangular plunger of 4 cm. The plunger was disengaged and driven in a vertical direction in contact with the fruit, which was horizontally positioned. The set parameters of each test were the following: pre-load 5 g; pre-test speed 2 mm/s; probe speed 0.5 mm/s; force max 500 N. The test was stopped at 15% of deformation after the auto-triggering signal. The hardness, cohesiveness (which accounts for the strength of the bonds in the olive flesh), gumminess (defined as the energy required to disintegrate a semisolid food product to a state ready for swallowing), chewiness (total amount of work necessary to chew a sample to a state ready for swallowing) and springiness (the degree or rate at which the sample returns to its original size/shape after partial compression between the tongue and palate), as reported by Szczesniak (1963) and Friedman et al. (1963), were evaluated and calculated using the instrument software. 2.12. Sensory evaluation The sensory evaluation of fermented table olives after 75 and 90 days of fermentation was performed by panelists chosen through a selection process, which was implemented according to the COI/ OT/MO No 1/Rev. 2 2011 Method for the sensory analysis of table olives, and based on his/her sensitivity and discriminatory power with regard to the organoleptic characteristics of table olives. The first step was performed to provide an identification of the sensory descriptors, to develop a common vocabulary for the description of the sensory attributes of olives and to familiarize the panelists with the scales and procedures used. Each attribute term was extensively described and explained to avoid any doubt in the relevant meaning. Based on the citation frequency (>60%), seven descriptors were selected to be inserted in the card: “rheological characteristics,” such as crunchiness (a property related to the noise produced by friction or fracture between two surfaces), “taste” (bitter, acid, sweet, and salty), and “flavor” (olive flavor and off-flavor). The panelists were also asked to provide their overall appreciation. A descriptive panel of eight judges, aged between 25 and 50 years, was employed and trained by preliminary tasting sessions using standard solutions to ensure that each judge could interpret the same descriptor in the same way as the other members of the panel. In particular, 3% and 9% NaCl aqueous solutions were used for salty taste, 0.2% and 0.8% lactic acid aqueous solutions were used for acid taste, an aqueous solution of quinine was used for bitter taste, Leerdammer cheese (soft) and raw carrots (hard) were used to establish hardness, and peach compote and celery were used to establish minimum and maximum values for crunchiness, respectively. With this aim, the panelists were asked to

repeatedly taste the same product without their knowledge to assess their ability to always equally detect the intensity of each descriptor. For the profile test, the related identified sensations were quantified on a non-structured line scale from 0 (absence of the attribute) to 10 (strong presence of the attribute). Each sample was served in a cupping-glass with an alpha-numeric code to the panelists, who were in separated booths subjected to conditions of normal daylight and room temperature at the sensory laboratory of the DISSPA Department (University of Bari, Apulia). Water at room temperature was used to rinse the samples before tasting. The results were averaged prior to data analysis.

2.13. Statistical analysis

The data (at least three replicates) were subjected to one-way analysis of variance (ANOVA), and pairwise comparison of the treatment mean values was achieved by Tukey's procedure with a P value of less than 0.05 using the statistical software Statistica for Windows (Statistica 7.0).

3. Results

3.1. Acidification and cell density of cultivable microorganisms

After one day of fermentation, the pH of the brines ranged from 4.96 ± 0.16 for the Ctrl olives to 4.49 ± 0.12 for the olives fermented with SYL (Fig. 1A). The pH values decreased during fermentation. However, a difference of approximately 0.58 pH units was found between the Ctrl and SYL samples. According to the pH values, the highest concentrations of lactic and acetic acids were found in the SYL samples (Fig. 1B). After one day of fermentation, the cell density of the lactic acid bacteria ranged from 5.70 ± 0.15 for the Ctrl olives to 8.52 ± 0.28 log CFU/g for the SYL samples (Fig. 2A). The yeast density ranged from approximately 3.84 for the Ctrl samples and the olives started with a commercial starter (S) to approximately 6.90 log CFU/g for the SY and SYL samples. With the exception of the SYL samples, all of the samples showed a density of approximately 3.2 log CFU/g of presumptive coliforms. Similar data ($P > 0.05$) were found for the brine samples (Fig. 2B). After 75 days of fermentation, the cell density of presumptive lactic acid bacteria in Ctrl olive samples reached 7.65 ± 0.12 CFU/g. The density of presumptive coliforms decreased after 75 and 90 days of fermentation. The lowest values were found in the S and, particularly, SYL samples.

3.2. Pyrosequencing analysis of the bacterial microbiota of the olives and brines

The DNA extracted from the brine samples (Ctrl, S, SY, and SYL) after 1, 75 and 90 days of fermentation were subjected to pyrosequencing. In addition, the DNA extracted from un-fermented olives and at the end of the fermentation (90 days) was studied. To compare the total bacteria with the metabolically active bacteria, the RNA extracted from the brines and olives after 90 days of fermentation was pyrosequenced. The bacterial community was analyzed using rarefaction curves, a species-level measure (OTU), a richness estimator (Chao1) and a diversity index (Shannon). Moreover, satisfactory coverage of the diversity was found for all of the samples analyzed with Good's coverage values above 99% (Table 1). The un-fermented olives showed the lowest OTU, Chao1 and Shannon index values. After one day of fermentation, the highest level of biodiversity was found in the Ctrl and S brines. With few exceptions, the OTU, Chao1 and Shannon index values of the brines samples increased from 1 to 75 days of fermentation. Different trends were observed among the brine samples after 90 days of fermentation. The lowest biodiversity was found for SY and, especially, SYL brine samples. This result was also found for the metabolically active bacteria. Compared with the brine samples, olives showed lower biodiversity. The microbial differences between the brines and olives were confirmed using three phylogeny based beta-diversity measures. The olive and brine samples were differentiated based on a bacterial principal coordinate analysis using an unweighted UniFrac distance matrix (Fig. S1). In addition, both Adonis and Anosim statistical tests indicated that the brine and olive samples had a significant ($P < 0.05$) influence on the microbial diversity. The surface of the un-fermented olives and the

Ctrl brines after one day of fermentation was characterized by a high level of Proteobacteria (mainly belonging to the Enterobacteriaceae and Methylobacteriaceae families, with relative abundances of 99.66% and 99.15%, respectively; Fig. 3A and Fig. S2).

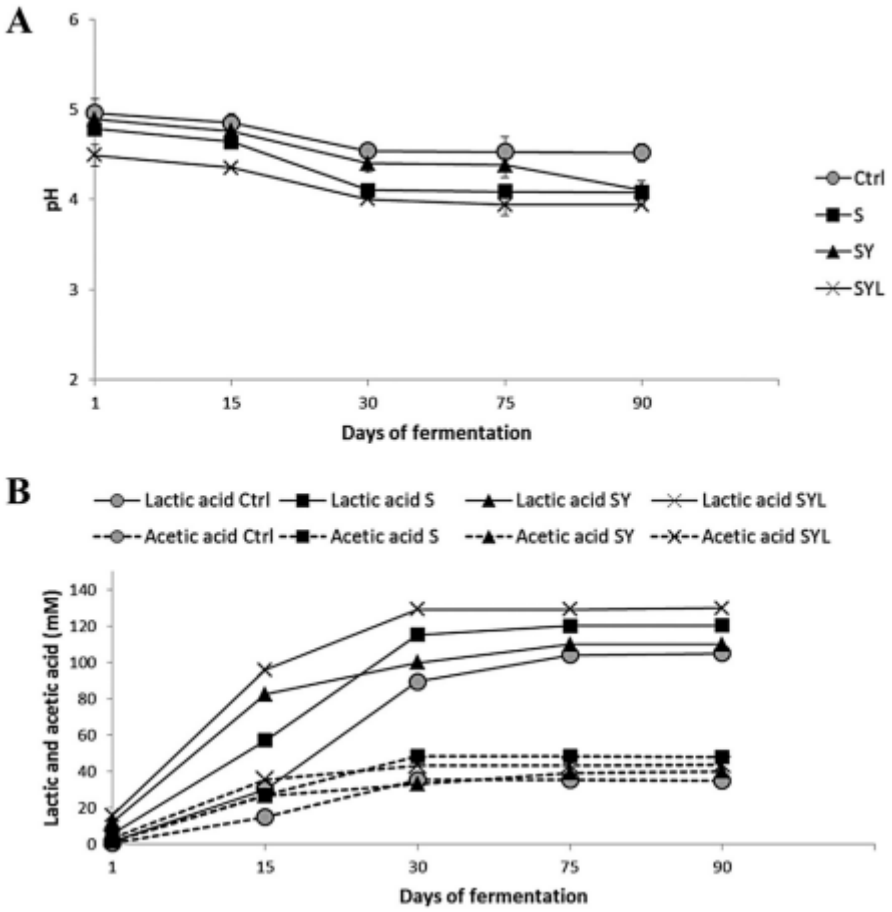

Fig. 1. Acidification of table olives. Decrease of pH (A) and concentrations of lactic and acetic acid (B) in the brines after 1, 75 and 90 days of table olive fermentation. Ctrl, table olives fermented by indigenous bacteria and yeasts; S, table olives fermented using commercial Lactobacillus plantarum as a starter; SY, table olives fermented using commercial L. plantarum and the selected autochthonous Wickerhamomyces anomalus DiSSPA73 as a starter; SYL, table olives fermented using commercial L. plantarum starter, autochthonous W. anomalus DiSSPA73, L. plantarum DiSSPA1A7, and L. pentosus DiSSPA7 as a starter

Among Proteobacteria, the Enterobacteriaceae and Methylobacteriaceae families dominated in the un-fermented olive surface and in the brines after one day of fermentation, and as expected, species belonging to Firmicutes (Lactobacillaceae family) together with Proteobacteria dominated in the started brine samples (S, SY, and SYL). With the exception of Ctrl, Firmicutes dominated in all of the samples after 90 days of fermentation (Fig. 3A and Fig. S2). At the genus and species levels, the un-fermented olives showed high levels of Hafnia (mainly Hafnia alvei, approximately 32% of the relative OTU abundance) and, particularly, Methylobacterium sp. (approximately 53.3%; Fig. 4 and Table S1). On the contrary, a very low abundance of lactic acid bacteria (mainly L. plantarum and L. pentosus) was found. Compared with un-fermented olives, the Ctrl samples showed a dominance of H. alvei after one day of fermentation. As expected, L. plantarum dominated in the S, SY, and SYL started samples with approximately 53, 42, and 76%, respectively. Within the set of lactic acid bacteria, L. plantarum, L. coryniformis, L. pentosus, L. brevis, Lactobacillus paracasei, L. paracollinoides, L. vaccinostercus and Lc. lactis were detected by analyzing the 16S rDNA in all of the brine samples after 90 days of fermentation. With few exceptions, all of the above-mentioned species were found in the metabolically active microbiota. The Ctrl brine samples also showed Propionibacterium acidipropionici after 90 days of fermentation.

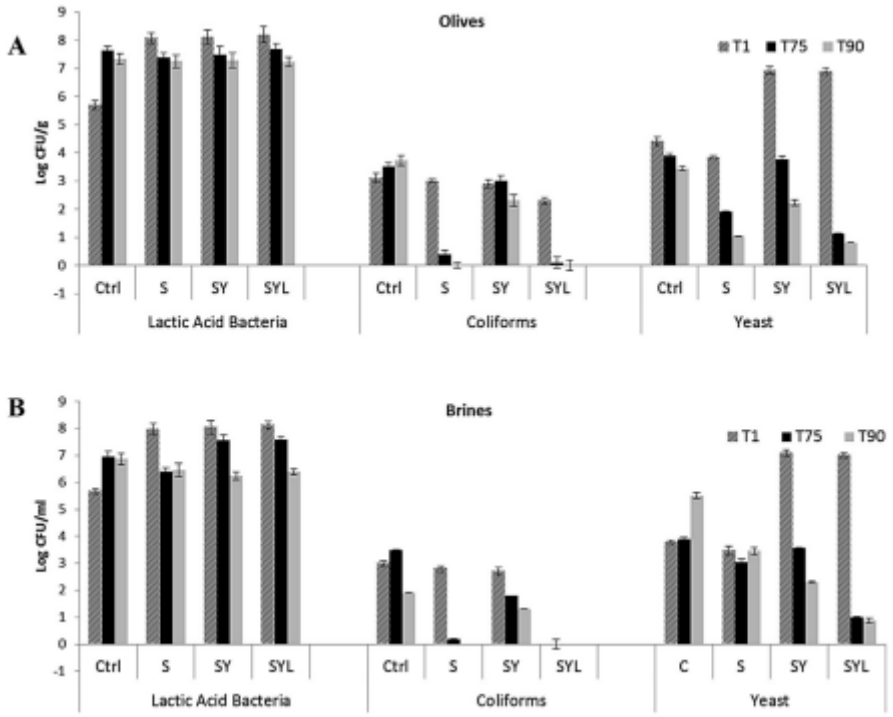

Fig. 2. Enumeration of cultivable bacteria and yeasts. Cell density (log cfu/g) of presumptive lactic acid bacteria, coliforms and yeasts in the table olives (A) and brines (B) after 1, 75 and 90 days of fermentation. Ctrl, table olives fermented by indigenous bacteria and yeasts; S, table olives fermented using commercial Lactobacillus plantarum as a starter; SY, table olives fermented using commercial L. plantarum and selected autochthonous Wickerhamomyces anomalus DiSSPA73 as a starter; SYL, table olives fermented using commercial L. plantarum, autochthonous W. anomalus DiSSPA73, L. plantarum DiSSPA1A7, and Lactobacillus pentosus DiSSPA7 as a starter

3.3. Phenolic compounds

The total phenolics in the Ctrl and S, SY and SYL samples after 75 and 90 days of fermentation were determined (Table 2). After 75 days of fermentation, oleuropein, the bitter-tasting secoroid glucoside that is the most abundant biologically active coumarinlike phenolic compound in olives, was not detected. Eight of the twelve detected phenolic compounds were identified as benzoic acid derivatives (vanillic, hydroxybenzoic and 3,4 hydroxybenzoic acids), cinnamic acid derivatives (hydroxycaffeic acid), phenylalcohols (hydroxytyrosol, tyrosol and 3,4 hydroxyphenylacetic acid) and glucosides (verbascoside). Compared with the Ctrl olives, the S, SY and SYL samples showed a significantly ($P < 0.05$) higher amount of phenyl-alcohols. Compared with the concentration obtained after 75 days of fermentation, the concentration of all of the detected phenolic compounds showed a significant decrease at the end of the fermentation.

3.4. Concentration of free amino acids and volatile organic compounds

Overall, no statistically significant ($P > 0.05$) differences in the total and individual FAAs were found between the brine samples after one day of fermentation. On the contrary, the amount of FAAs markedly differed between the brine samples after 75 and 90 days of fermentation (Table 3). Compared with the Ctrl sample, the S and SYL samples showed a higher amount of total FAAs. Overall, Ala was the FFA found at the highest concentration in all of the samples. In addition, g-amino butyric acid, a decarboxylation product of Glu, was also found at high level in all of the samples. The volatile compounds of the Bella di Cerignola table olive variety fermented after 90 days of brining were analyzed by GCeMS (Table 4). Eighty-two compounds encompassing several chemical classes, namely alkans (4), acids (12), aldehydes (8), alcohols (15), esters (27), ketones (3), aromatic compound (11), terpenes (1) and furans (1), were characterized. Among these, 47

volatile compounds showed a significant ($P < 0.05$) difference between the unstarted (Ctrl) and started table olives (S, SY, and SYL).

Table 1
Biodiversity measures of total (16S rDNA) and metabolically active bacteria (16S rRNA) in table olives.

	OTUs	Chao 1	Shannon index	Good's coverage (%)
Olives				
16S rDNA				
Un-fermented olives	21	21.75	1.39	99.97
Brines				
16S rDNA				
T1 Ctrl	31	45.00	1.85	99.89
T1 S	29	34.00	1.86	99.96
T1 SY	20	23.00	1.58	99.98
T1 SYL	21	21.50	1.31	99.97
T75 Ctrl	37	49.00	2.90	99.88
T75 S	51	62.00	2.96	99.68
T75 SY	38	38.17	2.15	99.95
T75 SYL	40	43.50	2.53	99.86
T90 Ctrl	69	52.50	3.57	99.82
T90 S	56	54.50	3.39	99.77
T90 SY	47	37.75	2.83	99.92
T90 SYL	44	50.50	1.89	99.94
16S rRNA				
T90 Ctrl	66	72.43	3.04	99.78
T90 S	47	61.86	2.79	99.86
T90 SY	46	55.25	1.94	99.70
T90 SYL	32	32.60	1.66	99.80
Olives				
16S rDNA				
T90 Ctrl	37	36.33	1.94	99.89
T90 S	37	36.33	1.97	99.94
T90 SY	31	35.67	1.57	99.83
T90 SYL	28	26.00	1.31	99.91
16S rRNA				
T90 Ctrl	30	43.33	1.87	99.60
T90 S	34	44.20	1.73	99.88
T90 SY	24	39.67	1.41	99.88
T90 SYL	24	29.00	1.32	99.83

Ctrl, table olives by indigenous bacteria and yeasts; S, table olives fermented by commercial *Lactobacillus plantarum* starter; SY, table olives fermented by commercial *L. plantarum* starter and selected autochthonous *Wickerhamomices anomalus* DiSSPA73; SYL, table olives fermented by commercial *L. plantarum* starter and autochthonous *W. anomalus* DiSSPA73, *L. plantarum* DiSSPA1A7, and *Lactobacillus pentosus* DiSSPA7; T1, 1 day of fermentation; T75, 75 days of fermentation; T90, 90 days of fermentation.

3.5. Texture profile analysis

The hardness, which is defined as the measurement of the maximum force of the first bite, did not show significant ($P > 0.05$) differences among the tables olives fermented under the different conditions (Table 5). The same trend was observed for cohesiveness and gumminess. Significantly lower values of chewiness and springiness were observed for the Ctrl sample. 3.6. Sensory evaluation A preliminary sensory evaluation was performed after 75 days of fermentation; nevertheless, the results are not reported because the olives were judged to be scarcely acceptable for direct consumption. The only exception was the SYL sample, which was judged acceptable for direct consumption after 75 days of fermentation. After 90 days of fermentation (Fig. 5), the SYL sample showed the highest overall appreciation. Compared with the S, SY and SYL samples, the Ctrl olives were characterized by lower values of crunchiness and olive flavor and the highest values for the perception of bitter, acid and off-flavor taste. Sweeter taste was perceived in the SYL olives compared with the other table olives. The salty sensation did not show any evident differences among the different samples.

4. Discussion

A correct fermentation of Greek-type table olives requires the presence and rapid growth of yeasts and, especially, lactic acid bacteria (Hurtado et al., 2012; Arroyo-Lopez et al., 2012). However, autochthonous lactic acid bacteria and/or yeasts prevail in the community of endogenous Gram-negative bacteria (e.g., Enterobacteriaceae) only after the second step of fermentation (Corsetti et al., 2012; Cocolin et al., 2013; Arroyo-Lopez et al., 2012). The abbreviation/elimination of the fermentative step dominated by Gram-negative bacteria may accelerate the fermentation processes and reduce the risk of developing unpleasant odors and flavors (Bevilacqua et al., 2015). Overall, the majority of starter preparations for olive fermentation are L. plantarum and/or L. pentosus (Hurtado et al., 2010). However, yeasts enhance the growth of lactic acid bacteria that release nutritive compounds (Arroyo-Lopez et al., 2008). The results of this study showed that the use of all of the strains together (SYL) allowed a rapid acidification of the brine, which reached a pH value of 4.49 after one day of fermentation. This finding was consistent with other published data obtained using lactic acid bacteria during Greek- or Spanish-style processing (e.g., L. plantarum, L. pentosus, L. paracasei, and L. rhamnosus), which reached a pH of 4.5 after more than 10 days (Benincasa et al., 2015; Blana et al., 2014; Aponte et al., 2012; De Bellis et al., 2010; Randazzo et al., 2014). pH values lower than 4.5 inhibit the growth of Proteobacteria and other acid-sensitive bacteria that protect table olives from spoilage and pathogens during fermentation/storage (Perricone et al., 2010). Compared with the brine samples, the olive samples harbored the highest cell density of lactic acid bacteria, confirming the ability of L. plantarum and L.

pentosus to colonize the surface of olives (Blana et al., 2014; Sisto and Lavermicocca, 2012). With the exception of the SY and SYL samples, the cell density of yeasts was 2e3 log CFU/ml lower than that of lactic acid bacteria (Panagou et al., 2008; Blana et al., 2014). The lactobacilli used in this study inhibited W. anomalus DiSSPA73 and indigenous yeasts after 75 and, especially, 90 days of fermentation. Some studies have shown the inhibition of the growth/ survival of yeasts in fermented salads through the use of lactic acid bacteria (Bonestroo et al., 1993). According to previous findings (Blana et al., 2014), presumptive coliforms were not detected in both the S and SYL olives and brines after 75 days of fermentation. The Spanish-style fermentation was associated with Enterobacteriaceae, such as Enterobacter, Citrobacter, Escherichia and Klebsiella (Cocolin et al., 2013). This study provides the first demonstration of the application of next-generation sequencing for investigating the incidence of a selected starter on the microbiota of table olives. The microbiota of brines after one day of fermentation was dominated by H. alvei and Methylobacterium. Previously, the genus Hafnia was not detected in table olives, including the Bella di Cerignola cultivar. However, H. alvei has been associated with post harvest green olive deteriorations in Morocco (Faid et al., 1994). Methylobacterium was recently detected in table olives by pyrosequencing (Cocolin et al., 2013). At the end of the fermentation, L. plantarum and L. pentosus dominated in all of the samples tested in this study. These results confirm the findings in previous studies of the ecology of lactic acid bacteria in table olives, which showed that the first step of fermentation was dominated by Enterobacteriaceae and that the following steps are mainly carried out by L. plantarum and L. pentosus (Hurtado et al., 2012). The use of lactobacilli and W. anomalus DiSSPA73 as starters markedly changes the microbiota of brines after one day of fermentation. Analyses of the number of species (OTU) and alpha diversity indicated that the microbial diversity of the brines was markedly simplified in the S, SY and, especially, SYL samples. This finding confirms the antimicrobial activities of W. anomalus DiSSPA73 and, especially, L. plantarum DiSSPA1A7 and L. pentosus DiSSPA7. According to culture-dependent data, the lowest out level belonging to Proteobacteria, including Enterobacteriaceae, was found for the SYL brine and olive samples. This finding is of particular relevance for the sensorial quality of table olives because Enterobacteria are involved in gas pockets (Arroyo-Lopez et al., 2010; Lanza, 2013). Interestingly, the use of commercial L. plantarum as a starter in the S, SY, and SYL samples inhibited the growth of Lc. lactis and P. acidipropionici. In addition, the Ctrl samples showed approximately 11% of metabolically active Clostridium in the brine after 90 days of fermentation. Clostridium and Propionibacterium can promote zapatera spoilage of table olives (Lanza, 2013). The lactobacilli and W. anomalus DiSSPA73 used in this study markedly affected the amount of FAAs and phenolic and volatile organic compounds. Two of the most representative volatile compounds found in inoculated olives, particularly in the SY olives, were ethanol and acetic acid. The major content of ethyl acetate found in the S, SY, and SYL samples was due to heterolactic fermentation, in which the acetic acid produced is esterified in the aqueous phase with ethanol. It is well known that acetate esters are synthesized by an alcohol-acyl-transferase that catalyzes the esterification of volatile alcohols with acetyl CoA molecules to produce volatile esters and free CoAeSH (Salas, 2004). It has been shown that ethyl acetate is the major flavor compound in heterofermentative metabolism (Hansen and Hansen, 1993; Hansen et al., 1989). The highest amount of propionic acid was found in the Ctrl olives, confirming the inhibition of Clostridium and Propionibacterium by the selected starters. According to the Ehrlich pathway, higher alcohols, such as isobutanol and isoamyl and amyl alcohols, are obtained through the decarboxylation and deamination of leucine, isoleucine and valine during alcoholic fermentation (Pietruszka et al., 2010). These three amino acids were found at low values in the SY samples, which showed the highest amounts of isobutanol and isoamyl and amyl alcohols. Moreover, a higher proportion of 1-hexanol and 3-hexen-1-ol, which are higher alcohols in the lipoxygenase pathway, was found in the S, SY, and SYL samples compared with the Ctrl sample. Previous studies have suggested that lipoxygenases, after their release due to the disruption of fruit cells during milling in olive oil, produce 9- and 13-hydroperoxides of linolenic and linoleic acids (Angerosa, 2002). In agreement with previous studies (Sabatini and Marsilio, 2008a; Sabatini et al., 2008b), the appreciable presence of 1-hexanol and 3- hexen-1-ol in the inoculated olives may be due to a lipoxygenaselike metabolism of polyunsaturated fatty acids affected by enzymes produced in the brine by lactic acid bacteria and yeasts together with other

different microorganisms. D-limonene was the only compound found in the S and SYL samples. Some researchers have demonstrated that D-limonene is naturally present in citrus and other fruits, and it has been recognized to exhibit insecticidal and antimicrobial properties (it is registered in 15 pesticide products) (Malheiro et al., 2011). Both a texture profile analysis and a sensory evaluation showed the highest appreciation for the started table olives. Interestingly, the SYL sample was judged ready-to-eat after 75 days of fermentation.

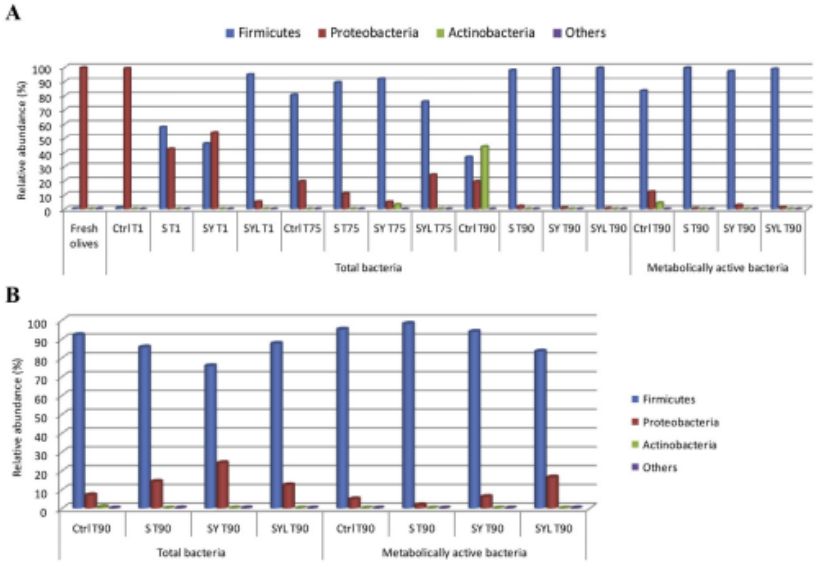

Fig. 3. Relative abundance (%) of phyla obtained by pyrosequencing. A, un-fermented olives (fresh olives), brines and olives after 1 (T1), 75 (T75) and 90 (T90) days of fermentation; B, olives after 90 days of fermentation; Ctrl, table olives fermented by indigenous bacteria and yeasts; S, table olives fermented using commercial Lactobacillus plantarum as a starter; SY, table olives fermented using commercial L. plantarum and selected autochthonous Wickerhamomyces anomalus DiSSPA73 as a starter; SYL, table olives fermented using commercial L. plantarum starter, autochthonous W. anomalus DiSSPA73, L. plantarum DiSSPA1A7, and Lactobacillus pentosus DiSSPA7 as a starter; total bacteria, data related to the pyrosequencing of DNA; metabolically active bacteria, data related to the pyrosequencing of RNA.

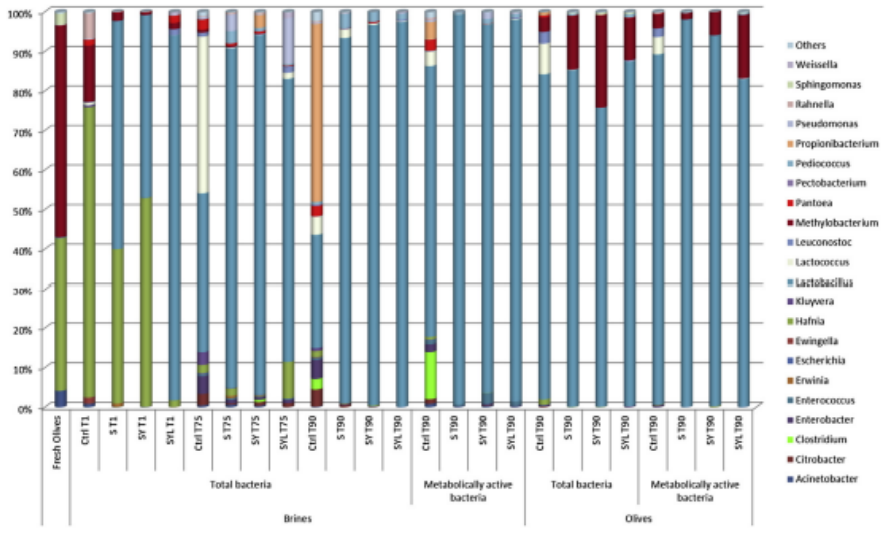

Fig. 4. Relative abundance (%) of genera obtained by pyrosequencing. A, un-fermented olives (fresh olives), brines and olives after 1 (T1), 75 (T75) and 90 (T90) days of fermentation; B, olives after 90 days of fermentation; Ctrl, table olives fermented by indigenous bacteria and yeasts; S, table olives fermented using commercial Lactobacillus plantarum as a starter; SY, table olives fermented using commercial L. plantarum and selected autochthonous Wickerhamomyces anomalus DiSSPA73 as a starter; SYL, table olives fermented using commercial L. plantarum starter, autochthonous W. anomalus DiSSPA73, L. plantarum DiSSPA1A7, and Lactobacillus pentosus DiSSPA7 as a starter; total bacteria, data related to the pyrosequencing of DNA; metabolically active bacteria, data related to the pyrosequencing of RNA.

Table 2
Phenolic compounds (mg kg^{-1} gallic acid) found in *Bella di Cerignola* table olives fermented by indigenous bacteria and yeasts (Ctrl) or by commercial *Lactobacillus plantarum* starter (S), commercial *L. plantarum* starter and autochthonous *Wickerhamomices anomalus* DiSSPA73 (SY), commercial *L. plantarum* starter and autochthonous *W. anomalus* DiSSPA73, *L. plantarum* DiSSPA1A7, and *Lactobacillus pentosus* DiSSPA7 (SYL) after 75 and 90 days of fermentation.

Phenolic compounds	Ctrl	S	SY	SYL
75 days of fermentation				
Hydroxytyrosol	125.25 ± 6.92 b	139.00 ± 4.74 b	249.35 ± 35.34 a	171.49 ± 3.23 b
Tyrosol	2.61 ± 0.35 c	4.71 ± 0.65 b	6.51 ± 0.54 a	4.36 ± 0.30 b
Hydroxycaffeic acid	4.58 ± 0.49 b	2.49 ± 0.34 c	8.04 ± 0.11 a	1.05 ± 0.22 d
3.4 Hydroxyphenylacetic acid	n.d	0.74 ± 0.21 b	1.19 ± 0.19 a	n.d
3.4 Hydroxybenzoic acid	n.d	n.d	n.d	0.23±-5
Hydroxybenzoic acid	0.15 ± 0.21 c	1.47 ± 0.49 a	0.68 ± 0.10 bc	1.27 ± 0.14 ab
Vanilic acid	1.73 ± 0.07 d	3.10 ± 0.18 c	5.99 ± 0.84 a	5.16 ± 0.26 b
Verbascoside	3.89 ± 0.32 c	2.44 ± 0.45 c	6.10 ± 0.17 b	10.79 ± 0.88 a
N.I.	2.33 ± 0.25 c	36.99 ± 1.93 a	13.90 ± 1.66 b	35.21 ± 5.80 a
N.I	0.05 ± 0.07 c	25.50 ± 1.95 b	18.54 ± 1.54 b	42.23 ± 2.79 a
N.I	2.76 ± 0.15 c	25.57 ± 3.05 a	5.08 ± 1.17 b	33.22 ± 2.77 a
N.I	n.d	4.01 ± 1.06 a	5.37 ± 0.19 a	3.16 ± 0.26 a
90 days of fermentation				
Hydroxytyrosol	80.70 ± 2.05 c	108.74 ± 14.30 bc	160.47 ± 5.82 a	128.52 ± 15.11 b
Tyrosol	2.22 ± 0.42 b	3.42 ± 0.46 a	3.28 ± 0.22 a	2.44 ± 0.11 b
Hydroxycaffeic acid	1.17 ± 0.18 d	2.57 ± 0.30 c	8.00 ± 0.16 a	3.57 ± 0.45 b
3.4 Hydroxyphenylacetic acid	n.d	5.41 ± 0.29 a	4.13 ± 0.21 b	n.d
3.4 Hydroxybenzoic acid	n.d	1.05 ± 0.10 a	0.85 ± 0.10 b	n.d
Hydroxybenzoic acid	1.14 ± 0.13 b	1.23 ± 0.16 b	2.51 ± 0.21 a	1.23 ± 0.22 b
Vanilic acid	1.82 ± 0.10 b	2.15 ± 0.35 ab	2.70 ± 0.33 a	1.84 ± 0.13 b
Verbascoside	4.60 ± 0.83 a	1.41 ± 0.42 c	3.08 ± 0.14 b	3.70 ± 0.41 b
N.I	0.78 ± 0.16 d	27.48 ± 1.28 a	6.86 ± 0.24 b	3.51 ± 0.39 c
N.I	0.52 ± 0.05 c	32.70 ± 3.46 a	6.23 ± 0.56 b	6.57 ± 0.51 b
N.I	2.36 ± 0.29 b	2.21 ± 0.32 b	5.14 ± 0.19 a	2.35 ± 0.32 b
N.I	1.59 ± 0.26 c	3.09 ± 0.09 b	4.48 ± 0.48 a	1.97 ± 0.18 c

a-d: different letters indicate significant differences for $P \leq 0.05$.
N.I: not idenfied.
n.d: not detected.

Table 3
Mean values[a] of free amino acids (mg L^{-1}) found in brines of *Bella di Cerignola* table olives fermented by indigenous bacteria and yeasts (Ctrl) or by commercial *Lactobacillus plantarum* starter (S), commercial *L. plantarum* starter and autochthonous *Wickerhamomices anomalus* DiSSPA73 (SY), commercial *L. plantarum* starter and autochthonous *W. anomalus* DiSSPA73, *L. plantarum* DiSSPA1A7 and *Lactobacillus pentosus* DiSSPA7 (SYL) after 1, 75 and 90 days of fermentation.

	Days of fermentation								
	1	75				90			
	Means[b]	Ctrl	S	SY	SYL	Ctrl	S	SY	SYL
Asp	1.41	26.09	4.58	104.05	15.54	18.39	158.68	15.32	5.53
Thr	0	4.69	0	3.26	5.25	5.34	0	1.69	0
Ser	11.37	0	82.18	1.28	4.20	0	78.45	2.59	124.82
Glu	3.84	12.03	21.00	14.64	17.53	13.20	15.50	13.67	13.74
Gly	0.32	7.98	6.33	3.67	9.26	3.02	6.89	5.70	7.01
Ala	6.04	48.16	137.47	55.47	166.20	44.96	44.65	115.32	92.67
Cys	0	6.33	4.59	4.17	5.45	6.46	4.30	4.06	4.47
Val	0.87	20.89	18.62	10.84	22.52	16.36	19.47	15.59	17.81
Met	0	3.60	3.05	2.04	3.53	2.82	3.55	2.94	2.76
Ile	0	14.18	12.69	7.67	16.16	7.90	13.17	9.86	12.00
Leu	0.27	11.88	10.95	6.34	14.33	6.80	11.77	8.92	11.30
Phe	0	2.89	1.16	0	1.49	3.42	0	0	0
GABA	0.94	118.36	101.94	75.48	127.06	85.74	117.02	91.39	98.27
His	0	4.52	3.64	0.95	1.23	2.96	4.05	0	1.06
Trp	0	4.52	3.64	0.95	1.23	2.96	4.05	0	1.06
Orn	0	0.90	0	0	0	0	0	0	0
Lys	0	1.55	0	0	0	0	0	0	0
Pro	1.30	20.41	17.74	17.27	22.10	18.30	20.15	16.93	19.19
Total	26.35 ± 0.60	308.98 ± 9.62	429.60 ± 14.22	308.07 ± 10.75	433.08 ± 13.55	238.64 ± 6.98	501.70 ± 17.55	303.96 ± 9.78	411.69 ± 13.41

[a] Means values ± SD for three batches of each variant of brines analyzed in triplicate.
[b] Mean values in brines in the begin of fermentation.

Table 4
Volatile components (percentage of the integrated area) that mainly differentiated *Bella di Cerignola* olives samples fermented by indigenous bacteria and yeasts (Ctrl) or by commercial *Lactobacillus plantarum* starter (S), commercial *L. plantarum* starter and autochthonous *Wickerhamomices anomalus* DiSSPA73 (SY), commercial *L. plantarum* starter and autochthonous *W. anomalus* DiSSPA73, *L. plantarum* DiSSPA1A7, *Lactobacillus pentosus* DiSSPA7 (SYL) (SYL) after 90 days of fermentation.

Chemical classes	Ctrl	S	SY	SYL
Alkans				
Octane	0.11 ± 0.01a	0.11 ± 0.05a	0.13 ± 0.01a	0.17 ± 0.09a
Hexadecane	0.10 ± 0.01b	0.08 ± 0.01c	0.13 ± 0.02a	0.09 ± 0.01bc
2.2.4.4.6.8.8-Heptamethyl-nonane	0.05 ± 0.00b	0.10 ± 0.05a	0.14 ± 0.07b	0.15 ± 0.02a
3.7-Dimethyl-1.3.6-octatriene (Z)	0.17 ± 0.04b	0.26 ± 0.05b	0.47 ± 0.08a	0.30 ± 0.14b
Acids				
Acetic acid	9.46 ± 0.06b	15.17 ± 1.22a	15.73 ± 1.97a	15.25 ± 2.43a
Propanoic acid	6.90 ± 0.51a	1.25 ± 0.17b	0.67 ± 0.39bc	0.51 ± 0.08c
Butanoic acid	2.06 ± 0.11a	0.21 ± 0.03b	0.09 ± 0.01c	0.08 ± 0.00c
Pentanoic acid	1.09 ± 0.05a	0.19 ± 0.06b	0.20 ± 0.06b	0.15 ± 0.01b
Hexanoic acid	1.19 ± 0.19a	1.38 ± 0.14a	1.50 ± 0.33a	1.29 ± 0.04a
Heptanoic acid	3.72 ± 0.04b	4.85 ± 0.67a	4.55 ± 0.26a	4.38 ± 0.08 ab
Octanoic acid	0.44 ± 0.01a	0.51 ± 0.15a	0.46 ± 0.01a	0.45 ± 0.02a
Nonanoic acid	0.39 ± 0.07a	0.42 ± 0.20a	0.34 ± 0.01a	0.39 ± 0.03a
2-Methyl-propanoic acid	0.36 ± 0.20a	0	0.13 ± 0.03b	0.13 ± 0.03b
2-Methyl-butanoic acid	0.50 ± 0.03c	0.53 ± 0.01bc	0.67 ± 0.16 ab	0.71 ± 0.09a
2-Ethyl-hexanoic acid	0.39 ± 0.16a	0.33 ± 0.05a	0.38 ± 0.11a	0.34 ± 0.06a
2 Methyl-2-propenoic acid	0.13 ± 0.02a	0.12 ± 0.02a	0.14 ± 0.04a	0.16 ± 0.08a
Aldehydes				
Hexanal	0.11 ± 0.01a	0.04 ± 0.00b	0.05 ± 0.01b	0.02 ± 0.03b
Octanal	0.06 ± 0.03a	0.04 ± 0.00 ab	0	0.02 ± 0.03b
Nonanal	0.57 ± 0.04a	0.03 ± 0.01b	0	0
2-Methyl-propanal	0	0.07 ± 0.01a	0.10 ± 0.07a	0.07 ± 0.02a
2-Methyl-butanal	0	0	0.57 ± 0.27a	0.40 ± 0.04a
3-Methyl-butanal	0.41 ± 0.13a	0.35 ± 0.05a	0.67 ± 0.38a	0.65 ± 0.40a
Benzaldehyde	0.19 ± 0.51a	0.11 ± 0.01b	0.13 ± 0.01b	0.11 ± 0.02b
Benzeneacetaldehyde	0.14 ± 0.02a	0.14 ± 0.04a	0.15 ± 0.08a	0.12 ± 0.01a
Alcohols				
Ethanol	7.64 ± 0.20c	11.16 ± 1.08b	15.49 ± 0.72a	11.50 ± 1.48b
1-Butanol	0.12 ± 0.09a	0	0.20 ± 0.05b	0
1-Hexanol	0.31 ± 0.02b	0.70 ± 0.11a	0.82 ± 0.09a	0.73 ± 0.03a
1-Octanol	0.11 ± 0.01a	0.10 ± 0.04a	0.11 ± 0.01a	0.07 ± 0.07a
2-Butanol	16.71 ± 2.49 ab	21.55 ± 2.23a	11.61 ± 5.05b	21.48 ± 0.07a
2-Hexen-1-ol (E)-	0.07 ± 0.01b	0.06 ± 0.01b	0.11 ± 0.01a	0
2-Ethyl-1-hexanol	0.13 ± 0.00a	0.06 ± 0.00a	0	0
2-Methyl-1-propanol	0	0.09 ± 0.00c	0.17 ± 0.01a	0.11 ± 0.07b
3-Methyl-1-butanol	2.14 ± 0.49b	2.21 ± 0.55 ab	3.33 ± 0.59a	2.81 ± 0.85 ab
3-Hexen-1-ol (Z)-	1.62 ± 0.08b	3.58 ± 0.55a	3.53 ± 0.50a	3.54 ± 0.33a
3-Methyl-3-buten-1-ol	0.14 ± 0.02b	0.22 ± 0.02 ab	0.36 ± 0.19a	0.28 ± 0.11 ab
3-Methyl-2-buten-1-ol	0.15 ± 0.01c	0.33 ± 0.09b	0.57 ± 0.06a	0.47 ± 0.12 ab
3.7-Dimethyl-1.6-octadien-3-ol	0	0.04 ± 0.06b	0.14 ± 0.09a	0.12 ± 0.01a
Benzyl Alcohol	0.26 ± 0.02b	0.38 ± 0.07a	0.38 ± 0.02a	0.32 ± 0.06 ab
Phenylethyl Alcohol	0	0	0	0.16 ± 0.05
Esters				
Methyl acetate	0.75 ± 0.13c	1.79 ± 0.02b	2.09 ± 0.19a	1.68 ± 0.23b
Methyl propionate	1.47 ± 0.28	0	0	0
Methyl pentanoate	0.59 ± 0.05a	0	0	0.04 ± 0.01b
Methyl hexanoate	0.41 ± 0.09a	0.09 ± 0.13c	0.24 ± 0.01b	0
Ethyl Acetate	4.53 ± 0.25c	11.29 ± 0.15b	14.66 ± 1.83a	10.55 ± 1.52b
Ethyl propanoate	6.76 ± 0.91a	1.84 ± 0.27b	1.98 ± 0.55b	1.39 ± 0.00b
Ethyl butanoate	5.15 ± 0.08a	1.55 ± 0.15c	0.96 ± 0.20d	2.29 ± 0.09b
Ethyl pentanoate	1.61 ± 0.12a	0.10 ± 0.05b	0.13 ± 0.06b	0.04 ± 0.02b
Ethyl hexanoate	0.91 ± 0.05a	0.17 ± 0.00b	0	0.14 ± 0.19b
Ethyl octanoate	0.07 ± 0.03a	0.08 ± 0.02a	0.06 ± 0.01a	0.08 ± 0.04a
Ethyl benzenepropanoate	0.22 ± 0.10a	0.15 ± 0.04a	0.16 ± 0.01a	0.14 ± 0.03a
Butyl acetate	0	0.04 ± 0.00a	0.04 ± 0.00a	0.03 ± 0.00b
n-Propyl acetate	0.65 ± 0.22b	1.54 ± 0.45a	1.02 ± 0.34 ab	1.20 ± 0.04 ab
Hexyl acetate	0.05 ± 0.03b	0.12 ± 0.01a	0.08 ± 0.07 ab	0
Phenyl-methyl acetate	0.03 ± 0.01a	0.02 ± 0.01a	0.06 ± 0.05a	0.02 ± 0.00a
1-Methyl-propyl acetate	2.53 ± 0.62a	3.39 ± 0.81a	1.27 ± 0.31b	3.32 ± 0.50a
1-Methyl-propyl butanoate	0.04 ± 0.00b	0.05 ± 0.00a	0	0
1-Butanol-3-methyl acetate	0.44 ± 0.06a	0.43 ± 0.06a	0.51 ± 0.09a	0.47 ± 0.02a
2-Methyl-propyl acetate	0.03 ± 0.01c	0.05 ± 0.01b	0.09 ± 0.01a	0.06 ± 0.01b
2-Methyl-methyl butanoate	0.10 ± 0.04a	0.10 ± 0.02a	0.14 ± 0.01a	0.12 ± 0.01a
2-Methyl-ethyl butanoate	1.89 ± 0.80a	0.83 ± 0.25b	1.00 ± 0.11b	0.99 ± 0.20b
3-Methyl-ethyl butanoate	0.31 ± 0.01b	0.46 ± 0.08 ab	0.61 ± 0.18a	0.48 ± 010 ab
2-Hydroxy-ethyl benzanoate	0.09 ± 0.03a	0	0.12 ± 0.04a	0.12 ± 0.03a
2-Hydroxy-methyl propanoate	1.02 ± 0.23b	2.04 ± 1.24 ab	3.02 ± 1.33a	1.76 ± 0.81 ab
2-Hydroxy-3-methyl-ethyl butanoate	0	0	0.11 ± 0.02a	0.10 ± 0.01a
3-Hexen-1-ol-acetate (Z)-	0.16 ± 0.05b	0.27 ± 0.06a	0.23 ± 0.06 ab	0.23 ± 0.03 ab
Methyl salicylate	0.03 ± 0.01a	0.02 ± 0.00a	0.04 ± 0.01a	0.04 ± 0.02a

(*continued on next page*)

Table 4 (*continued*)

Chemical classes	Ctrl	S	SY	SYL
Ketones				
2-Butanone	5.83 ± 0.61a	2.55 ± 0.13b	2.98 ± 1.25b	3.07 ± 0.16b
3-Hydroxy-2-butanone	0.06 ± 0.00 ab	0	0.08 ± 0.02a	0.05 ± 0.01b
6-Methyl-5-hepten-2-one	0.04 ± 0.01b	0.33 ± 0.09a	0	0.06 ± 0.01b
Aromatic compounds				
Phenol	0.31 ± 0.01a	0.10 ± 0.03b	0.06 ± 0.02bc	0.06 ± 0.03c
3-Methyl-phenol	0	0.04 ± 0.03a	0.04 ± 0.00a	0.04 ± 0.01a
4-Ethyl-phenol	1.01 ± 0.02a	0.23 ± 0.07b	0.21 ± 0.00b	0.17 ± 0.07b
4-Ethyl-2-methoxy-phenol	0.21 ± 0.00a	0	0.04 ± 0.00b	0.04 ± 0.01b
2-Methoxy-4-methyl-phenol	2.01 ± 0.83a	1.77 ± 0.43a	2.13 ± 0.03a	2.14 ± 0.07a
2-Methoxy-phenol	1.31 ± 0.29a	0.33 ± 0.04b	0.15 ± 0.04b	0.10 ± 0.03b
1.3-Dimethyl-benzene	0.06 ± 0.02b	0.07 ± 0.01 ab	0.09 ± 0.02a	0.07 ± 0.01a
1.2.3-Trimethyl-benzene	0.04 ± 0.06a	0.03 ± 0.02a	0.03 ± 0.01a	0.02 ± 0.00a
1-Methoxy-benzene	0.15 ± 0.02	0	0	0
1.2-Dimethoxy-benzene	0.07 ± 0.02a	0.07 ± 0.06a	0.11 ± 0.09a	0.04 ± 0.00a
Acetophenone	0.16 ± 0.07a	0.03 ± 0.01b	0.03 ± 0.07b	0.04 ± 0.00b
Terpenes				
D-Limonene	0	0.22 ± 0.02b	0	0.20 ± 0.03b
Furans				
cis-Linaloloxide	0	0.19 ± 0.01a	0.09 ± 0.01b	0.08 ± 0.00b

a–c. Data in the same row with different superscript letters are significantly different ($P < 0.05$).
Mean values ± SD for 3 batches of each variant of olives analyzed in duplicate.

Table 5
Texture profile analysis of *Bella di Cerignola* table olives fermented by indigenous bacteria and yeasts (Ctrl) or by commercial *Lactobacillus plantarum* starter (S), commercial *L. plantarum* starter and autochthonous *Wickerhamomices anomalus* DiSSPA73 (SY), commercial *L. plantarum* starter and autochthonous *W. anomalus* DiSSPA73, *L. plantarum* DiSSPA1A7, *Lactobacillus pentosus* DiSSPA7 (SYL) after 90 days of fermentation.

Sample	Hardness (N)	Cohesiveness	Gumminess (N)	Chewiness (N mm^{-1})	Springiness
Ctrl	31.62 ± 5.34 a	0.23 ± 0.03 a	7.12 ± 1.37 a	3.05 ± 0.65 b	0.43 ± 0.04 b
S	35.94 ± 5.27 a	0.24 ± 0.01 a	8.53 ± 1.15 a	3.82 ± 0.53 a	0.45 ± 0.04 ab
SL	30.49 ± 10.91 a	0.25 ± 0.03 a	7.37 ± 2.39 a	3.58 ± 1.26 ab	0.49 ± 0.05 a
SYL	32.06 ± 5.16 a	0.23 ± 0.02 a	7.47 ± 1.23 a	3.31 ± 0.60 ab	0.45 ± 0.04 ab

*Texture has been determined with a TPA test (see the Materials and Methods section).
Data with different letters indicate significant differences for $P \leq 0.05$.

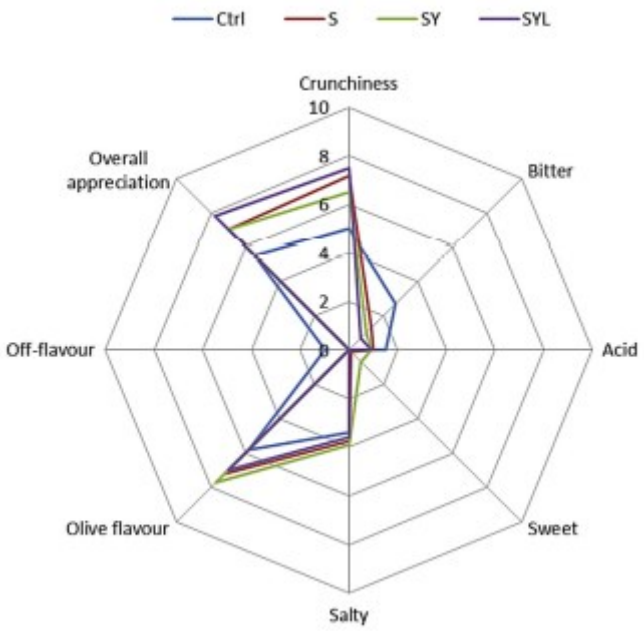

Fig. 5. Sensory analysis of table olives. Ctrl, table olives fermented by indigenous bacteria and yeasts; S, table olives fermented using commercial Lactobacillus plantarum as a starter; SY, table olives fermented using commercial L. plantarum and selected autochthonous Wickerhamomyces anomalus DiSSPA73 as a starter; SYL, table olives fermented using commercial L. plantarum starter, autochthonous W. anomalus DiSSPA73, L. plantarum DiSSPA1A7, and Lactobacillus pentosus DiSSPA7 as a starter. The data are the mean values from three independent experiments.

5. Conclusions

Under the experimental conditions of this study, the use of the selected lactobacilli and yeast strains provided more controlled and consistent fermentation and positively impacted the overall table olive quality. As

shown through microbiological, biochemical, and sensory analyses, the accelerated fermentation of Bella di Cerignola table olives was achieved using the selected lactobacilli and yeast strains.

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Appendix A. Supplementary data Supplementary data related to this article can be found at http:// dx.doi.org/10.1016/j.fm.2015.06.002.

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