



Use of selected yeast starter cultures in industrial-scale processing of brined Taggiasca black table olives

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ABSTRACT

The aim of this study was to evaluate the effects of six selected yeast starters on natural Taggiasca black table fermentations, in different brine solutions. The olives were subjected to fermentation in 8% (w/v) NaCl, 12% (w/v) NaCl and 12% (w/v) NaCl brine solutions with 0.3% (w/v) citric acid and inoculated with selected yeast starter strains belonging to the following species: *Candida adriatica* 1985, *Candida diddensiae* 2011, *Cytoromyces matritensis* 2005, *Nakazawaea molendini-olei* 2004, *Saccharomyces cerevisiae* 2046 and *Wickerhamomyces anomalus* 1960. Samples of brines and olives were analysed in the initial phase, then again after 30 and 120 days of fermentation. The yeast starters survived differently during the first 30 days of brine fermentation, depending on the NaCl concentration. After 120 days of fermentation *N. molendini-olei* 2004 and *C. matritensis* 2005 failed in the brines with 12% NaCl, while the yeast starter cultures *C. diddensiae* 2011, *C. adriatica* 1985 and *W. anomalus* 1960 showed the best performances in terms of survival and competitiveness towards wild yeasts of the brines. The physicochemical and sensorial analysis suggest a potential positive role of these yeasts during the debittering process of the Taggiasca table olives. Considering the combination between yeast starters and the fermentation conditions, the best indication occurred with the brines containing 12% NaCl acidified with citric acid.

1. Introduction

Table olives are the most important fermented vegetables in Mediterranean countries. Spain is the largest producer of olives, producing 563,400 tons in 2018, followed by Greece (261,000 tons) and Italy (60,000 tons) (IOOC, 2018). In general, the methods of producing table olives include the Sevillian and Californian systems, which incorporate a chemical treatment of the fruits with lye, and the natural system, which involves natural fermentation of olives in brine and produces olives known as natural black olives (Garrido Fernández et al., 1997). In the natural black table olive processing system, olives are directly placed in the brine solution without any prior debittering pretreatment. During the debittering process, olives are subjected to spontaneous fermentation involving a microbial community, including members of Lactic Acid Bacteria (LAB) and yeasts. Factors such as the salt concentration and pH affect the presence and dominance of LAB and yeasts during the fermentation period (Durán Quintana et al., 1997; Özyay and Borcakli, 1996). Salt concentrations greater than 10% (w/v) favour yeasts, whereas those under 8% (w/v) induce mixed fermentation, where LAB dominate the initial stage of fermentation (Leventdurur et al., 2016; Nisiotou et al., 2010). LAB produce enzymes

engaged in the table olive debittering process and lactic acid, which decrease the pH of the brine (Ciafardini et al., 1994). During the later stage of fermentation, LAB inhibit the brine through a high phenolic concentration and yeasts become the microorganisms responsible for fermentation. Yeasts play critical roles in table olive fermentation and have the ability to enhance the aroma and taste of fermented olives through the activity of specific enzymes, such as lipase and esterase, in the formation of several aromatic compounds (Arroyo-López et al., 2008; Ciafardini et al., 2005). Furthermore, through the production of β -glucosidase, yeasts degrade some bitter phenolic compounds, enhance the growth of LAB by the synthesis of various vitamins and act as biocontrol agents, inhibiting the growth of non-desirable fungi (Arroyo-López et al., 2012; Viljoen, 2006). Moreover, some yeast strains degrade anti-nutritional compounds, such as phytate complexes, by their phosphatase and phytase activities, which release inorganic phosphorous from the cells (Olstorpe et al., 2009). However, yeasts may have undesirable effects during the fermentation process, as they can soften the fruit, and produce a “gas pocket” formation, as well as a biofilm production on the top layer of the brine. The natural table olive processing system is the main, traditional debittering system used in Italy, with some variations depending on the cultivar, stage of fruit

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ripening and brine composition. The Taggiasca cultivar is typical of the Liguria region (North of Italy), where it is cultivated for the production of both oil and table olives. To produce Taggiasca table olives, the fruits are harvested when they become black and are sorted, rinsed with water, and placed in 160–200 L polyethylene (PE) or polyvinyl chloride (PVC) plastic barrels. The PVC barrels are practical and economic since they perform multiple functions, including olive transport from one company to another (sometimes over large distances), and for fruit processing and storage of the processed product. The barrels are filled with freshly prepared brine with a salt concentration of 8–12% (w/v). For safety reasons, the practice requires the reduction of pH by the addition of lactic or citric acid. Fruits are marinated in the brine until they lose their bitter taste, and after 6 or more months, they are placed in jars, filled with fresh brine and pasteurized. During the processing period, the barrels are stored at room temperature under sun-protected roofs or for short periods outdoors. This system of black table olive processing, characterized by the high salt concentration of the brine, is often referred to as the Greek-style. In this system, the removal of bitter compounds, mainly represented by oleuropein and its aglycons, occurs due to the enzymatic activity (β -glucosidase and esterase) of the fruits and the microorganisms (mainly yeasts) 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 that are naturally directly processed in brine has led to the rapid expansion of Taggiasca table olive production in recent years. However, producers often encounter difficulties in supplying the market throughout the year due to long processing periods, which necessitate approximately 5–6 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 long periods, in some cases, between 7 and 8 months. Studies on the natural fermentation of Taggiasca black table olives processed on an industrial-scale in PE or PVC barrels are limited, and knowledge of the microbial populations involved in the initial phase of fermentation is scarce. The present study focused on the microbiological changes that occur during the initial spontaneous fermentation of brined Taggiasca black table olives treated with starters and processed on an industrial-scale. The main aim of the work was to study the physicochemical and sensory parameters of the brined olives and the survival of six selected yeast starter cultures under different fermentation conditions.

2. Materials and methods

2.1. Yeast selection

This study was conducted with 21 yeast strains of *Nakazawaea molendini-olei* (7 strains), *Cyteromyces matritensis* (5 strains), *Candida diddensiae* (3 strains), *Candida adriatica* (2 strains), *Wickerhamomyces anomalus* (2 strains), and *Saccharomyces cerevisiae* (2 strains). The yeasts had been previously isolated from brined black table olives and oil of Leccino and other cultivars, identified by sequencing of the D1/D2 region of the 26S rDNA gene (Kurtzman and Robnett, 1997) and stored long-term in the collection kept in the Agricultural Department of the University of Molise (Italy). The physicochemical characteristics were used to identify the yeast strains that performed best, which were subsequently used to produce starters for inoculating the 200 L industrial PVC barrels containing Taggiasca black table olives in brine. For physicochemical and enzymatic characterization, the yeasts were cultured in a Malt, Yeast, Glucose, Peptone (MYGP) agar medium with the following composition: 3 g of yeast extract (Biolife, Milan, Italy), 3 g of malt extract (BBL, Cockeysville, Maryland, USA), 2.5 g casein bacto tryptone (BD, Sparks, Maryland, USA), 2.5 g of soy peptone (Biolife, Milan, Italy), 10 g of D-glucose (Merck, Germany), and 1000 mL of distilled water, pH 7 (Ciafardini and Zullo, 2018). After two days of incubation at 30 °C the cultures were used in the trials described below.

2.1.1. β -glucosidase assay

All the yeast strains belonging to the six species described above were used to set up master cultures (Maniatis et al., 1982). Each master culture 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) FeCl₃ (Carlo Erba, Milan, Italy). After 48 h 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) and each test was performed in duplicate.

2.1.2. Esterase activity

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

2.1.3. Lipase activity

The lipase activity was assessed as described by Ciafardini et al. (2005). In detail, 5 mL of O/N stock culture of each yeast strain (O.D. adjusted to ca. 0.8) after 5 min centrifugation at 9000 \times 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).

2.1.4. Protease activity

The protease activity of the yeasts was evaluated by setting up the master cultures using the same method as above with a MYGP agar medium supplemented with 1% (w/v) skimmed milk (Sigma-Aldrich). After 12 h of incubation at 30 °C, the enzymatic activity was recorded with the appearance of transparent halos around the colonies. The test was repeated twice.

2.1.5. Growth at 22 °C

Yeast growth capacity at 22 °C, which represents the temperature most widely used in industrial-scale processing of brined Taggiasca black table olives, was assessed by adding 100 μ L of yeast sample, suspended in sterile water (O.D.₆₀₀ = 0.7) to 10 mL of MYGP broth in Pyrex tubes fitted with screw caps. For each yeast culture, 6 test tubes were prepared. After slight agitation of the tubes, they were divided into two groups: one group was incubated at 22 °C and a second (control) group was incubated at the optimal temperature of 30 °C. After two days of incubation, the tubes were stirred with a vortex for 1 min and then analysed with a spectrophotometer (Jenway model 6300, UK) at a wavelength of 600 nm. The growth at 22 °C recorded for each group of tubes (3 repetitions) was expressed as a percentage of that of the control.

2.1.6. Sedimentation capacity

The sedimentation capacity of a yeast makes it possible to simplify the recovery of cell biomass during the preparation of the starters without resorting to centrifugation. The sedimentation capacity test was performed according to the procedures described above with some

modifications. A total of 100 μL of yeast sample ($\text{O.D.}_{600} = 0.7$) was inoculated into Pyrex tubes with 10 mL of MYGP broth medium. After brief agitation, 3 tubes for each strain were incubated for 2 days at 30 °C. At the end of the incubation period, 1 mL of each tube was carefully withdrawn and analysed with the spectrophotometer as before. Then the sample was transferred back to its tube of origin, agitated for 1 min with a vortex and analysed as before. The sedimentation of the sample was expressed as the percentage O.D._{600} of each sample before and after agitation.

2.1.7. Salt resistance assay

The NaCl resistance of the yeast strains 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 were centrifuged at 10000 $\times g$ (Hettich Instruments, Tuttlingen, 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.): $\text{G.I.} = (\text{O.D.}_{\text{sample}}/\text{O.D.}_{\text{control}} \times 100)$, where $\text{O.D.}_{\text{sample}}$ represents the absorbance at 600 nm of cultures grown in MYGP with NaCl, $\text{O.D.}_{\text{control}}$ represents the absorbance at 600 nm of cultures grown in MYGP lacking NaCl.

2.2. Starter production

The starter production was performed by selecting the strain of each yeast species that showed the best physicochemical performance in the previous tests. The selected strains were *N. molendini-olei* 2004, *C. matritensis* 2005, *C. diddensiae* 2011, *C. adriatica* 1985, *W. anomalus* 1960, and *S. cerevisiae* 2046. The starters were produced using the laboratory-scale MYGP medium, and a less expensive substrate composed of ingredients which are widely used in the market for other purposes.

2.2.1. Starter production with MYGP medium

After verifying the purity of each culture through several streaks on MYGP agar medium sterilized at 121 °C for 20 min, the yeasts were transferred into 100 mL of sterile MYGP and incubated at 30 °C for 48 h. At the end of the incubation, the individual cultures were used to prepare the stock cultures in 1000 mL Pyrex flasks. A volume of 800 mL of sterile MYGP medium was inoculated with 10 mL of yeast per culture ($\text{O.D.}_{600} = 0.7\text{--}0.8$), stirred briefly by hand and incubated for 48 h at 30 °C. Subsequently, 50 mL of each stock culture was transferred to a 5 L Pyrex flask containing 4 L of sterile MYGP medium and incubated at 30 °C for 5 days, shaking the flask by hand every 12 h. At the end of the incubation period, the cell biomasses were collected by centrifuging at 9000 $\times g$ for 10 min or by discarding the supernatant clear liquid from the sediment depending on the physicochemical characteristics of each yeast species. The concentrated biomass of each yeast collected from the various flasks was further concentrated by centrifugation at 9000 $\times g$ for 10 min.

2.2.2. Starter production with low-cost medium

The reproduction of yeast starters in low-cost medium was carried out following the same procedure as described above. The substrate composition was as follows: soy protein flour extract, 200 mL; brewer's yeast extract, 200 mL; glucose syrup, 200 mL; and distilled water, 400 mL. The substrate had a pH of 7. The soy flour extract was obtained from 20% (w/v) protein flour (44% protein) of soy suitable for animal feed (Lameri S.p.A., Cremona, Italy) mixed with water. The soy protein flour mixture was heated to 90 °C for 10 min, cooled, and stirred for

30 min. Then, the aqueous fraction was separated by centrifugation at 9000 $\times g$ for 10 min and used after a few hours. The brewer's yeast extract (*S. cerevisiae*) was produced by suspending 20% of baking yeast in water (Zeus Iba, Florence, Italy). After heating at 90 °C for 10 min, the mixture was stirred for 60 min, and the aqueous fraction was separated as above by centrifugation. The commercial glucose syrup for food use (Cameo, Brescia, Italy) from the enzymatic hydrolysis of starch was used directly without any pre-treatment. Before inoculation with yeasts in the same way as described above, the substrate was sterilized at 110 °C for 15 min and cooled rapidly. The biomass of cells produced was recovered, concentrated through centrifugation, and weighed.

2.2.3. Inoculation of the 200 L PVC barrels

The above starter cultures were washed in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4) of pH 7.4 and suspended in a final volume of 1000 mL of physiological solution (0.9% NaCl), adjusting the concentration with a hemocytometer to 10^8 yeast cells per mL. After 24 h, each 200 L barrel, used separately for each treatment, was inoculated with 100 mL of yeast starter, filled with fresh brine according to the treatment, and closed with a full-sized lid.

2.3. Olive processing in industrial PVC barrels

Taggiasca black olives produced in the Liguria region were harvested at the pigmented stage of ripeness ready for processing using the Greek method. Fermentation was undertaken under ambient conditions (15–20 °C) in commercial, 200 L volume, closed PVC barrels containing 120 kg of olives and 80 L of freshly prepared brine. The brine treatments were as follows: 8% (w/v) NaCl in non-acidified brine, 12% (w/v) NaCl in non-acidified brine and 12% (w/v) NaCl in brine acidified with 0.3% (w/v) citric acid. The different brines were inoculated with the above described yeast starters *N. molendini-olei* 2004, *C. matritensis* 2005, *C. diddensiae* 2011, *C. adriatica* 1985, *W. anomalus* 1960, and *S. cerevisiae* 2046, with 100 mL of each starter distributed in each 200 L PVC barrel. For each type of brine, an uninoculated control was established. After inoculation with the respective starters, the barrels were filled completely with brine, and then sealed with a lid. All treatments were performed with three repetitions, and the barrels were maintained for an overall period of 120 days. At the moment of the barrel inoculation, the brine was microbiologically analysed and the initial population size of the yeasts was recorded. In the early stage of the process corresponding to 30 days of fermentation at room temperature, a microbiological analysis of the brines was performed, while after 120 days of fermentation that in this case represent the end of the process, the microbiological and the physicochemical analysis of the brines and the processed olives was accomplished.

2.3.1. pH, titratable acidity and brine salinity

The pH of the commercial-scale olive brine was measured periodically using a pH-meter with an In Lab Routine Pro probe (Mettler, USA). Titratable acidity assays and the salt concentration in the brine were measured according to Garrido-Fernández et al. (1997).

2.3.2. Total polar phenols

A total of 1.5 mL of each brine was centrifuged at 12000 $\times g$ for 5 min and 1 mL of supernatant was diluted in 9 mL of distilled water. Subsequently, 0.1 mL of 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) diluted to 1:10 (v/v). In addition, 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 diluted (1:10, v/v) was established. The colorimetric reaction proceeded with the samples in the dark at room temperature for 2 h. Subsequently, after briefly stirring, the samples were analysed with a spectrophotometer using a wavelength of 765 nm. The total

polar phenols of olive samples were extracted five times with methanol using 6 g of olive pulp. After adding 30 mL of acetone to remove the oil fraction, the samples were homogenized with a Turrax model T25 homogenizer (IKA, Milan, Italy) for 2 min and then centrifuged at $9000 \times g$ for 10 min. The supernatant was removed, and 12 mL of methanol was added to the pellet, followed by homogenization and centrifugation as described above. The extracts were combined, methanol was evaporated under a vacuum and the residue was dissolved in 12 mL methanol then diluted with distilled water to a ratio of 1:10 and analysed using a spectrophotometer at a wavelength of 765 nm.

2.3.3. Microbiological analysis

The microbiological analyses were conducted using both brine and Taggiasca olives sampled under sterile conditions, near the surface, at mid-depth and near the bottom of each tank, after 30 and 120 days of fermentation. A total sample of 1000 mL of brine, containing approximately 300 g of olives, was collected through the mix of the samples taken at three different depths in each barrel, poured into a sterile Pyrex glass flask and transported immediately to the laboratory. The table olive microbiota was evaluated in brine samples collected after 30 days of fermentation. At 120 days of fermentation, when the salt concentration and other physicochemical parameters were more balanced between the olives and brine, both the stoned olives and corresponding brines were analysed. The olives were separated, rinsed twice in 500 mL sterile distilled water and analysed separately. The table olive microbiota was evaluated by randomly selecting 25 olives from each sample in which the pit was removed using sterile scalpels and forceps under axenic conditions. A total of 10 g of olive pulp was aseptically added to 90 mL of sterile $\frac{1}{4}$ Ringer's solution and homogenized with a Turrax model T25 homogenizer (IKA, Milan, Italy) for 60 s. The resulting suspension was serially diluted with a factor 10 using the same diluents and analysed as below. The brine samples were collected after 30 and 120 days of fermentation and then serially diluted by a factor of 10 using $\frac{1}{4}$ Ringer's solution, and the different dilutions were spread on to agar media as described below. For the enumeration of total culturable yeasts, serial dilutions of the olive suspension and brines were plated onto the same type of MYGP agar medium described previously and supplemented with 100 $\mu\text{g}/\text{mL}$ of chloramphenicol and incubated at 30 °C for 3 days. Lactic acid bacteria (LAB) were enumerated after incubation at 25 °C for 72 h on de Man-Rogosa-Sharpe (MRS) agar medium (Biolife, Milan, Italy) adjusted to pH 5.7 and supplemented with 0.05% (w/v) cycloheximide (AppliChem GmbH, Darmstadt, Germany). Total aerobic bacteria (TAB) were enumerated after 24 h of incubation at 30 °C on Nutrient Agar (CM0003, Oxoid, UK) supplemented with 0.05% (w/v) cycloheximide to prevent the growth of yeasts. *Enterobacteriaceae* were evaluated on Violet Red Bile Glucose Agar (VRBGA) (Biolife, Milan, Italy), with incubation at 37 °C for 24 h. All plates were examined visually for typical colony types and morphological characteristics, which were recorded along with the corresponding growth medium. The bacteria grown in each selective medium were evaluated by microscopic examination of smears prepared from randomly selected colonies and Gram staining. The results are expressed as Log values of colony forming units (CFU) per gram of olives (Log CFU/g) or mL of brine (Log CFU/mL).

2.3.4. Yeast isolation and identification

The yeast colonies obtained from microbiological analysis of the brines after 30 and 120 days of fermentation and those from olives after 120 days of fermentation, were counted, then the colony from the brine samples were used to prepare a series of masters using MYGP agar medium. All the colonies from each analysed brine sample, corresponding approximately to 600 units, were distributed in master cultures established in Petri dishes containing 100 colonies each. After 3 days of incubation at 30 °C, the masters were replicated on CHROM agar *Candida* medium (BBL 4354093) and tested as described by Tornai-Lehoczki et al. (2003). The yeast cultures grown on MYGP agar

medium were analysed after 10 days incubation at 30 °C, whereas the corresponding cultures grown on CHROM agar *Candida* medium were observed after 7 days of incubation at 30 °C, and their chromogenic characteristics were recorded. Yeasts grown on MYGP agar medium were analysed individually under an optical microscope (Olympus, Milan, Italy) to ascertain the presence of pseudohyphae and cell shape and size. The main characteristics suitable for distinguishing the different chromogenic yeast groups were cell shaped (round vs. elongated), the presence of pseudohyphae, the morphology and colour of the colony. To assess the survival rates of the yeast starter cultures in the brines of the industrial barrels, we assessed population size and identity of yeasts isolated from the brines after 30 and 120 days of fermentation, taking into consideration the specific homogeneous chromogenic groups of the single starter cultures as well as the other groups of wild yeasts in the brines. From each chromogenic group of each analysed sample, 10 yeast isolates were randomly selected and analysed 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 yeast species in the public gene database using a BLAST search of call GenBank + EMBL + DDBJ + PDB sequence, on the NCBI website <http://www.ncbi.nlm.nih.gov/blast>.

2.3.5. Sensory evaluation

The sensory analysis of olive samples was performed by official, fully trained, analytical tasters after 120 days of fermentation in 200 L industrial PVC barrels. Olive samples of 1500 g obtained by collection near the surface (500 g), middle (500 g), and bottom (500 g) of each barrel were analysed by the panelists in accordance with the International Olive Oil Council method (IOOC, 2011). The test was performed by a panel of 8 judges between the ages of 25–50. Each tasting section entailed the sensory analysis of no more than three samples. The judges were trained for identification of gustatory attributes (salty, bitter, acid) and for kinaesthetic attributes (hardness, fibrousness and crunchiness). Sensory data were elaborated by calculating the median 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.

2.3.6. Statistical analysis

Data are expressed as mean value \pm standard deviation. The similarity index (S.I.) between strains, refer to dimorphism (pseudohyphae production) and enzymes of the strain of each species selected for starter production. The similarity index was calculated using the Sorensen's method as follows: $S.I. = \frac{2H}{A + B}$, where H corresponds to the positive characteristics common to A and B; A, represents positive characteristics of A; B, corresponds to the positive characteristics of B (Sorensen, 1948). The Statistical software (version 7.0) was used for data processing (Statsoft for Windows, Tulsa, USA). Comparisons among means were performed with Duncan's multiple-range tests (one-way ANOVA), and differences were considered significant at $p < 0.05$.

3. Results and discussion

The use of starter cultures during spontaneous fermentation is not a common practice in the production of brined Taggiasca black olives. This type of naturally fermented table olives is produced at an industrial-scale according to traditional artisanal methods that allow the use of 160–200 L PVC barrels, where olives are preserved for several months in brine. Fermentation of the brined olives starts when the fruits are placed in the brine and continues more or less rapidly depending on external factors, such as temperature, and internal factors related to the chemical composition of the fruits and brine (Bonatsou et al., 2017).

3.1. Yeast starter production

The composition of the microbiota brines in the first months of fermentation was modified through the use of starters developed from selected yeast strains. The results of the selection tests carried out at the laboratory-scale are shown in Table 1. The yeasts selected for starter production in increasing order of enzymatic activity were as follows: *C.*

matritensis 2005, *C. diddensiae* 2011, *S. cerevisiae* 2046, *C. adriatica* 1985, *W. anomalus* 1960, and *N. molendini-olei* 2004. The tests of yeast starters grown on CHROM agar *Candida* medium allowed the identification of characteristic morphological and chromogenic groups reflecting different yeast species, whereas the strains of the same species did not differ from one another. Furthermore, *C. diddensiae* and *C. adriatica* produced pseudohyphae on MYGP agar medium. These

Table 1
Physiological characteristics of yeasts subjected to selection before preparation of the starters.

Yeast Strain	Pseudohyphae production	β -glucosidase	Esterase	Lipase	Protease	Similarity index ^b	Chromogenic group	Growth at 22 °C	Sedimentation after two days incubation at	Salt tolerance	
										G.I. (%) ^c	30 °C (%)
<i>N. molendini-olei</i>											
2004 ^a	-	+	+	-	+		smooth red	99 ± 3 ^a	45 ± 2 ^a	93 ± 4 ^a	5 ± 1 ^{bc}
2013	-	+	-	-	+	0.8	smooth red	43 ± 2 ^c	2 ± 0 ^c	1 ± 0 ^d	1 ± 0 ^d
2014	-	+	-	+	+	0.7	smooth red	52 ± 2 ^b	3 ± 0 ^c	2 ± 0 ^d	1 ± 0 ^d
2019	-	+	-	+	+	0.7	smooth red	53 ± 3 ^b	5 ± 1 ^c	8 ± 1 ^d	0
2021	-	+	-	+	+	0.7	smooth red	67 ± 5 ^b	2 ± 0 ^c	1 ± 0 ^d	0
2022	-	+	-	-	+	0.8	smooth red	63 ± 1 ^b	3 ± 0 ^c	1 ± 0 ^d	0
2023	-	+	-	-	+	0.8	smooth red	63 ± 5 ^b	12 ± 1 ^c	5 ± 1 ^d	2 ± 0 ^d
<i>C. matritensis</i>											
2005 ^a	-	+	-	-	+		smooth brown	90 ± 2 ^a	35 ± 2 ^a	100 ± 7 ^a	7 ± 1 ^{bc}
2006	-	-	-	-	+	0.7	smooth brown	71 ± 5 ^b	38 ± 4 ^a	99 ± 5 ^a	9 ± 1 ^b
2016	-	-	-	+	+	0.5	smooth brown	80 ± 3 ^{ab}	37 ± 3 ^a	100 ± 10 ^a	7 ± 1 ^{bc}
2017	-	-	-	+	+	0.5	smooth brown	44 ± 5 ^c	36 ± 2 ^a	83 ± 3 ^a	3 ± 0 ^{cd}
2018	-	-	-	+	+	0.5	smooth brown	99 ± 3 ^a	35 ± 1 ^a	98 ± 2 ^a	17 ± 2 ^a
<i>C. diddensiae</i>											
2007	+	+	+	-	-	1	bordeaux center white exterior	83 ± 4 ^{ab}	2 ± 0 ^c	53 ± 4 ^b	8 ± 2 ^b
2008	+	+	+	-	-	1	bordeaux center white exterior	88 ± 3 ^a	2 ± 0 ^c	72 ± 6 ^{ab}	4 ± 0 ^c
2011 ^a	+	+	+	-	-		bordeaux center white exterior	99 ± 5 ^a	15 ± 1 ^c	74 ± 4 ^{ab}	6 ± 1 ^{bc}
<i>C. adriatica</i>											
1985 ^a	+	+	+	+	+		dark bordeaux uniform	62 ± 3 ^b	35 ± 2 ^a	48 ± 3 ^{bc}	8 ± 1 ^b
2036	-	+	+	+	+	0.9	dark bordeaux uniform	54 ± 2 ^b	28 ± 1 ^b	41 ± 3 ^c	4 ± 0 ^c
<i>W. anomalus</i>											
1960 ^a	-	+	+	+	+		light bordeaux uniform	56 ± 2 ^b	2 ± 0 ^c	80 ± 4 ^{ab}	2 ± 0 ^d
2032	-	+	+	+	+	1	light bordeaux uniform	43 ± 5 ^c	3 ± 0 ^c	72 ± 3 ^{ab}	2 ± 0 ^d
<i>S. cerevisiae</i>											
2046 ^a	-	+	+	-	+		intense bordeaux	52 ± 5 ^b	45 ± 2 ^a	5 ± 1 ^d	1 ± 0 ^d
2070	-	+	+	-	+	1	intense bordeaux	47 ± 3 ^{bc}	43 ± 1 ^a	5 ± 2 ^d	1 ± 0 ^d

^a Yeast strain selected for starter preparation.

^b Sorensen's index of similarity refer to the dimorphism and the enzymes of the strain of yeast species selected as starter.

^c Growth Index. Data are reported as mean and standard deviation obtained from triplicate trials. Different letter in the same column indicate significant differences by Duncan's multiple-range tests ($p < 0.05$). +, positive; -, negative.

Table 2
Production of starters in the laboratory with selected yeasts.

Starter	Biomass produced in MYGP medium (g per Liter)	Biomass produced in low-cost medium (g per Liter)	% compared to MYGP	Biomass recovery System medium	Yeast content of the starter used as inoculum (Log CFU/mL)
<i>N. molendini-olei</i> 2004	1.95 ± 0.10 ^{ab}	1.05 ± 0.07 ^{ab}	54	Sedimentation	8.20 ± 0.20
<i>C. matritensis</i> 2005	1.34 ± 0.22 ^b	0.80 ± 0.05 ^b	59	Sedimentation	8.30 ± 0.14
<i>C. diddensiae</i> 2011	2.20 ± 0.31 ^b	1.20 ± 0.09 ^{ab}	55	Centrifugation	8.70 ± 0.20
<i>C. adriatica</i> 1985	1.72 ± 0.10 ^b	1.30 ± 0.13 ^{ab}	76	Sedimentation	8.80 ± 0.15
<i>W. anomalus</i> 1960	3.22 ± 0.18 ^a	1.70 ± 0.19 ^a	53	Centrifugation	8.00 ± 0.33
<i>S. cerevisiae</i> 2046	2.50 ± 0.20 ^{ab}	1.40 ± 0.10 ^{ab}	56	Sedimentation	8.80 ± 0.16

Data are reported as mean and standard deviation obtained from triplicate trials. Different letter in the same column indicate significant differences by Duncan's multiple-range tests ($p < 0.05$).

Table 3
Yeast and bacteria population sizes in the brine of Taggiasca black olives after 30 days of fermentation.

Starter	Initial yeast population ^a			8% NaCl			12% NaCl			12% NaCl with 0.3% citric acid		
	Yeast	LAB ^b	TAB ^c	Yeast	LAB	TAB	Yeast	LAB	TAB	Yeast	LAB	TAB
<i>N. molendini-olei</i> 2004	5.70 ± 0.40	5.25 ± 0.15 ^a	5.80 ± 0.13 ^b	5.96 ± 0.21 ^a	5.25 ± 0.15 ^a	5.80 ± 0.13 ^b	5.90 ± 0.15 ^a	3.40 ± 0.20	4.90 ± 0.10 ^b	6.10 ± 0.11 ^a	-	3.20 ± 0.10 ^b
<i>C. matritensis</i> 2005	5.50 ± 0.15	5.70 ± 0.21 ^a	6.12 ± 0.19 ^{ab}	5.55 ± 0.07 ^a	5.70 ± 0.21 ^a	6.12 ± 0.19 ^{ab}	5.70 ± 0.10 ^a	3.90 ± 0.10	5.00 ± 0.11 ^{ab}	5.80 ± 0.10 ^a	-	3.60 ± 0.09 ^{ab}
<i>C. diddensiae</i> 2011	5.80 ± 0.22	5.10 ± 0.17 ^a	6.50 ± 0.15 ^a	6.17 ± 0.12 ^a	5.10 ± 0.17 ^a	6.50 ± 0.15 ^a	6.70 ± 0.06 ^a	3.00 ± 0.15	5.40 ± 0.17 ^{ab}	6.00 ± 0.12 ^a	-	4.00 ± 0.08 ^{ab}
<i>C. adriatica</i> 1985	6.00 ± 0.30	5.96 ± 0.25 ^a	6.00 ± 0.12 ^{ab}	5.96 ± 0.25 ^a	5.20 ± 0.09 ^a	6.00 ± 0.12 ^{ab}	6.50 ± 0.07 ^a	3.85 ± 0.12	5.80 ± 0.09 ^a	5.90 ± 0.11 ^a	-	3.50 ± 0.12 ^{ab}
<i>W. anomalous</i> 1960	5.30 ± 0.40	5.89 ± 0.10 ^a	6.80 ± 0.08 ^a	5.89 ± 0.10 ^a	5.90 ± 0.22 ^a	6.80 ± 0.08 ^a	6.10 ± 0.07 ^a	4.00 ± 0.18	5.00 ± 0.10 ^{ab}	6.12 ± 0.17 ^a	-	3.00 ± 0.22 ^{ab}
<i>S. cerevisiae</i> 2046	5.60 ± 0.32	6.25 ± 0.21 ^a	5.80 ± 0.09 ^b	6.25 ± 0.21 ^a	5.98 ± 0.13 ^a	5.80 ± 0.09 ^b	5.90 ± 0.14 ^a	3.80 ± 0.17	5.20 ± 0.14 ^{ab}	6.00 ± 0.12 ^a	-	4.10 ± 0.15 ^a
Control	1.20 ± 0.52	5.02 ± 0.07 ^b	5.70 ± 0.15 ^b	5.02 ± 0.07 ^b	4.10 ± 0.28 ^b	5.70 ± 0.15 ^b	4.80 ± 0.10 ^b	3.20 ± 0.09	5.10 ± 0.16 ^{ab}	5.05 ± 0.11 ^b	-	3.80 ± 0.13 ^{ab}
x ± sd		5.82 ± 0.42	6.10 ± 0.41	5.82 ± 0.42	5.32 ± 0.64	6.10 ± 0.41	5.94 ± 0.62	3.59 ± 0.39	5.20 ± 0.31	5.85 ± 0.37	-	3.60 ± 0.40

^a Yeast population size in the brine at the beginning of fermentation.^b LAB, Lactic Acid Bacteria.^c TAB, Total Aerobic Bacteria. Data are reported as mean and standard deviation of Log CFU mL⁻¹ obtained from triplicate trials. Different letter in the same column indicate significant differences by Duncan's multiple-range tests (p < 0.05).

characteristics, along with others, such as the size and shape of cells detected under the microscope, were selective markers capable of distinguishing thousands of colonies of the selected yeast starters that survived in the brine after inoculation of the 200 L PVC barrels. Cell sedimentation that permitted the recovery of the yeast biomass directly from the medium occurred for all of the *C. matritensis* and *S. cerevisiae* strains and only some of the *N. molendini-olei* and *C. adriatica* strains. This result was confirmed during starter preparation (Table 2). The yield of the yeast biomass to be used for the production of starters was higher for *W. anomalous* 1960 than for all other strains in both media, whereas the lowest yields were recorded for *C. matritensis* 2005. The results listed in Table 2 indicate that all of the tested yeast starters grew quite well on the low-cost medium, with yields of 50% or higher than those obtained with the MYGP medium. These results are interesting from an economic point of view, since the cost of the low-cost medium is approximately 1/40th that of the MYGP medium (data not shown). The use of appropriate low-cost medium, which was associated with simplified biomass recovery through sedimentation, can provide significant reductions in time and production costs of starters, which hinder the spread of starters for the inoculation of brined Taggiasca black table olives.

3.2. Industrial-scale fermentation of black table olives in barrels at 30 days

In the initial phase of testing, the size of the yeast population ranged from 5.3 Log CFU per mL to 6.0 Log CFU per mL in the brines inoculated with the starters and 1.2 Log CFU per mL in the uninoculated control (Table 3). During the first month of fermentation the number of indigenous yeasts in the brine of the control increased significantly, reaching values close to those of the inoculated brines. The microbiological analysis of the brines performed after 30 days of fermentation showed a different concentration of yeasts, LAB and TAB depending on the chemical composition of the brines and the type of starter used, while the *Enterobacteriaceae* was absent in all samples. The use of starters caused a significant increase (p < 0.05) in the total number of yeasts in those brines treated with starters compared to the uninoculated-control brines regardless of salt concentration (Table 3). LABs were significantly more numerous in the starter-treated brines with 8% NaCl than in the other brines and in the uninoculated control brines. The discovery that LAB concentration was highest in the starter-treated brine with 8% NaCl might have been due to greater numbers of yeasts in this treatment than in the control treatment that produce vitamins, amino acids and other compounds that promote the growth of LAB (Segovia Bravo et al., 2007; Viljoen, 2006). The inhibition of LAB growth in the brine with 12% NaCl and acidified with citric acid agree with the results reported by Romeo et al. (2010) for the Moresca variety, which is processed in the same manner as the Taggiasca variety. On the other hand, Landete Irazo et al. (2010) demonstrated that diffused olive phenols exerted a significant inhibitory effect on LAB during brining when they were associated with NaCl. Unlike LAB numbers, TAB numbers in the brine after 30 days of fermentation was reduced by approximately 40% only in the brine with 12% of NaCl and acidified with citric acid (Table 3). The results of the microbiological analyses, shown in Fig. 1, demonstrated the survival of the yeast starters in the brines after 30 days of fermentation, with a minimum of 15% (*C. matritensis* 2005) to a maximum of 68% (*W. anomalous* 1960). *N. molendini-olei* 2004 was negatively influenced by a high concentration of NaCl, ranging in prevalence from 65% (8% NaCl) to approximately 20–31% (12% NaCl), consistent with the results of the selection tests shown in Table 1. The other yeast starters were not significantly influenced by NaCl concentration and increased in prevalence in the following order: *S. cerevisiae* 2046 (30–33%), *C. diddensiae* 2011 (52–63%), *C. adriatica* 1985 (54–60%), and *W. anomalous* 1960 (40–68%).

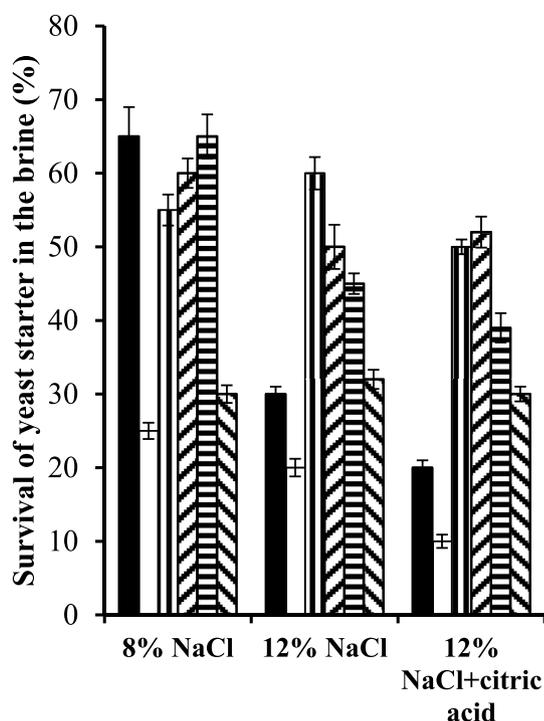


Fig. 1. Survival of starters inoculated in the brine of Taggiasca black olives after 30 days of fermentation. (■), *N. molendini-olei*; (□), *C. matritensis*; (▨), *C. diddensiae*; (▧), *C. adriatica*; (▩), *W. anomalus*; (▪), *S. cerevisiae*.

3.3. Microbiota characteristics after 120 days of processing black brined olives

The yeast population size after 120 days of fermentation had increased slightly from that recorded after 30 days in all brines regardless of starter treatment. As shown in Table 4, the average yeast population size ranged from 6.01 Log CFU mL⁻¹ in the brine with 12% NaCl to 6.64 Log CFU mL⁻¹ in that with 8% salt. Since the LABs, at this phase of fermentation had disappeared from all the brines, the yeasts coexisted with TABs in the brines containing 8 and 12% NaCl, whereas in the brine with 12% NaCl it acidified with citric acid, the TAB had disappeared and yeasts were the only surviving evaluated microorganisms. As demonstrated by Tassou et al. (2002) and Bautista-Gallego et al. (2011), the reduction of the LAB number after 30 days of fermentation and subsequently of the TAB number is linked not only to the high salt concentration but also to the higher concentration of polar phenolic compounds from the drupe, which does not seem to influence the yeast population. However, although the total number of yeasts was similar across all the brines, the composition of the yeast microbiota varied with the chemical composition of the brine and the type of starter used. From the results shown in Fig. 2 it is possible to note how in the fermentation conducted after 120 days and under three different conditions of salt and presence or not of citric acid, some yeast starters disappeared from the brine while others survived. The yeast starters with the best survival capacity in all conditions were *C. diddensiae*, *C. adriatica*, and *W. anomalus*. In the brines with 8% NaCl the predominance of the starters over the spontaneous yeasts, in increasing order of abundance, were as follows: *C. adriatica* 1985 (40%), *W. anomalus* 1960 (42%), and *C. diddensiae* 2011 (45%) (Fig. 2A). In contrast, *N. molendini-olei* 2004 was absent from the brine with 12% NaCl (Fig. 2B), and both *N. molendini-olei* 2004 and *C. matritensis* 2005 were absent from the brines with 12% NaCl acidified with citric acid (Fig. 2C). In the salt-rich acidified brine, the predominant yeasts, in increasing order of abundance, were as follows: *S. cerevisiae* 2046 (7%), *W. anomalus* 1960 (16%), *C. adriatica* 1985 (28%) and *C. diddensiae*

Table 4
Yeast and bacteria population sizes in brine and olives of Taggiasca after 120 days of fermentation.

Starter	8% NaCl						12% NaCl						12% NaCl with 0.3% citric acid						
	Yeast			TAB			Yeast			TAB			Yeast			TAB			
	Brine ^a	Olive ^b		Brine	Olive		Brine	Olive		Brine	Olive		Brine	Olive		Brine	Olive		
<i>N. molendini-olei</i> 2004	6.70 ± 0.30	5.50 ± 0.37	5.36 ± 0.30	5.50 ± 0.20	5.50 ± 0.30	5.10 ± 0.10	3.36 ± 0.10	3.10 ± 0.21	6.10 ± 0.10	5.45 ± 0.32	-	6.10 ± 0.10	5.45 ± 0.32	-	6.10 ± 0.10	5.45 ± 0.32	-	6.10 ± 0.10	5.45 ± 0.32
<i>C. matritensis</i> 2005	6.70 ± 0.10	5.60 ± 0.45	5.01 ± 0.20	5.30 ± 0.27	6.40 ± 0.20	5.10 ± 0.32	4.10 ± 0.20	4.00 ± 0.30	6.90 ± 0.20	5.80 ± 0.20	-	6.90 ± 0.20	5.80 ± 0.20	-	6.90 ± 0.20	5.80 ± 0.20	-	6.90 ± 0.20	5.80 ± 0.20
<i>C. diddensiae</i> 2011	6.60 ± 0.20	5.10 ± 0.20	5.64 ± 0.30	5.30 ± 0.10	6.30 ± 0.35	4.90 ± 0.22	4.13 ± 0.40	4.10 ± 0.10	6.60 ± 0.06	5.75 ± 0.16	-	6.60 ± 0.06	5.75 ± 0.16	-	6.60 ± 0.06	5.75 ± 0.16	-	6.60 ± 0.06	5.75 ± 0.16
<i>C. adriatica</i> 1985	7.00 ± 0.06	5.90 ± 0.50	5.10 ± 0.20	5.70 ± 0.22	5.50 ± 0.10	5.00 ± 0.20	3.25 ± 0.10	3.40 ± 0.10	6.70 ± 0.20	5.70 ± 0.30	-	6.70 ± 0.20	5.70 ± 0.30	-	6.70 ± 0.20	5.70 ± 0.30	-	6.70 ± 0.20	5.70 ± 0.30
<i>W. anomalus</i> 1960	6.40 ± 0.10	5.10 ± 0.25	4.90 ± 0.30	5.40 ± 0.19	6.00 ± 0.20	5.30 ± 0.36	3.92 ± 0.20	3.80 ± 0.22	6.60 ± 0.20	5.50 ± 0.45	-	6.60 ± 0.20	5.50 ± 0.45	-	6.60 ± 0.20	5.50 ± 0.45	-	6.60 ± 0.20	5.50 ± 0.45
<i>S. cerevisiae</i> 2046	6.60 ± 0.10	5.50 ± 0.15	5.05 ± 0.20	5.20 ± 0.37	6.30 ± 0.10	5.00 ± 0.17	4.02 ± 0.10	3.87 ± 0.10	6.40 ± 0.35	5.10 ± 0.22	-	6.40 ± 0.35	5.10 ± 0.22	-	6.40 ± 0.35	5.10 ± 0.22	-	6.40 ± 0.35	5.10 ± 0.22
Control	6.50 ± 0.25	5.30 ± 0.10	5.05 ± 0.06	5.00 ± 0.35	5.70 ± 0.20	5.20 ± 0.20	3.43 ± 0.30	3.10 ± 0.10	6.60 ± 0.06	5.42 ± 0.15	-	6.60 ± 0.06	5.42 ± 0.15	-	6.60 ± 0.06	5.42 ± 0.15	-	6.60 ± 0.06	5.42 ± 0.15
x ± sd	6.64 ± 0.19	5.43 ± 0.29	5.16 ± 0.25	5.34 ± 0.22	6.01 ± 0.34	5.08 ± 0.13	3.74 ± 0.38	3.62 ± 0.42	6.56 ± 0.25	5.53 ± 0.24	-	6.56 ± 0.25	5.53 ± 0.24	-	6.56 ± 0.25	5.53 ± 0.24	-	6.56 ± 0.25	5.53 ± 0.24

Data are reported as mean and standard deviation obtained from triplicate trials; ^a Log CFU mL⁻¹; ^b Log CFU g⁻¹ pulp.

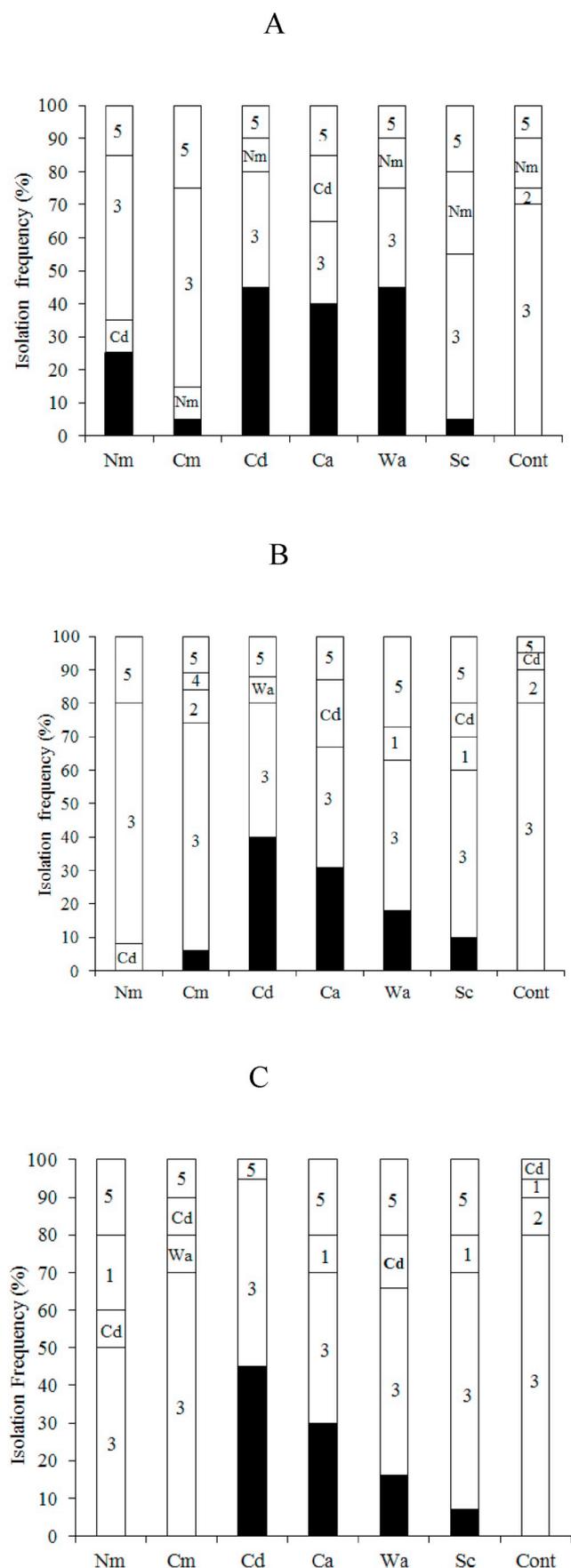


Fig. 2. Isolation frequency of yeasts from brines of Taggiasca black olives after 120 days of fermentation. A, Brines with 8% NaCl; B, Brines with 12% NaCl; C, Brines with 12% NaCl acidified. Nm, *N. molendini-olei*; Cm, *C. matritensis*; Cd, *C. diddensiae*; Ca, *C. adriatica*; Wa, *W. anomalus*; Sc, *S. cerevisiae*; Cont, Uninoculated control. 1, *Candida boidinii*; 2, *Candida auringsensis*, 3, *Pichia manshurica*; 4, *Meyerozyma guilliermondii*; 5 undetermined minor species. The black histogram represents the corresponding yeast starter.

2011 (45%) (Fig. 2C). Considering the survival rate of the above starters in all the conditions tested, after 120 days of fermentation, the best combination yeast starter-fermentation conditions, in increasing order, was as follows: *S. cerevisiae* 2046 (7–13%), *W. anomalus* 1960 (16–50%), *C. adriatica* 1985 (28–40%), and *C. diddensiae* 2011 (45–48%). *C. diddensiae* together with other species of the genus *Candida* was reported to occur at high frequency in the early phases of fermentation in several experimentations (Bautista-Gallego et al., 2011). In general, this species is described as a strong fermentative yeast that adapts well to the first fermentation stage (Hurtado et al., 2008; Nisiotou et al., 2010). Information on *C. adriatica* is scarce as this species was discovered only a few years ago in olive oil and subsequently isolated from olives of the Leccino variety (Čadež et al., 2012). *W. anomalus* has been detected in several olive fermentations, and Hernández et al. (2007) reported the relevance of this species in table olive fermentation as a starter culture, with it adapting well to the environmental conditions that govern table olive fermentation. The results in Fig. 2 agree with these previous findings demonstrating the presence of *C. diddensiae* and *W. anomalus* in the brines, specifically brines with 12% NaCl (Fig. 2 B.C). Some of the yeast species used as starters in this study were also found as wild contaminants in brines inoculated with other yeast species. Controls were also contaminated in this way. In fact, *N. molendini-olei* was isolated as contaminant only from the brine with 8% (w/v) NaCl (Fig. 2A) while *C. diddensiae* and *W. anomalus* yeast species contaminated also some brines with 12% (w/v) NaCl and 12% (w/v) NaCl acidified with citric acid (Fig. 2). The results demonstrated that NaCl-rich Taggiasca brines (12%) represent a suitable habitat for the growth of *C. diddensiae*, *C. adriatica*, and *W. anomalus* yeast species, while *N. molendini-olei* seem to be favored by brines with a lower salt content. However, as shown in Fig. 2, in the starter-treated brines with 12% NaCl, the predominance of each best-performing yeast starters was significantly reduced during the first 120 days of fermentation, whereas the numbers of autochthonous species, represented mainly by *Pichia manshurica*, increased. This wild yeast showed a predominance of 70–80% in the control brine regardless of salt concentration. However, in brines treated with starter, this yeast reached the same level of predominance as recorded for the control brine only in those cases where the yeast starters did not survive (Fig. 2). *P. manshurica* is considered a halophilic yeast and is observed at low concentrations in naturally brined Aloreña green table olives and natural black Kalamata and Conservolea olives (Abriouel et al., 2011; Bonatsou et al., 2017; Nisiotou et al., 2010). Golomb et al. (2013) isolated polygalacturonase-producing strains of *P. manshurica* from spoiled olives that are responsible for olive softening in the directly brined, naturally fermented, colossal Sevillian olives. However, *P. manshurica* has been reported to significantly improve the flower and fruit notes of brewing and other traditional Chinese liquors (Zhang et al., 2017). The high presence of *P. manshurica* has so far not been reported in the brine of other varieties of table olives. The high competitiveness of wild yeast development, such as *P. manshurica*, in the brines of the Taggiasca variety, seems to limit the use of starters. However, if selected starters are used to improve the competitiveness of yeasts and some important biochemical processes, such as the rapid debittering process of the fruits in the initial phase of fermentation, the

Table 5
Salt, total acidity and pH of the brines after 120 days of fermentation.

Starter	Salt			Total acidity ^a			pH		
	8%	12%	12% + citric acid	8%	12%	12% + citric acid	8%	12%	12% + citric acid
<i>N.molendini-olei</i> 2004	6.43 ± 0.30 ^a	7.60 ± 0.60 ^b	8.13 ± 0.30	5.14 ± 0.12 ^a	4.18 ± 0.21	5.61 ± 0.18	4.32 ± 0.12 ^b	4.37 ± 0.15	4.25 ± 0.21
<i>C.matritensis</i> 2005	5.06 ± 0.15 ^b	7.90 ± 0.40 ^b	7.78 ± 0.22	5.24 ± 0.55 ^a	4.28 ± 0.30	5.62 ± 0.40	4.35 ± 0.18 ^b	4.37 ± 0.15	4.23 ± 0.18
<i>C.diddensiae</i> 2011	5.50 ± 0.22 ^b	7.66 ± 0.60 ^b	7.90 ± 0.12	4.88 ± 0.70 ^{ab}	4.04 ± 0.20	5.55 ± 0.27	4.36 ± 0.15 ^b	4.39 ± 0.22	4.27 ± 0.16
<i>C.adriatica</i> 1985	5.60 ± 0.20 ^b	9.70 ± 0.62 ^a	7.02 ± 0.40	5.16 ± 0.32 ^a	4.36 ± 0.70	5.53 ± 0.30	4.35 ± 0.20 ^b	4.40 ± 0.10	4.24 ± 0.15
<i>W.anomalus</i> 1960	5.85 ± 0.32 ^b	8.95 ± 0.71 ^{ab}	8.07 ± 0.27	5.15 ± 0.10 ^a	4.38 ± 0.42	5.61 ± 0.20	4.33 ± 0.15 ^b	4.42 ± 0.15	4.20 ± 0.12
<i>S.cerevisiae</i> 2046	5.87 ± 0.15 ^b	7.84 ± 0.42 ^b	7.84 ± 0.25	5.16 ± 0.14 ^a	4.16 ± 0.40	5.49 ± 0.20	4.35 ± 0.10 ^b	4.38 ± 0.22	4.29 ± 0.17
Control	5.55 ± 0.18 ^b	9.60 ± 0.50 ^a	7.66 ± 0.31	4.94 ± 0.80 ^b	4.34 ± 0.78	5.35 ± 0.20	4.42 ± 0.12 ^a	4.34 ± 0.20	4.27 ± 0.16
x ± sd	5.68 ± 0.42	8.46 ± 0.92	7.77 ± 0.37	5.09 ± 0.13	4.24 ± 0.12	5.53 ± 0.09	4.35 ± 0.03	4.38 ± 0.03	4.25 ± 0.03

^a The total acidity refers to g of lactic acid per 1000 mL. Data are reported as mean and standard deviation obtained from triplicate trials. Different letter in the same column indicate significant differences by Duncan's multiple-range tests ($p < 0.05$).

lack of high starter prevalence over lengthy periods is no longer a problem.

3.4. Physicochemical changes

After 120 days of fermentation, the salt concentration reached stable levels in all the brines with initial NaCl concentrations of 8 and 12% (w/v) NaCl, with average values of 6 and 8% of NaCl, respectively which, as previously reported, limited the numbers of LAB and TAB (Table 5). The higher total acidity and the lower pH showed by the brines with 8% NaCl, can be attributed to the acidification caused by the higher numbers of LAB compared to those of brines with 12% NaCl (Tables 3 and 5). In a previous study (Bleve et al., 2015), Conservolea and Kalamata olives were fermented naturally, and the minimum pH value at the end of fermentation was 4.20–4.30, consistent with the pH values reported in this work. However, in fermentation carried out mainly by yeasts, acidification of 12% NaCl brine with 0.3% (w/v) citric acid seemed to provide the best results. Table 5 shows that the final values of pH and total acidity are within the limits of the standard trade for table olive oil (IOOC, 2004) of 4.30. Regarding phenolic compounds, after 120 days of fermentation, the total concentration of polar phenolic compounds was lower in the brines treated with the yeast starters *C. adriatica* 1985, *C. diddensiae* 2011, and *W. anomalus* 1960 than in the other brines (Table 6). The sensory profile of Taggiasca black olives processed for 120 days in industrial-barrels is reported in

Table 6
Total polar phenols of brined Taggiasca black table olives after 120 days of fermentation.

Starter	8% NaCl		12% NaCl		12% NaCl + 0.3% citric acid	
	Brine ^a	Mesocarp ^b	Brine	Mesocarp	Brine	Mesocarp
<i>N. molendini-olei</i> 2004	3.21 ± 0.08 ^a	6.72 ± 0.22 ^{ab}	3.15 ± 0.13 ^a	5.43 ± 0.32 ^a	3.34 ± 0.17 ^{ab}	6.75 ± 0.35 ^a
<i>C. matritensis</i> 2005	3.04 ± 0.11 ^{ab}	8.90 ± 0.16 ^a	2.64 ± 0.07 ^{ab}	5.90 ± 0.15 ^a	4.01 ± 0.07 ^a	6.70 ± 0.18 ^a
<i>C. diddensiae</i> 2011	2.59 ± 0.07 ^b	5.45 ± 0.00 ^b	2.50 ± 0.08 ^b	4.93 ± 0.22 ^b	2.62 ± 0.04 ^b	4.62 ± 0.13 ^b
<i>C. adriatica</i> 1985	2.04 ± 0.18 ^b	4.80 ± 0.18 ^b	2.39 ± 0.09 ^b	4.65 ± 0.16 ^b	2.84 ± 0.11 ^b	4.90 ± 0.15 ^b
<i>W. anomalus</i> 1960	2.28 ± 0.03 ^b	4.22 ± 0.13 ^b	2.43 ± 0.02 ^b	4.52 ± 0.12 ^b	2.73 ± 0.06 ^b	4.83 ± 0.32 ^b
<i>S. cerevisiae</i> 2046	3.03 ± 0.16 ^{ab}	6.65 ± 0.00 ^{ab}	2.71 ± 0.01 ^{ab}	6.81 ± 0.13 ^a	3.41 ± 0.05 ^{ab}	7.10 ± 0.21 ^a
Control	3.10 ± 0.19 ^a	6.80 ± 0.15 ^{ab}	2.90 ± 0.05 ^a	7.44 ± 0.18 ^a	3.94 ± 0.07 ^a	7.55 ± 0.08 ^a
x ± sd	2.76 ± 0.45	6.22 ± 1.55	2.67 ± 0.27	5.67 ± 1.11	3.27 ± 0.56	6.10 ± 1.23

^a mg caffeic acid/mL.

^b mg caffeic acid/g. Data are reported as mean and standard deviation obtained from triplicate trials. Different letter in the same column indicate significant differences by Duncan's multiple-range tests ($p < 0.05$).

Table 7. Significant differences among treatments was observed for only some parameters, such as bitter, salty and hardness, while no significant statistic difference was found for the remaining sensory attributes. The intensity of bitter taste compared to the control, was significantly ($p < 0.05$) lower for the fruits processed with the yeast starters *C. diddensiae* 2011, *C. adriatica* 1985, and *W. anomalus* 1960, regardless of salt concentration. The values of bitter attribute were in accordance with the low concentration of the total polar phenols found in this olive mesocarp (Table 6). The salty flavor of the fruits, in accordance also with the chemical analysis of the brines reported in Table 5, compared to the brine with 8% NaCl, was slightly higher for the olives processed with 12% NaCl. The hardness parameter was high in all samples, with some small variations depending on the starter or the NaCl concentration of the brines. No explicit defects were detected in any of the samples and on the basis of the Defect Predominantly Perceived (DPP), the product was classified in the standard category (IOOC, 2011) (data not shown).

4. Conclusions

The trials showed the positive roles played by some selected yeast starter cultures in the initial phase of fermentation of the brined Taggiasca black table olives, processed in industrial-scale. The use of low-cost mediums and the simplification of the recovery of cell biomass, through sedimentation, indicated the possibility of significantly

Table 7
Sensory attributes of brined Taggiasca table olives after 120 days of fermentation.

Starter	8% NaCl					12% NaCl					12% NaCl + 0.3% citric acid							
	Bitter ^a	Acid	Salty	Hardness	Fibrousness	Crunchiness	Bitter	Acid	Salty	Hardness	Fibrousness	Crunchiness	Bitter	Acid	Salty	Hardness	Fibrousness	Crunchiness
<i>N. molenndini-olei</i> 2004	6 ± 0.5 ^a	4 ± 0.3	3 ± 0.1	5 ± 0.3 ^b	5 ± 0.4	5 ± 0.7	7 ± 0.4 ^a	5 ± 0.3	5 ± 0.2	5 ± 0.4 ^b	6 ± 0.3	5 ± 0.6	5 ± 0.5 ^a	5 ± 0.2	6 ± 0.3	6 ± 0.2	6 ± 0.5	6 ± 0.3
<i>C. maritensis</i> 2005	5 ± 0.2 ^a	4 ± 0.4	3 ± 0.3	6 ± 0.3 ^a	5 ± 0.5	6 ± 0.3	6 ± 0.4 ^a	5 ± 0.4	5 ± 0.3	6 ± 0.3 ^a	6 ± 0.4	6 ± 0.4	5 ± 0.4 ^a	5 ± 0.3	5 ± 0.3	6 ± 0.1	6 ± 0.5	6 ± 0.4
<i>C. diddensiae</i> 2011	2 ± 0.4 ^b	4 ± 0.2	2 ± 0.1	6 ± 0.2 ^a	6 ± 0.4	6 ± 0.4	3 ± 0.2 ^b	4 ± 0.3	5 ± 0.3	6 ± 0.3 ^a	5 ± 0.7	6 ± 0.4	3 ± 0.2 ^b	5 ± 0.2	5 ± 0.4	6 ± 0.4	5 ± 0.7	6 ± 0.5
<i>C. adriatica</i> 1985	3 ± 0.3 ^b	5 ± 0.3	3 ± 0.1	6 ± 0.1 ^a	5 ± 0.3	6 ± 0.4	3 ± 0.3 ^b	4 ± 0.2	4 ± 0.2	6 ± 0.2 ^a	6 ± 0.4	6 ± 0.5	2 ± 0.3 ^b	4 ± 0.3	5 ± 0.3	6 ± 0.2	6 ± 0.2	6 ± 0.3
<i>W. anomalous</i> 1960	2 ± 0.2 ^b	4 ± 0.3	2 ± 0.2	6 ± 0.1 ^a	6 ± 0.6	6 ± 0.5	2 ± 0.4 ^c	4 ± 0.3	4 ± 0.2	6 ± 0.4 ^a	6 ± 0.2	6 ± 0.3	2 ± 0.2 ^b	5 ± 0.4	6 ± 0.2	6 ± 0.2	6 ± 0.2	6 ± 0.4
<i>S. cerevisiae</i> 2046	6 ± 0.2 ^a	4 ± 0.5	3 ± 0.1	5 ± 0.2 ^b	6 ± 0.5	5 ± 0.8	6 ± 0.5 ^a	4 ± 0.4	5 ± 0.3	5 ± 0.2 ^b	6 ± 0.3	5 ± 0.6	5 ± 0.4 ^a	4 ± 0.2	5 ± 0.3	6 ± 0.2	5 ± 0.7	6 ± 0.5
Control	6 ± 0.4 ^a	5 ± 0.4	3 ± 0.2	5 ± 0.3 ^b	6 ± 0.4	6 ± 0.3	7 ± 0.5 ^a	4 ± 0.4	4 ± 0.1	4 ± 0.2 ^{ab}	5 ± 0.6	5 ± 0.7	6 ± 0.5 ^a	5 ± 0.2	5 ± 0.4	5 ± 0.3	6 ± 0.4	6 ± 0.3

^a Median of the values. Different letter in the same column indicate significant differences by Duncan's multiple-range tests ($p < 0.05$).

lowering the production costs of some yeast starters. The selected yeast starter cultures named *C. diddensiae* 2011, *C. adriatica* 1985 and *W. anomalous* 1960 showed the best performance in terms of survival and competitiveness towards wild yeasts of the brines, respectively during the first 30 and 120 days of fermentation. The physicochemical and sensorial analysis of the fruits assessed after 120 days of fermentation, suggest a potential positive role played by these yeasts during the initial phase of the debittering process of the Taggiasca table olive. Considering the combination of the three best selected yeast starters and the fermentation conditions, the best indication occurred with the brines containing 12% NaCl acidified with citric acid. Tolerance towards the high salt concentration and low pH shown by these yeast starters makes it possible to improve not only the efficiency of the Taggiasca debittering process, but also to safeguard the product under hygienic conditions, respecting the safety precautions suggested by the IOOC (2011).

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