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Yeast dynamics in the black table olives processing using fermented brine as starter

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Abstract

Spontaneous fermentation with autochthonous yeasts is the most traditional method used for the production of black table olives, which requires minimal intervention. The aim of this study was to evaluate the effects of adding a brine starter, developed from one-year spontaneously fermented black table olives, on the microbiological, chemical, and sensory characteristics of naturally fermented Taggiasca table olives. The olives were fermented in brine containing 12% (w/v) NaCl with 0.6% (w/v) citric acid and inoculated with a selected brine starter containing primarily *Pichia manshurica* (87% predominance) and *Saccharomyces cerevisiae* (10% predominance). Brine and olives samples were analysed at the start of the experiment and after 3, 6, 9, and 12 months of fermentation. The *P. manshurica* and *S. cerevisiae* present in the brine starter drove the entire fermentation process, along with other autochthonous species that were not included in the starter, including *Zygosaccharomyces mrakii*. Spoilage microorganisms and undesired off-odours and off-flavours were not present at the end of fermentation. When compared to the control, the use of the brine starter increased the total yeast population in the brine during the first 6 months of fermentation, thus shortening the debittering time of the fruits and improving the taste complexity of the final product.

1. Introduction

The majority of the world's olive tree (*Olea europea* L.) cultivation is concentrated in the Mediterranean, where the olives are used for both the production of oil and table olives. In the 2019-2020 season, table olive production exceeded 2.9 million tons, and the main producers were Spain, Egypt, Turkey, Algeria, Italy, Greece, and Portugal (IOC, 2020). Table olives are highly appreciated for their sensory characteristics and nutritional value. They also have potential health benefits in humans since they are rich in antioxidant phenols, which are strong free-radical scavengers (Tataridou and Kotzekidou, 2015). However, olive drupes cannot be eaten just after harvest due to the presence of oleuropein, a bitter phenolic glucoside consisting of glucose, elenolic acid, and *o*-diphenol hydroxytyrosol compounds (Ciafardini *et al.,* 1994). There are large differences in the sensory and nutritional characteristics of olives depending on their genotype and processing method (Alagna *et al.,* 2012; Kiai and Hafidi, 2014; Ambra *et al.,* 2017). Processing methods for table olives include the Sevillian and Californian methods, which involve chemical treatment of the fruits, and the natural fermentation method, where the olives are processed in brine without pre-treatment with chemical products. In the case of black olives, these approaches are also termed natural black table olive processing systems (Garrido-Fernàndez *et al.,* 1997). During the debittering process, the olives are subjected to spontaneous fermentation by a microbial community that includes lactic acid bacteria (LAB) and yeasts. Factors, such as the salt concentration and pH, affect the presence and dominance of LAB and yeasts during fermentation (Durán Quintana *et al.,* 1997). High phenolic content and a salt concentration greater than 10% are associated with brine acidification by citric acid, which favours yeast during the fermentation period (Tassou *et al.,* 2002; Nisiotou *et al.,* 2010; Ciafardini and Zullo, 2019). The natural black table olives processing systems are the debittering systems traditionally used in different areas of Italy. The Taggiasca olive variety is typical of the Liguria region in Northern Italy, where it is cultivated for the production of both oil and table olives. To produce traditional naturally fermented table olives, the fruits are harvested when they become black. Then, they are sorted, rinsed with water, and placed in 200 L plastic barrels. The barrels are filled with freshly prepared brine containing 10-12% (w/v) salt acidified

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with lactic or citric acid, sealed, and stored at room temperature in sheds to protect them from the sun and outdoor conditions. The fruits are marinated in the brine until they lose their bitter taste. After 6 months or more, they are placed in jars, filled with fresh brine, and pasteurized. This type of black table olive processing system, characterized by the high salt concentration of the brine, is often referred to as the Greek style. In this system, the bitter compounds, mainly oleuropein and its aglycons, are removed via the enzymatic activities (β-glucosidase and esterase) of the fruits and microorganisms, as well as the diffusion of the phenolic compounds into the brine (Corsetti *et al.,* 2012). Consumer appreciation of the sensory qualities of Taggiasca table olives has led to the rapid expansion of table olive production in recent years. However, producers often encounter difficulties in supplying the market throughout the year due to the long processing periods, which require approximately 6-7 months of fermentation. During cold winters, the debittering process often slows down, thus, to be marketed effectively, the olives must be stored in barrels for longer periods, in some cases, 7-8 months. The duration of the debittering process for Taggiasca black table olives can be shortened by inoculating the brines with a starter containing selected oleuropeinolytic yeasts produced in the laboratory. In a previous study conducted of Taggiasca olives processed via the Greek-style method, there was a significant reduction in the duration of fermentation when the fruits were processed with the yeast starters *Candida diddensiae*, *Candida adriatica* and *Wickerhamomyces anomalus*, regardless of the salt concentration (Ciafardini and Zullo, 2019). However, when a given starter preparation, composed of a limited number of strains is used to ferment olives from different geographic areas, a flattening of the taste of the final product may occur. The microbial diversity in fermentation by commercial or autochthonous starters does not represent the complexity and variability of the microbial population that is characteristic of natural Taggiasca olive fermentation. Such microbial diversity plays an important role in terms of the flavour characteristics of the final product (Arroyo-López *et al.,* 2012). In recent years, the demand for Taggiasca table olives with rich sensorial profiles has increased considerably, and the number of companies producing table olives processed using traditional methods and typical autochthonous microorganisms has also increased. Brine inoculation with a naturally fermented brine starter can maintain certain typicality of Taggiasca table olives processed in the original geographic area by the Greek-style method. Inoculation of fresh brines with microbiologically controlled brine from the same variety of olives processed in the previous year under controlled conditions, in some ways, resembles the "pied de cuve" method used in the production of wine with spontaneous yeasts capable of conditioning the composition of the final product by providing wines with unique regional characters (Fleet, 2003, 2008). Research investigating the use of "pied de cuve" technology developed with LAB on the microbiological characteristics of green fermented table olives has reported an improvement of the taste complexity (Martorana *et al.,* 2015). However, similar technology has not been developed for the yeasts involved in the natural fermentation of Taggiasca black table olives using the Greek-style method. Since Taggiasca olives are produced by traditional methods using spontaneous fermentation without inoculating the brine with single cultures of selected yeasts, inoculation of the brine with a starter consisting of previously fermented brine, with controlled chemical and microbiological properties, could be a way to guarantee a certain typical characteristics of the final product and shorten the duration of the debittering process, while preventing defects related to fruit spoilage. Given the lack of knowledge in this sector, this work aimed to study the effects of using a starter consisting of a naturally fermented brine on the microbiological and physicochemical characteristics of Taggiasca table olives during fermentation in an industrial-scale plant.

2. Materials and methods

2.1 Experimental design

Trials were performed in two consecutive years at a processing plant for Taggiasca olives produced in the Liguria Region of northern Italy. Taggiasca black olives were processed in industrial-scale barrels using the Greek-style system. For both years, the tests were conducted at the same processing company using fruits collected from the same orchard in the same way as the rest of the fruit destined for the market. The tests in the first year aimed to study the chemical and microbiological characteristics of brines for their potential use as a starter in the second-year tests. The tests in the second year aimed to investigate the effects of the inoculum, brine from a controlled natural fermentation, on the microbiological and physicochemical characteristics of Taggiasca table olives during fermentation.

2.1.1 Starter preparation

In the first-year experiment, Taggiasca black table olives were processed using 200 L industrial polyvinylchloride (PVC) barrels stored at room temperature in a covered area. Ten numbered PVC barrels were set up with Taggiasca olives harvested in November, when the maturation phase was suitable for

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processing. The fruits were washed with tap water, classified, and poured into the PVC barrels (120 kg of olives, each) filled with 80 L of brine containing 12% (w/v) NaCl, and then acidified with 0.6% (w/v) citric acid. At preparation, the barrels were completely filled with brine, closed with a full-sized lid containing a cap for visual checks, and stored for 12 months. During the fermentation period, the level of the brine in all barrels was checked every month. When necessary, the barrels were topped up with fresh brine. Every month, a visual check was performed, and the number of barrels contaminated by yeast film was recorded. After both 6 and 11 months of fermentation, one sample of brine was taken at mid-depth from each barrel under sterile conditions. One olive sample was taken as well. The brine samples were subjected to chemical and microbiological analyses, while the olive samples were subjected to sensory analyses as described below for the second-year experiment. Barrels with a microbial film on the brine surface during fermentation were excluded from the first-year experiment. The brine starter for the second-year experiment was selected from the barrel that showed the best physicochemical and microbiological characteristics at the end of fermentation. In fact, the criteria that guided this choice were based on the hygienic-sanitary characteristics, the lack of mold, the predominance of yeast species provided with technologically important enzymes useful for the fermentation of Taggiasca olives, and the absence of sensory defects on the fruit. Based on the results obtained from the chemical, microbiological, and sensorial analyses of the brines and fruits performed after 11 months of fermentation, one barrel was identified as having the most suitable fermented brine for starter production. The selected brine was then analyzed to microbiologically characterize their autochthonous yeast species. Ten strains of each yeast species isolated in the previous microbiological analysis were randomly selected, cultured on MYGP agar medium for 5 days at 30°C, and then tested for salt tolerance (7.5% and 15% NaCl), pseudohyphae production on MYGP agar, enzymatic activity (β-glucosidase, esterase, lipase, and protease), and the chromogenic characteristics of colonies, as described below. The biodiversity among the autochthonous yeast strains mentioned above was evaluated by analyzing the fatty acid profiles of each strain and comparing them with those belonging to the same yeast species isolated from commercial starters. The data obtained were statistically analyzed.

2.1.2 Experimental olive production

In the second-year experiment, table olive production was performed using Taggiasca black olives. After harvesting, the olives were prepared for the trials using the same procedures described for the previous year. Bulk olive fruits were washed, classified, and placed in modified plastic barrels, which were jacketed inside with a food-grade high-density polyethylene (HDPE) bag appropriate for brining. Each HDPE bag had a diameter of 960 mm and a height of 1500 mm, which was compatible with the internal measurements of the barrels as described previously (Ciafardini and Zullo, 2020). The upper part of each HDPE bag that exceeded the height of the barrel transiently protruded outside the opening of the container. In the second-year tests, modified barrels were used in order to ensure the best conditions for anaerobiosis, which are optimal for the fermentation of the product. Finally, the olives were placed in 10 barrels (120 kg of fruit, each) with approximately 60 L of brine containing 12% (w/v) NaCl and acidified with 0.6% (w/v) citric acid. The barrels were divided into two groups and numbered. The first group, consisting of five barrels, was inoculated with a starter consisting of Taggiasca fermented brine without olives, described above (10 L for each barrel). The second group of barrels included 10 L of fresh brine containing 12% salt and 0.6% citric acid and was used as a control. Subsequently, the upper part of each HDPE bag that exceeded the height of the modified barrel was wrapped to expel the internal air and then sealed tightly with plastic bands. All barrels were filled completely with brine, closed with a full-sized lid containing a cap for visual checks, and stored for 12 months at room temperature. During the fermentation period, the brine level in all barrels was checked every month, and correct coverage with brine was carried out. During the fermentation period, at the start and at three-month intervals, 1000 mL of brine and approximately 1500×*g* of fruit were collected, under sterile conditions, from the mid-depth of each barrel by opening the upper part of the HDPE bags, which were closed immediately after sampling. After sampling, the barrels were refilled with fresh brine and checked each month. The samples were poured into sterile Pyrex glass flasks and transferred immediately to the laboratory for chemical and microbiological analyses as described below.

2.1.3 Physico-chemical analysis

2.1.3.1 pH, titratable acidity, and salinity

The pH of the olive brine was measured periodically using a pH meter with an In Lab Routine Pro probe (Mettler, Toledo, USA). The measurement was performed three times. Titratable acidity assays were performed with 5 mL of brine in 45 mL of distilled water and 20 µL of a 1% phenolphthalein indicator dissolved in isopropanol (Thermo Fisher Scientific, Waltham, MA, USA). NaOH (0.1 N) (Thermo Fisher Scientific Scientific) was added until the solution retained a light

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pink colour (approximately pH 8) for 30 s. Titratable acidity was expressed as g of lactic acid per 1 L of brine and analysis was repeated three times. The sodium chloride content of the brine was assessed according to the Mohr method. 1 mL of brine was first diluted with 50 mL of distilled water and then titrated with $AgNO_3$ 0.1 N using K_2CrO_4 as the indicator (Garrido-Fernández *et al.,* 1997). The results were expressed as a percentage (w/v) of NaCl. The test was repeated three times.

2.1.3.2 Free carbon dioxide (CO_2) content in brine

The free CO_2 content in the brine was assessed by the titration method reported by the American Public Health Association with some modifications (APHA, 2005; Ciafardini and Zullo, 2020). After collection, the brine samples were stored in closed bottles and transported to the laboratory. The analysis was performed by dividing each sample into two 50 mL fractions. The first fraction was immediately analysed, while the second was placed in a beaker and stored overnight at −20°C. Subsequently, the CO_2 remaining in the sample was removed by establishing a slight flow of N_2 gurgle in the brine for 30 mins followed immediately by analysis. The brine samples were treated with Na_2CO_3 (0.0454 N) (Sigma-Aldrich) until a pH value of 8.30 was reached as judged using a pH meter (Mettler). The free CO_2 content in brine was calculated by subtracting the free CO_2 content of untreated brine from that obtained by the treated brine. The free CO_2 content was expressed as mg CO_2 per L of brine. The chemical analysis was repeated three times.

2.1.3.3 Total polar phenols

1.5 mL of brine was centrifuged at 12,000 g for 5 mins, then 1 mL supernatant was diluted in 9 mL of distilled water. Subsequently, 0.1 mL diluted brine was added to a screw cap tube containing 0.9 mL of 0.5 N sodium bicarbonate solution (pH 8.5) and 1 mL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Saint Louis. Missouri, USA) diluted to 1:10 (v/v). A treatment consisting of 0.1 mL of distilled water, 0.9 mL of sodium bicarbonate and 1 mL of Folin-Ciocalteu's phenol reagent (1:10, v/v) was used as a control. The colourimetric reaction proceeded with the samples in the dark at room temperature for 2 hrs. After briefly stirring, the samples were analysed with a model 6300 spectrophotometer at a wavelength of 765 nm (Jenway, Staffordshire, UK). The results are expressed as mg of caffeic acid equivalent/mL of brine. The analysis of total phenols was repeated three times.

2.1.4 Detection of microorganism populations

The brine microbiota was evaluated in the brine samples collected at 6- and 11-months fermentation in the first year of trials, and at the beginning of the experiment (November) and after 3, 6, 9, and 12 months of fermentation in the second year of experimentation. The microorganisms analysed were yeasts, total aerobic bacteria (TAB), lactic acid bacteria (LAB), enterobacteria, and moulds (Heperkan, 2013). The brine samples were serially diluted by a factor of 10 using sterile Ringer's solution 0.9% (w/v). Aliquots of each dilution were spread on agar media. For the enumeration of total culturable yeasts, serial dilutions of the brines were plated onto a malt yeast glucose peptone (MYGP) agar medium (pH 7) with the following composition (per L distilled water): 3 g of yeast extract (Biolife, Milan, Italy), 3 g of malt extract (BBL, Cockeysville, Maryland, USA), 2.5 g of casein bacto tryptone (BD, Sparks, Maryland, USA), 2.5 g of soy peptone (Biolife), 10 g of glucose (Sigma-Aldrich) (Ciafardini and Zullo, 2018). Petri dishes containing MYGP agar medium supplemented with 100 µg/mL of chloramphenicol were inoculated with 0.2 mL of sample and incubated at 30°C for 5 days. Counting of yeast colonies grown in the same plates was performed at two different times. After two days of incubation, the fast grow yeasts were counted and recorded, marking the colonies on the plates, while, after 5 days of incubation, the slow grow yeasts and the total yeasts were assessed. TAB was enumerated after 24 hrs of incubation at 30°C on nutrient agar (CM 0003, Oxoid, Basingstoke, UK) supplemented with 0.05% (w/v) cycloheximide to prevent the growth of yeasts. LAB was enumerated on de Man-Rogosa Sharpe (MRS) medium (Biolife), containing 0.05% (w/v) cycloheximide (Sigma-Aldrich) and incubated at 30°C for 4 days under anaerobic conditions. *Enterobacteria* were evaluated on violet red bile glucose agar (VRBGA; Biolife) incubated at 37°C for 24 h. Moulds were evaluated after 7 days of incubation at 28°C using glucose yeast extract agar (GYEA; Oxoid). All plates were examined visually for typical colony types and morphological characteristics, which were recorded along with the corresponding growth medium. The results are expressed as Log values of colony forming units/mL of brine (Log CFU/mL).

2.1.5 Yeast biodiversity assessment

The yeast colonies obtained from microbiological analysis of the Taggiasca brine were used to prepare a series of masters using MYGP agar medium (Maniatis *et al.*, 1982). Each master containing 100 colonies. After 3 days of incubation at 30°C, the masters were replicated on CHROM agar Candida medium (BBL 4354093) and tested as previously described (Tornai-Lehoczki *et al.,* 2003). The masters were prepared in duplicate using small sterile wooden sticks to transfer the single colonies into Petri dishes with the two media. The yeast colonies

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grown on MYGP agar medium were analyses after 10 days incubation at 30°C, whereas the corresponding colonies grown on CHROM agar *Candida* medium were analysed after 7 days incubation at 30°C, and their chromogenic characteristics were recorded. Yeasts grown on MYGP agar medium were analysed individually by examination using an optical microscope (Olympus, Milan, Italy) to ascertain the presence of pseudohyphae and cell shape and size. On the base of some characteristics as cell shaped (round vs. elongated), presence of pseudohyphae, and colony morphology and colour, the entire yeast population was divided into homogenous chromogenic yeast groups. From each chromogenic yeast colony group, 10 yeast isolates were randomly chosen and used for subsequent identification of yeast species. The selected yeast colonies, belonging to different chromogenic groups, were subjected to genetic analysis and identified at the species level by sequencing the approximately 600 base-pair D1/D2 region of the large (26S) ribosomal subunit using primers NL1 and NL4, as described by Kurtzman and Robnett (1997). The ribosomal sequence obtained from the NL1 primer was compared to those of published yeast species available in the public gene database using a BLAST search of call GenBank+EMBL+DDBJ+PDB sequence on the NCBI website http://www.ncbi.nlm.nich.gov/blast.

2.1.6 Salt resistance assay

The NaCl resistance of the yeast strains (10 randomly selected strains from each yeast species of the starter) was evaluated in Pyrex tubes equipped with screw caps and containing sterile MYGP medium with 0, 7.5% or 15% (w/v) NaCl. Yeast cultures grown overnight in MYGP were centrifuged at 10,000×*g* (Hettich Instruments, Tüttlingen, Germany) and then re-suspended in sterile distilled water, reaching an initial $O.D._{600}$ of approximately 0.7. A total of 100 µL yeast inoculum was dispensed into the corresponding tubes with 10 mL MYGP medium without NaCl (control) or with 7.5 or 15% (w/v) NaCl. The test was performed in triplicate. The tubes were incubated for 2 days at 30°C, and the contents were stirred for 3 s after each day of incubation. At the end of the incubation period, the yeast cultures were stirred with a vortex for 1 min and analysed with the spectrophotometer at 600 nm. The growth of individual yeast strains was expressed according to the following Growth Index (G.I.): G.I. = ($O.D._{sample}/O.D._{control} \times 100$), where $O.D._{sample}$ represents the absorbance at 600 nm of cultures grown in MYGP with NaCl, $O.D._{control}$ represents the absorbance at 600 nm of cultures grown in MYGP lacking NaCl. Finally, the average of the values obtained from the 10 strains of each yeast species analyzed was recorded.

2.1.7 Enzymatic activity assays

2.1.7.1 β-glucosidase

The β-glucosidase test was performed by transplanting the yeast cultures with small sterile wooden sticks in the MYGP agar medium supplemented with 0.1% (w/v) esculin (Sigma-Aldrich, Milan, Italy) and 0.03% (w/v) $FeCl_3$ (Carlo Erba, Milan, Italy). After 48 hrs incubation at 30°C, β-glucosidase activity was monitored visually based on the presence or absence of a dark halo around the colony (Arévalo *et al.,* 2007). Each test was performed in duplicate.

2.1.7.2 Esterase

The esterase activity was evaluated in 96-well microplates using the 4-NitroPhenyl Acetate (4-NPA; Sigma-Aldrich) as substrate. Volumes of 70 µL alcohol solution (methanol) with 0.5% (w/v) 4-NPA, 70 µL of 0.1 M phosphate buffer (pH 7), and 70 µL of each yeast strain ($O.D._{600}$ = 0.8) were added to each well. Positive control was established by replacing the microbial cultures with 70 µL of a solution of porcine esterase (Sigma-Aldrich; 50 U/mL of phosphate buffer), and negative control was established that lacked both yeast and esterase. After 180 mins incubation at 30°C, the absorbance at 410 nm of the twice repeated tests was measured zeroing the microplate reader with the respective controls.

2.1.7.3 Lipase

The lipase activity was assessed using 5 mL of the stock culture of each yeast strain (O.D. adjusted to ca. 0.8) after 5 mins centrifugation at 9,000 g was suspended in 2 mL of phosphate buffer (0.1 M, pH 6) with 12% (w/v) NaCl and supplemented with 6 mL of filter-sterilized (Minisart NML-Sartorius, Göttingen, Germany) virgin olive oil. A test with all the components excluding the yeast was prepared. Three repetitions were performed for each yeast strain and all the samples were incubated at 30°C for 7 days, mixing the masses daily with a vortex for 1 min. The lipolytic activity was assessed through the titrimetric method for the determination of the olive oil free fatty acid content in accordance with the European Community Regulation 1348/2013 (EC, 2013).

2.1.7.4 Protease

The protease activity of the yeasts was evaluated by transplanting the yeast cultures in the MYGP agar medium supplemented with 1% (w/v) skimmed milk (Sigma-Aldrich). After 12 hrs of incubation at 30°C, the enzymatic activity was recorded with the appearance of transparent halos around the colonies. The test was repeated twice.

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2.1.8 Fatty acid methyl ester profiles

The fatty acid methyl esters (FAMES) were obtained by the yeast strains used in the above tests and two yeast species isolated from the commercial starter. The cultures were grown on MYGP broth at 30°C for 48 hrs, 0.3 g of wet cells were recuperated through centrifugation (6,000×*g* for 5 mins) and transferred respectively into 10 mL glass screw-caps tubes. The yeast fatty acids were saponified by adding 1 mL of a 1.125 M NaOH aqueous methanol solution (1:1, v/v) and heating at 100°C in a water bath (Argo Lab, mod. Wb-12, Italy) for 30 mins. The FAMES (fatty acid methyl esters) derivatives were prepared by adding 2 mL of a 6 N aqueous methanol HCl solution (46:54, v/v) and heating at 80°C in a water bath for 10 mins. FAMES were extracted from the aqueous phase with 1.25 mL of a hexane-methyl tert-butyl mixture (1:1, v/v), and 3 mL of a diluted solution of 0.3 M NaOH in water was used to remove residual reagents from the organic extracts. The upper solvent phase was transferred into 2 mL glass vials and evaporated for 12 hrs at room temperature. The dry extract of the FAMES was stored at -20°C until analysis. The dry extract was solubilized using heptane and individual FAMEs from yeast cells were separated and quantified using gas chromatography equipment (Perkin-Elmer) equipped with a flame ionization detector (FID) and an SP-2560 capillary column (100 m x 0.25 µm film x 0.20 d.i.) from SUPELCO (USA). Hydrogen was used a carrier gas with a flow rate of 25.0 mL/min. The oven was set to the following temperature program: 60°C (the initial temperature) for 3 mins, increased to 170°C at a rate of 5 °C/min for 9 mins, then to 230°C at a rate of 10°C/min for 5 mins. The injector and detector temperature were set at 250°C and 270°C, respectively, and the injection volume was 1 µL. Fatty acids peak identification was accomplished by comparing the peak retention times with standard compounds from Sigma-Aldrich, injected under the same chromatographic conditions. The results were expressed as relative percent of total area. Three replicates were prepared and analysed per sample.

2.1.9 Sensory evaluation

The evaluation of the sensory profiles was performed using olives taken at the end of fermentation (11 months) in the first-year experiment, and after 3, 6, 9, and 12 months of fermentation in the second-year experiment. The sensory analysis of olive samples was performed by official, fully trained, and analytical tasters. Olive samples of 1500 g obtained by the mid-depth of each barrel were analysed by the panellists in accordance with the International Olive Oil Council method with small changes (IOOC, 2011). The test was performed by a panel of 8 judges between the ages of 25 to 50. Each tasting section entailed the sensory analysis of no more than three samples. The judges were trained for the identification of gustatory attributes (saltiness, bitterness, complexity taste), kinaesthetic attributes (hardness and crunchiness) and off-odour or off-flavour defects. Sensory data were elaborated by calculating the average of the results, and the test supervisor chose a significance level of 5%. The Quantitative Descriptive Analysis (QDA) were utilized to define the typical sensory profile.

2.1.10 Statistical and explorative multivariate analyses

A priori one-way analysis of variance, using Tukey's honest significant difference (HSD) test, was performed using Statgraphics software (version 6; Manugistics, Inc. Rockville, MD). Statistical significance was defined at $p <$ value less than 0.05. In addition, explorative multivariate analysis was employed to investigate the relationships between the FAMES profiles of different yeast strains isolated from the fermented brine used as the starter. Hierarchical cluster analysis (HCA) of similarity was carried out to group yeast strains according to their FAMES composition. Furthermore, correlations were subjected to principal component analysis (PCA) to condense the information into fewer factors. The PCA was performed with JMP statistical software (version 15.1.0; SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1 Chemical and microbiological composition of the starter

The use of industrially produced starter cultures made from a mixture of yeast strains or a single selected yeast strain is not a common practice in the production of brined Taggiasca black olives. Instead, this type of naturally fermented table olives is produced on an industrial scale according to traditional artisanal methods, which allow the use of 200 L PVC barrels, where olives are preserved in brine for several months. A recent study suggested a potentially positive role for some starters containing selected strains of *Candida diddensiae*, *Candida adriatica*, and *Wickerhamomyces anomalus* yeasts (Ciafardini and Zullo, 2019). However, the cost of industrially produced starters and the need to shorten the duration of the transformation process and standardize the sensory profile of Taggiasca black olives produced through spontaneous fermentation have increased producers' interest in new production technologies, such as the use of brine starters developed from the spontaneously fermented brine of Taggiasca olives produced the previous year in the same

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geographical area and/or in the same industrial plant. However, the risk of generating off-flavour and off-odours due to the development of spoilage microorganisms during inoculation has to be considered. The use of this type of starter is similar to the "pied de cuve" technology used for wine production (Li *et al.,* 2012). However, to maintain a rich population of autochthonous yeasts, the starter used in this study, unlike the traditional "pied de cuve", was not regenerated by incubation in the presence of new olives and fresh brine before. To limit risks, such as the brine containing spoilage microorganisms, the brine that was used as a starter was selected based on its physicochemical and microbiological characteristics. The results of the chemical analysis performed on the brine starter before inoculation are shown in Table 1. The presence of medium-high salt contents and polar phenolic compounds associated with low pH values indicate that the starter habitat is likely unsuitable for the development of many spoilage bacteria, while the good level of free CO_2 content represents a suitable for fermentative yeasts. The results of the microbiological analysis of the starter confirmed the chemical results, as they indicated the presence of 5.92 log CFU/mL yeasts and the complete absence of enterobacteria, LAB, TAB and mould (Table 2). The microbiota in the brine starter consisted of predominantly (96%) two autochthonous yeast species, *Pichia manshurica* and *Saccharomyces cerevisiae*. Although both species have been shown to tolerate a salt concentration equal to 15% (w/v), *P. manshurica*, with a prevalence of 88%, showed better salt tolerance (Table 2). Good resistance to salt is associated with the activities of enzymes of technological interest, such as β-glucosidase and esterase, which are involved in the degradation of oleuropein. Autochthonous yeasts also possess good salt resistance, which contributes to the transformation of Taggiasca black table olive using the Greek-style method, where a brine with an average of 12% (w/v) NaCl and acidified with citric acid is used (Ciafardini and Zullo, 2019).

3.2 Biodiversity of the yeasts in the starter

In addition to the different physiological characteristics shown in Table 2, the autochthonous yeast strains isolated from the brine starter were also differentiated based on their fatty acid profiles. Fatty acids are a source of cellular energy and can serve as building blocks for a number of complex membrane lipids. Gas-chromatographic analysis of $FAME_S$ in yeasts requires several steps, such as lipid esterification, sample injection, separation, identification, and quantification (Eder, 1995). $FAME_S$ analysis using the gas-liquid chromatography method has been applied successfully for the identification of clinically important yeast species (Kutty and Philip, 2008). The average fatty acid composition of the yeasts isolated from the brine starter and the reference species isolated from the commercial starters are reported in Table 3. The numbers and concentrations of fatty acids in the analysed yeasts varied according to both species and strain. Unlike *P. manshurica*, all *S. cerevisiae* strains were found to be free of polyunsaturated fatty acids ($PUFA_s$), which is in agreement with our previous findings and those of other studies (Avery *et al.,* 1996; Bassan *et al.,* 2014; Ciafardini and Zullo, 2019). The mean relative percentage of fatty acid found in the cellular material of the brine-starter yeasts varied according to strain. Compared with *P. manshurica* strains, more than 90% of the *S. cerevisiae* strains showed higher myristic and palmitoleic acid contents, while the commercial *S. cerevisiae* 2070 strain and autochthonous strains 12, 13, and 14 showed lower palmitic acid contents (Table 3). The HCA discriminated autochthonous strains of *P. manshurica* and *S. cerevisiae* from brine starter based on their $FAME_S$ profiles. It is an unsupervised method that recognizes and distributes $FAME_S$ data groupings according to their affinity in clusters of progressive dissimilarity, as described in a dendrogram. Analysis of the hierarchical tree showed two different macro-clusters in which *P. manshurica* strains tended to be more dissimilar than the *S. cerevisiae* strains (Figure 1). The *P. manhsurica* strains were grouped into three clusters within which the $FAME_S$ profile of the commercial strain *P. manshurica* 2045 differed markedly from the remaining native strains from the brine starter. The autochthonous *S. cerevisiae* strains were divided into two main clusters based on their $FAME_S$ profile; however, unlike *P. manshurica*, the commercial strain *S. cereviasiae* 2070 was associated with one of the autochthonous yeast strains isolated from the brine starter, *S. cerevisiae* strain 12 (Figure 1). Correlation analysis showed that there were many significant relationships. Thus, the data were appropriate for principal component analysis (PCA) to condense the information into fewer factors. The results of the PCA showed a clear difference between the $FAME_S$ profiles of the *P. manshurica* strains and those of the *S.*

Table 1. Chemical characteristics of the brine used as starter

pH	Titratable acidity (g lactic acid/L)	NaCl (%)	CO_2 (mg/L)	Total Phenol (mg CAE*/mL)
4.35±0.20	4.41±0.32	6.90±0.37	490±40	2.42±0.28

*CAE, caffeic acid equivalent

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Table 2. Physiological characteristics of the yeasts found in the Taggiasca olive brines used as starter

Total yeasts (Log CFU/mL)	Yeast species	Predominance (%)	Salt tolerance 7.5%	(% G.I.) 15%	Pseudohyphae production	β-glucosidase	Esterase	Lipase	Protease	Chromogenic Group
5.92±0.30	*Pichia manshurica*	88	47±8	35±6	-	+	-	+	+	Bordeaux center white esterior
	Saccharomyces cerevisiae	8	12±3	6±1	-	+	+	-	+	Bordeaux
	Other	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

G.I..: Growth Index. Negative symbol (-) indicates absence of production referred to 10 randomly selected strains; Positive symbol (+) indicates positive activity of 10 randomly selected strains, with various production levels; n.d.: not detected.

Table 3. Average fatty acids composition of the yeast strains found in the starter.

Yeast Strain	Myristic acid	Palmitic acid	Palmitoleic acid	Heptadecanoic acid	Heptadecenoic acid	Stearic acid	Oleic acid	Linoleic acid	Arachic acid	Linolenic acid	Eicosenoic acid	Behenic acid	Lignoceric tR acid	SFA	MUFA	PUFA
P.m. 2045	0.27^{b}	11.27^{ab}	18.30^{c}	0.40	0.13	0.88	40.43^{a}	14.61^{a}	0	4.92	0.14	0.69	3.6	17.11^{ab}	59.00^{a}	19.53^{b}
P.m. 2	0.37^{b}	13.18^{a}	18.53^{c}	0.24	1.17	1.64	33.88^{ab}	17.76^{a}	0.65	9.83	0.24	0.06	0.27	16.41^{ab}	53.84^{ab}	27.59^{a}
P.m. 3	1.05^{b}	14.13^{a}	14.08^{c}	0.27	0.79	1.46	25.21^{bc}	12.99^{ab}	0.47	5.79	0.14	0.05	0	17.43^{ab}	40.22^{b}	18.78^{b}
P.m. 4	1.33^{b}	16.78^{a}	13.05^{c}	0.20	0.87	1.57	21.09^{c}	11.87^{ab}	0.49	6.19	0.09	0.05	0	20.42^{a}	35.10^{b}	18.06^{b}
P.m. 5	0.50^{b}	10.38^{ab}	18.63^{c}	0.18	1.46	1.29	24.33^{bc}	14.20^{a}	0.41	7.05	0.14	0.02	0	12.78^{b}	44.56^{b}	21.25^{ab}
P.m. 6	1.19^{b}	15.00^{ab}	10.00^{c}	0.42	1.20	1.82	22.10^{c}	10.60^{b}	0.52	7.07	0.17	0.05	0	13.20^{b}	38.10^{b}	20.18^{b}
P.m. 7	0.40^{b}	14.10^{a}	15.60^{c}	0.25	0.80	1.45	40.35^{a}	13.30^{ab}	0.60	9.05	0.22	0.60	0	17.40^{ab}	56.97^{a}	22.35^{ab}
P.m. 8	1.00^{b}	16.00^{a}	15.00^{c}	0.37	0.72	1.51	21.10^{c}	15.20^{a}	0.65	5.70	0.27	0.05	0	19.58^{ab}	37.09^{b}	20.90^{b}
P.m. 9	0.50^{b}	10.08^{b}	10.00^{c}	0.40	1.40	1.55	40.22^{a}	14.05^{a}	0.58	6.40	0.25	0.05	0	13.16^{b}	51.87^{ab}	20.45^{b}
P.m. 10	0.70^{b}	16.90^{a}	18.08^{c}	0.52	1.20	1.27	21.10^{c}	12.07^{ab}	0.52	8.20	0.41	0.04	0	19.95^{ab}	40.79^{b}	20.27^{b}
S.c. 2070	0.28^{b}	7.32^{b}	40.94^{b}	0.14	0.52	2.93	30.52^{b}	0	0	0	0	0	0	10.67^{b}	71.98^{a}	0
S.c. 12	0.20^{b}	7.13^{b}	49.24^{ab}	0.15	0.26	3.76	27.97^{b}	0	0	0	0	0	0	11.24^{b}	77.47^{a}	0
S.c. 13	4.15^{a}	8.41^{b}	46.84^{ab}	0.06	0.27	1.71	21.24^{c}	0	0.04	0	0.03	0	0	14.37^{ab}	68.38^{a}	0
S.c. 14	5.36^{a}	8.48^{b}	42.92^{b}	0.13	0.14	2.44	22.38^{c}	0	0.04	0	0.10	0.09	0	16.54^{ab}	65.54^{ab}	0
S.c. 15	4.02^{a}	11.56^{ab}	47.68^{ab}	0.00	0.00	3.01	33.71^{ab}	0	0	0	0	0	0	18.59^{a}	81.39^{a}	0
S.c. 16	5.38^{a}	11.29^{ab}	50.34^{a}	0.00	0.00	3.84	29.14^{b}	0	0	0	0	0	0	20.51^{a}	79.48^{a}	0
S.c. 17	4.87^{a}	11.89^{ab}	47.01^{ab}	0.00	0.00	3.67	32.54^{ab}	0	0	0	0	0	0	20.43^{a}	79.55^{a}	0
S.c. 18	5.76^{a}	11.63^{ab}	55.22^{a}	0.00	0.00	2.18	25.18^{bc}	0	0	0	0	0	0	19.57^{a}	80.40^{a}	0
S.c. 19	5.87^{a}	10.89^{ab}	53.58^{a}	0.00	0.00	2.98	26.65^{bc}	0	0	0	0	0	0	19.74^{a}	80.23^{a}	0
S.c. 20	4.41^{a}	12.78^{ab}	46.41^{ab}	0.00	0.00	3.89	32.51^{ab}	0	0	0	0	0	0	21.08^{a}	78.92^{a}	0

P.m. 2045, *Pichia manshurica* strain 2045 from commercial starter; *P.m.* from 2 to 10, *Pichia manshurica* strains isolated from the brine used as starter before barrels inoculation; *S.c.* 2070, *Saccharomyces cerevisiae* strain 2070 from commercial starter; *S.c.* from 12 to 20, *Saccharomyces cerevisiae* strains isolated from brine used as starter before barrels inoculation. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid. Values with different superscript in the same column indicate significant difference by Tukey's test ($p < 0.05$).

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cerevisiae strains by distributing them into two separate groups. In the biplot shown in Figure 2, it is possible to observe how within each group, the autochthonous yeast strains isolated from the brine starter belonging to the same species lie close together on the same axis. In contrast, the commercial 2045 strain of *P. manshurica* and the commercial 2070 strain of *S. cerevisiae* are two different strains isolated from others of the same species. Thus, the multivariate statistical approaches (HCA and PCA) showed that both predominant yeast species in the brine starter from Taggiasca olives are made up of a multitude of autochthonous strains that clearly differ from those isolated from the commercial starters used in the transformation of a variety of other olives by methods different from the Greek-style method. The set of results reported above suggests brine-starter inoculation as a valuable method that favours the growth of autochthonous yeast strains with a technological aptitude that is able to drive the fermentation process of Taggiasca black table olives.

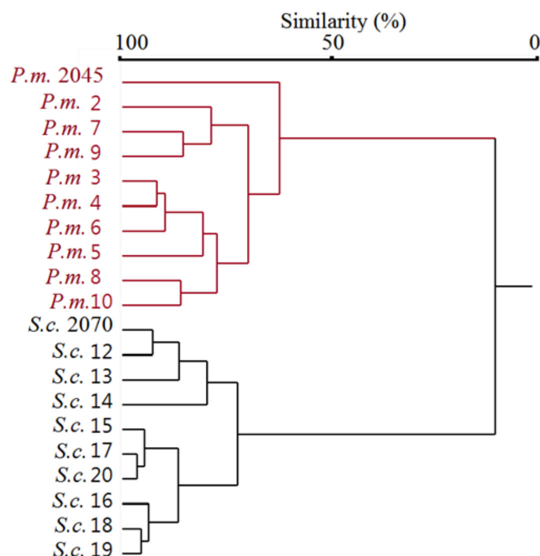

Figure 1. Dendrogram from the cluster analysis of the fatty acid profile of the yeast strains found in the fermented brine used as starter before barrel inoculation. (*P.m.* 2045, *Pichia manshurica* strain 2045 from commercial starter; *P.m.* from 2 to 10, *Pichia manshurica* strains isolated from the fermented brine used as starter before barrel inoculation; *S.c.* 2070, *Saccharomyces cerevisiae* strain 2070 from commercial starter; *S.c.* from 12 to 20, *Saccharomyces cerevisiae* strains isolated from the fermented brine used as a starter before barrel inoculation)

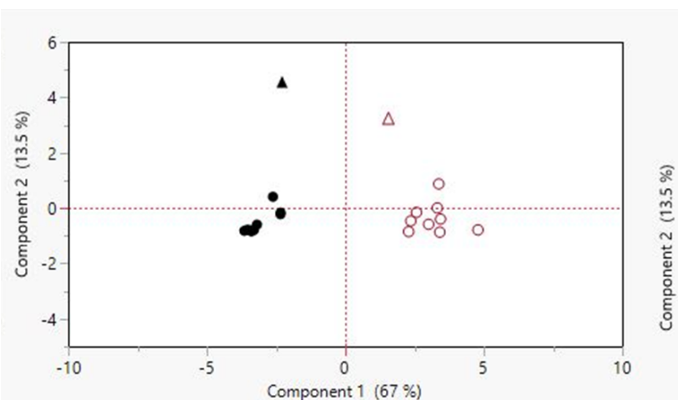

Figure 2. PCA analysis based on the values of FAMES profile of the yeast strains isolated from the fermented brine starter before barrel inoculation. ▲, *Saccharomyces cerevisiae* strain 2070 isolate from a commercial starter; ●, *Saccharomyces cerevisiae* strains isolated from the fermented brine used as a starter before barrel inoculation; Δ, *Pichia manshurica* strain 2045 isolate from a commercial starter; ○, *Pichia manshurica* strains isolated from the fermented brine used as a starter before barrel inoculation.

3.3 Industrial-scale fermentation of Taggiasca olives

Experimental olive production was performed in modified PVC barrels containing 120 kg of olives and 60 L of brine with 12% (w/v) NaCl and 0.6% (w/v) citric acid, inoculated with 10 L of either the brine starter described above or fresh brine (control). The chemical characteristics of the Taggiasca olive brines during 1 year of fermentation are shown in Tables 4 and 5. Chemical analysis of the brines performed set up indicated the absence of phenols, low CO_2 content, and a pH below three in both brines (Tables 4 and 5). However, a slightly lower salt concentration was noted in the inoculated brine, which was attributed to dilution of the starter, which possessed 6.90% (w/v) NaCl (Table 1). Beginning at the third month of fermentation, the pH and salt concentration similarly stabilized in both the inoculated brines and uninoculated controls. However, during fermentation, the pH values and titratable acidity were within the limits for the standard trade of table olives, which was 4.30 (IOOC, 2004). During fermentation, the phenolic contents and CO_2 concentrations were higher in the control brine than in the brines treated with the starter (Table 5). These results, which were also highlighted by subsequent microbiological tests, seemed to depend on the type of metabolism of the microbial species present and on the high prevalence of yeasts in the brines inoculated with the starter, which accelerated the degradation of the phenolic compounds released into the brine by the fruits.

3.4 Microbiological tests

The microbiological analysis of all analysed brine samples, which were taken regularly from the barrels during the fermentation of the Taggiasca olives, showed an absence of LAB, enterobacteria, and mould. In contrast, TAB was found, but only during the first 3 and 6 months of fermentation, respectively, in the starter-inoculated and control brines. Yeasts appeared to drive the fermentation throughout the incubation period (Table 6). According to previous studies, the absence of LAB and the disappearance of TAB after a few months of fermentation may be linked to the high salt concentration, increased concentration of polar phenolic

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Table 4. Chemical characteristics of the Taggiasca olive brines during fermentation

Treatment	0-month			3-month			6-month			9-month			12-month		
	pH	Titratable acidity (g lactic acid/L)	NaCl (%)	pH	Titratable acidity (g lactic acid/L)	NaCl (%)	pH	Titratable acidity (g lactic acid/L)	NaCl (%)	pH	Titratable acidity (g lactic acid/L)	NaCl (%)	pH	Titratable acidity (g lactic acid/L)	NaCl (%)
Inoculated brine	2.35±0.15	5.34±0.25	10.80±0.42	3.8±0.10	5.99±0.35^b	8.94±0.25	3.96±0.15^b	6.50±0.20^a	8.85±0.32^a	3.93±0.20^b	5.73±0.28^a	8.83±0.35	4.00±0.10^b	5.76±0.35^a	8.93±0.45
Uninoculated brine	2.19±0.17	5.29±0.13	11.26±0.50	3.92±0.13	6.41±0.45^a	9.48±0.35	4.11±0.17^a	6.00±0.30^b	8.08±0.22^b	4.13±0.30^a	5.06±0.25^b	8.31±0.30	4.20±0.12^a	5.10±0.25^b	8.10±0.22

Values with different superscript in the same column are significantly different by Tukey's test (P<0.05).

Table 5. Total phenols and carbon dioxide content of the Taggiasca olive brines during fermentation

Treatment	0-month			3-month			6-month			9-month			12-month		
	Total phenols[1]	CO2[2]	Δ[3]	Total phenols[1]	CO2[2]	Δ[3]	Total phenols[1]	CO2[2]	Δ[3]	Total phenols[1]	CO2[2]	Δ[3]	Total phenols[1]	CO2[2]	Δ[3]
Inoculated brine	0	72±12	-	1.85±0.21	178±36^b	-	2.54±0.22^b	349±68^b	-	2.95±0.12^b	192±47^b	-	2.60±0.08^b	320±86^b	-
Uninoculated brine	0	72±25	0	2.30±0.37	331±67^a	153	3.19±0.20^a	778±94^a	429	3.96±0.15^a	623±55^a	431	3.45±0.10^a	720±97^a	400

[1]Total phenols refer to mg of caffeic acid equivalent per mL of brine. [2]The free CO_2 content was expressed as mg CO_2 per L of brine. [3]Increase in CO_2 concentration in the uninoculated brines compared to the inoculated ones. Values with different superscript in the same column are significantly different by Tukey's test (P<0.05).

Table 6. Yeast and bacteria population size in brine of Taggiasca olives during fermentation

Treatment	0-month		3-month		6-month		9-month		12-month	
	Yeast	TAB	Yeast	TAB	Yeast	TAB	Yeast	TAB	Yeast	TAB
Inoculated brine	3.60±0.26	1.60±0.06	5.40±0.37^a	2.20±0.15	6.10±0.46^a	0	5.95±0.33	0	6.90±0.47	0
Uninoculated brine	d.l.	2.20±0.04	1.85±0.26^b	2.40±0.05	4.24±0.36^b	1.55±0.22	5.21±0.16	0	6.29±0.52	0

TAB: Total Aerobic bacteria, d.l.: detection limit. Values are expressed as mean±standard deviation (log CFU/mL) of triplicate trials. Values with different superscript in the same column are significantly different by Tukey's test (P<0.05).

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compounds from the fruits during fermentation (Table 6), and the high yeast populations (Tassou *et al.,* 2002; Bautista-Gallego *et al.,* 2011; Ciafardini and Zullo, 2019). In the initial phase of testing, the use of the brine-starter caused a significant increase in the total number of yeasts in the treated brines, which reached 3.60 log CFU/mL, while in uninoculated brine, the number of yeasts did not exceed the detection limit. A higher concentration of yeasts was observed in brines treated with the starter than in the untreated controls during the first 6 months of fermentation; however, in the subsequent 6 months, the values in both brines were almost identical. As shown in Table 6, during the first 6 months of incubation, the average yeast population size ranged from 3.60 log CFU/mL to 6.10 log CFU/mL in the brine treated with starter, while in the control, the average yeast population size was zero to 4.24 log CFU/mL. The total numbers of yeasts in the two types of brines were dissimilar, and the use of the starter also changed the composition of the yeast microbiota. The results shown in Table 7 indicate that for the 12-month fermentation, the predominant yeast species differed depending on whether the brine has been treated with the starter or not. More specifically, in the brine treated with starter, two yeasts *P. manshurica* and *S. cerevisiae*, which initially showed prevalence rates of 87% and 10%, respectively, were equal during the first 6 months of fermentation, while in the subsequent period, *S. cerevisiae* became more predominant. However, another autochthonous species, *Zygosaccharomyces mrakii*, which was not initially prevalent in the starter, reached 32% predominance by the end of fermentation. In contrast, in the control brines, starting from the third month of fermentation, *P. manshurica* and *Z. mrakii* were predominant with a clear prevalence of the latter species (Table 7). However, it is interesting to note that microbiological analysis of total yeasts after 5 days of incubation at 30°C revealed two types of yeasts, called fast and slow growing. Fast growing yeasts were able to produce colonies on MYGP agar medium after 2 days of incubation at 30°C, while slow-growing yeasts produced colonies on the same medium only after the fourth day of incubation. From Figure 3, it is possible to see how, during the fermentation of brines treated with the starter, the slow-growing yeasts increasingly entered into competition with the fast-growing yeasts present in the starter, going from an initial value of 0 to over 30% predominance, much to the detriment of fast-growing yeasts, which dropped from 100% to less than 70% predominance after 12 months of fermentation. In contrast, in the control brine, starting from the third month of fermentation, the ratio of fast to slow-growing yeasts remained virtually unchanged over time. Taxonomic analysis of the different types of yeasts showed that slow-growing yeasts refer to *Z. mrakii* species, while the other species mentioned above belong to the fast-growing yeasts. The set of results reported above demonstrate the positive role played by the brine starter on the total microbial load of the brine, especially in the first 6 months of fermentation, which are crucial for the rapid and appropriate spontaneous fermentation of Taggiasca olives.

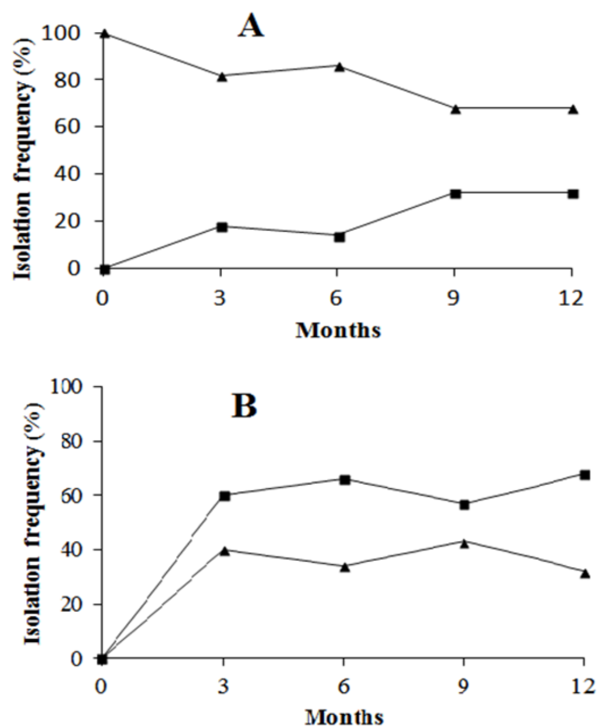

Figure 3. Isolation frequency of fast (▲) and slow (■) growing yeasts from inoculated (A) and uninoculated (B) brine of Taggiasca olives during fermentation

3.5 Sensory profile

The sensory profiles of Taggiasca black olives processed for 12 months in industrial barrels are reported in Table 8. Significant differences among the treatments were observed for some parameters, such as bitterness and taste complexity, while no significant statistical differences were found for all other tested sensory attributes. When compared to the uninoculated control, the intensity of bitterness was significantly ($p < 0.05$) lower for fruits processed in brine inoculated with the starter. This difference was highlighted starting from the third month of fermentation until its complete disappearance after 9 months of fermentation. However, in the sixth month of fermentation, olives processed in brine inoculated with the starter showed a low bitterness intensity, with a median index of 2, thus resulting in completely debittered olives that were ready for commercialization (Table 8). In contrast, in the sixth month of fermentation, control olives were definitely more bitter, with a median bitterness index of 4 and required further incubation before their complete

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Table 7. Frequency of isolation of the yeast species isolated from the brine of Taggiasca olives during fermentation

Treatment	0-month		3-month		6-month		9-month		12-month	
	Yeast species	(%)	Yeast species	(%)	Yeast species	(%)	Yeast species	(%)	Yeast species	(%)
Inoculated brine	*P. manshurica*	87	*P. manshurica*	68	*P. manshurica*	47	*P. manshurica*	22	*P. manshurica*	6
	S. cerevisiae	10	*S. cerevisiae*	14	*S. cerevisiae*	39	*S. cerevisiae*	46	*S. cerevisiae*	62
	Others	3	*Z. mrakii*	18	*Z. mrakii*	14	*Z. mrakii*	32	*Z. mrakii*	32
Uninoculated brine	-	-	*Z. mrakii*	60	*Z. mrakii*	66	*Z. mrakii*	57	*Z. mrakii*	68
			P. manshurica	15	*P. manshurica*	34	*P. manshurica*	43	*P. manshurica*	32
			Others	25						

Table 8. Sensory attributes of the brined Taggiasca table olives during fermentation

Treatment	3-month								6-month							
	Bitter	Acid	Salty	Hardness	Crunchiness	Off-flavours	Off-odours	Complexity taste	Bitter	Acid	Salty	Hardness	Crunchiness	Off-flavours	Off-odours	Complexity taste
Inoculated brine	5±0.2[b]	5±0.1	6±0.3	7±0.5	7±0.4	0	0	7±0.5[a]	2±0.2[b]	6±0.5	7±0.4	6±0.6	7±0.7	0	0	8±0.6[a]
Uninoculated brine	8±0.5[a]	5±0.4	5±0.5	8±0.9	8±0.6	0	0	5±0.2[b]	4±0.1[a]	6±0.4	7±0.5	7±0.7	7±0.8	0	0	6±0.3[b]

Values are expressed as median±standard deviation. Values with different superscript in the same column are significantly different by Tukey's test ($P<0.05$).

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processing. The salty flavour of the fruit, in accordance with the chemical analysis of the brines reported in Table 4, was slightly higher for olives processed with both methods. Hardness and crunchiness, two positive parameters, were high in all samples, regardless of the use of the brine starter. The intensity of taste complexity evaluated during fermentation was significantly ($p<0.05$) higher for fruit processed in the starter-inoculated brine than in the uninoculated control fruit (Table 8). No explicit defects were detected in all tested samples, based on defects predominantly perceived (DPP), and the products were classified in the standard category (IOOC, 2011). The results of the sensory analysis (Table 8) are in accordance with those of the microbiological analyses (Tables 6 and 7). In fact, the higher concentration of total yeasts recorded during the first 6 months of fermentation in the starter-treated brine with the starter (3.60-6.10 log CFU/mL) compared to that in uninoculated control (0-4.24 log CFU/mL), accelerated the hydrolysis of the bitter glycosides in the olives due to the oleuropeinolytic enzymes (β-glucosidase and esterase) from the autochthonous *P. manshurica* and *S. cerevisiae* strains in the starter (Table 2). Consequently, from a technological point of view, the use of a brine starter for the transformation of Taggiasca black table olives using the Greek-style method led to a shortening of the processing time of the product compared with the control (Table 8).

4. Conclusion

The use of a low-cost brine starter containing autochthonous yeast strains may be a traditional, low-intervention method for processing black table olives on an industrial scale using the Greek-style system. The trials showed the positive effects of brine starter on the debittering process of the product, shortening it by about 3 months when compared to the control. The possibility of marketing the product after only a few months of storage in barrels, and the maintenance of the typical taste of the black table olives, as well as the spontaneous fermentation achieved with native yeast strains, should meet the fundamental requirements of many olive producers. The use of brine starters is a new way to achieve these goals. However, to prevent the risk of spreading spoilage microorganisms or using brine starters with sensory defects, it is strongly advisable to choose brine starters only after performing in-depth physicochemical, microbiological, and sensory analyses.

Conflict of interest

The authors declare no conflict of interest.

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