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# Monitoring the yeasts ecology and volatiles profile throughout the spontaneous fermentation of Taggiasca cv. table olives through culture-dependent and independent methods

Chiara Traina [a], Ilario Ferrocino [a], Ambra Bonciolini [a], Vladimiro Cardenia [a], Xinping Lin [b], Kalliopi Rantsiou [a], Luca Cocolin [a,*]

[a] Department of Agricultural, Forest and Food Science (DISAFA), University of Torino, Grugliasco, Torino 10095, Italy
[b] Biotechnology National Engineering Research Center of Seafood, School of Food Science and Technology, Dalian Polytechnic University, Dalian 116034, PR China

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ABSTRACT

Taggiasca table olives are typical of Liguria, a Northwestern Italian region, produced with a spontaneous fermentation carried out by placing the raw drupes directly into brine with a salt concentration of 8–12 % w/v. Such concentrations limit the development of unwanted microbes and favor the growth of yeasts. This process usually lasts up to 8 months. Yeasts are found throughout the entire fermentation process and they are mainly involved in the production of volatile organic compounds, which strongly impact the quality of the final product. The aim of this study was to evaluate the dynamics of autochthonous yeasts in brines and olives in a spontaneous process with no lye pre-treatment or addition of acids in the fermenting brine with 10 % NaCl (w/v) in two batches during 2021 harvest. Three hundred seventy-three yeast colonies were isolated, characterized by rep-PCR and identified by the D1/D2 region of the 26S rRNA gene sequencing. Mycobiota was also studied by 26S rRNA gene metataxonomics, while metabolome was assessed through GC–MS analysis. Traditional culture-dependent methods showed the dominance of *Candida diddensiae*, *Wickerhamomyces anomalus*, *Pichia membranifaciens* and *Aureobasidium pullulans*, with differences in species distribution between batches, sampling time and type of sample (olives/brines). Amplicon-based sequencing confirmed the dominance of *W. anomalus* in batch 1 throughout the entire fermentation, while *Cyteromyces nyonsensis* and *Aureobasidium* spp. were most abundant in the fermentation in batch 2. Volatilome results were analyzed and correlated to the mycobiota data, confirming differences between fermentation stages. Given the high appreciation for this traditional food, this study helps elucidate the mycobiota associated to Taggiasca cv. table olives and its relationship with the quality of the final product.

## 1. Introduction

Taggiasca olive cultivar is typically produced in the Liguria region in the provinces of Imperia and Savona (North-West Italy), where it is cultivated for the production of both table olives and extra-virgin olive oil (EVOO) (Cecchi et al., 2023; Amelio and Demuro, 2000; Senizza et al., 2023). Although Taggiasca olives are not acknowledged of a PDO (Protected Denomination of Origin) certification, this product is considered of high-quality, as well as traditional and quite costly (Aceto et al., 2019; Rellini et al., 2022; Senizza et al., 2023). Its uniqueness and intrinsic value are conferred by the climatic conditions and the soil of the Ligurian Riviera, the type of cultivation taking place on terraces, where olive trees grow up to 600 a.s.l., but also by the distinctive flavor and fruit color which varies from dark black to brown with green and purple shades (Senizza et al., 2023). Traditionally, Taggiasca table olives are produced with the Greek-style fermentation method, a spontaneous process that consists of immersing the fruits into the brine with no lye pre-treatment. In general, olives are harvested when they turn black, they are sorted, rinsed with water, and placed into polyvinyl chloride (PVC) or polyethylene (PE) plastic barrels (160–200 L). Freshly prepared brine with a salt content of 8–12 % (w/v) is added to the barrels until coverage and olives are brined until their bitterness is partially or totally lost (Ciafardini and Zullo, 2019; Corsetti et al., 2012; Tofalo et al., 2013). For safety reasons, pH can be lowered by adding lactic or citric

* Corresponding author at: Department of Agricultural, Forest and Food Science (DISAFA), University of Torino, Italy.
*E-mail address:* lucasimone.cocolin@unito.it (L. Cocolin).

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acid to the brine, but practices may vary according to the company (Cecchi et al., 2023). The fermentation period is influenced by factors such as cultivar, temperature, salt concentration and physico-chemical conditions and can last for up to 8 months. Then, they are transferred into jars filled with fresh brine and pasteurized (Bleve et al., 2014; Cocolin et al., 2013; De Angelis et al., 2015; Tofalo et al., 2012). During the fermentation, barrels can be kept outdoors or maintained at room temperature. Enzymatic activities of the autochthonous microorganisms lead to the debittering of the fruits and the improvement of the overall sensory qualities (Cecchi et al., 2023; Tofalo et al., 2013). Fermented olives are characterized by a complex consortium of microorganisms, with lactic acid bacteria (LAB) and yeasts being the main representatives (Arroyo-López et al., 2008; Tofalo et al., 2013). However, specific environmental conditions such as low temperatures (<18 °C), high salt concentration (>8 %) and a limited content of reducing sugars in the brine can favor the growth of yeasts over LAB (Botta and Cocolin, 2012). Previous studies showed that the prevailing genera found in table olives are *Saccharomyces*, *Pichia*, *Debaryomyces* and *Candida* (Arroyo-López et al., 2008), however other species may be associated to spontaneously fermented olives such as *Metschnikowia pulcherrima*, *Wickerhamomyces anomalus* and *Aureobasidium pullulans* (Arroyo-López et al., 2012a, 2012b; Ciafardini et al., 2021).

Yeasts exert many beneficial functions throughout table olives fermentation. The main positive effect is the production of volatile compounds, which are end products of the yeasts' metabolism. They are essential for the development of the olives' flavor and contribute to the uniqueness of the finished product (Arroyo-López et al., 2008; Panagou and Tassou, 2006). Other desirable features are their enzymatic (i.e., lipolytic, β-glucosidase, catalase, pectinolase) and antagonistic activities towards pathogens and spoilage microorganisms (Arroyo-López et al., 2012a, 2012b; Hernández et al., 2008). On the other hand, yeasts may be responsible for defects such as package bulging, production on gas-pockets and film formation on the brine, thus it is important to monitor their dynamics throughout the entire process (Ciafardini and Zullo, 2020).

Recent molecular methods for the study of microbial populations involve the use of next-generation sequencing techniques (NGS), which currently allow a deeper investigation of the microbial diversity in fermented foods compared to traditional culture-dependent techniques (Botta and Cocolin, 2012; Jampaphaeng et al., 2018). Among them, sequencing of the D1/D2 domain of the 26S rRNA gene represents the most commonly used method for the identification of fungal communities and is considered a suitable genetic marker to overcome biases related to amplicon-based sequencing (Mota-Gutierrez et al., 2019). Given the complexity of the microbial interactions within fermented matrices, the combination of culture-based techniques and amplicon-based sequencing represents a good strategy to cover most of the diversity, considering both viable and non-viable microbial populations.

Due to the importance that yeasts play in the production of volatile compounds and their role in the final organoleptic profile of Taggiasca table olives, the present work aimed to define the yeast ecology of Taggiasca olives through culture-dependent and independent methods and to associate the mycobiota with the production of VOCs.

## 2. Materials and methods

### 2.1. Olives samples and fermentation conditions

Taggiasca olives and brines samples deriving from two separate fermentation batches, prepared from olives harvested in November 2021, were provided by a company located in the province of Imperia (North West of Italy) and analyzed from time 0 until the end of the fermentation period (month 6). The fermentations took place at the industrial plant and they were prepared as follows: about 130 kg of black and green olives were rinsed and placed into plastic bags where freshly prepared brine with 10 % NaCl (w/v) was added until olives were fully covered. Then, bags were placed into plastic tanks which were filled with further brine until immersion of the bags and were left ferment outdoors. When necessary, fresh brine was added. At the end of the fermentation, freshly prepared brine was added and the packaged product was pasteurized. Microbiological analysis and pH measurements were performed on brines and olives separately at the following fermentation times: 0, 3, 7, 28, 60, 90, 120 and 180 days. Each batch was analyzed in duplicate.

### 2.2. Microbiological analysis and pH measurements

For the microbiological analysis, 5 g of olives were washed twice with sterile Ringer solution (Oxoid, Basingstoke, UK) to remove all unattached microorganisms and homogenized in a Stomacher machine BAGMIXER 400 (Interscience, St Nom, France) with 45 mL of Ringer for 1.5 min, while brines (10 mL) were analyzed as such. Olives or brine samples were serially diluted with ten-fold dilutions and aliquots of each dilution were plated in duplicate as follows: i) 100 μL spread for yeasts and molds enumeration on Malt Extract agar (AMT; Generon, Modena, Italy) supplemented with tetracycline (5 mg/mL) and incubated at 25 °C for 48 h; ii) 100 μL inoculum spread for the determination of total viable counts (TVC) on a generic peptone medium (Lab M Limited, Heywood, UK) incubated at 30 °C for 48 h; ii) 1 mL inoculum with double-layer inclusion method for lactic acid bacteria (LAB) enumeration on de Man-Rogosa-Sharpe (MRS; Oxoid, Basingstoke, UK) agar supplemented with natamycin (2,5 mg/mL) and incubated at 30 °C for 48 h; iv) 1 mL by inclusion for *Enterobacteriaceae* on Violet Red Bile Glucose (VRBG; Oxoid, Basingstoke, UK) agar incubated at 37 °C for 24 h. After incubation, colonies were counted and means and standard deviations of the duplicates were calculated. For yeasts isolation, fifteen colonies from AMT at each sampling point were randomly picked and isolated on new plates. Olives and brines were also enriched in 20 mL sterile MRS broth to favor the growth of LAB. pH was measured in duplicates in olives and brines at time: 0, 3, 7, 28, 60, 90, 120 and 180 days. Olives were diluted in sterilized Ringer solution with a 1:10 ratio, whereas the pH of the brine was measured in the brine directly. A basic 20 pH-meter Five Easy F20 (Mettler Toledo, Greifensee, Switzerland) was used for the measurements.

### 2.3. Molecular analysis

#### 2.3.1. DNA extraction from pure cultures

For the DNA extraction from yeasts isolates, 1 mL of a fresh overnight culture in YPD was transferred into 1.5 mL sterile tubes (Resnova, Milan, Italy) filled with 0.3 g of silica beads (VWR, Milan Italy) and centrifuged at 13,000 ×*g* for 5 min to pellet the cells. DNA extraction was carried out with the phenol-chloroform-isoamyl alcohol protocol according to (Cocolin et al., 2001) with the following modifications: Three hundred μL of breaking buffer (2 % Triton X-100, 1 % sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris [pH 8], 1 mM EDTA [pH 8] (Sigma, Milan Italy)) were added to each pellet and were vortexed for 5 s. Three hundred μL of phenol-chloroform-isoamyl alcohol (25:24:1; pH 6.7; Sigma) were added to each tube, and three 30 s rounds at maximum speed, with 10 s intervals between treatments, were performed with a bead beader (Mini Bead Beader 8; Biospec Products, Inc., Bartlesville, Okla.). Tubes were then centrifuged at 12,000*g* at 4 °C for 10 min, 400 μL aqueous phases containing nucleic acids were collected in new tubes and 800 μL ice-cold absolute ethanol were added for the precipitation of nucleic acids. After 3 rounds of precipitation with 200 μL 70 % (v/v) ethanol, residues of ethanol were removed, and pellets were let dry at room temperature. Fifty μL of ultrapure sterile water were added and incubated at 37 °C for 30 min. DNA yield and quality were assessed with the NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Milan, Italy) and standardized to 100 ng/μL.

#### 2.3.2. Molecular characterization and identification of isolates

Biotyping of the isolates was carried out by means of rep-PCR and selected biotypes were then subjected to sequencing of the D1/D2 variable region of the large-subunit 26S rRNA gene for species identification. Rep-PCR was performed in a final volume of 25 μL containing 5 μL 1× PCRBIO Reaction Buffer (already provided with 15 mM $MgCl_2$ and 5 mM dNTPs), 1.5 μL 0.6 μM $(GTG)_5$ primer 0.25 μL 0,5 U PCRBIO Taq DNA polymerase (PCR Biosystems, UK) and 1 μL of template DNA (100 ng/μL) and amplification was carried out in a thermocycler (ProFlex PCR System, Applied Biosystems, Milan, Italy) with the following protocol: initial denaturation at 95 °C for 1 min; 30 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. PCR products were separated by gel electrophoresis in a 2 % agarose gel (Nippon Genetics Europe GmbH, Düren, Germany) in TBE 1× at 120 V for 2 h and gels were scanned in GelDoc Go Imaging System (Bio-Rad, USA). Fingerprinting analysis was performed with Bionumerics ver. 6.1 software (Applied Maths, Sint- Martens-Latem, Belgium) using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) for hierarchical cluster analysis. Isolates with identification percentage higher than 85 % were considered to belong to the same cluster. Representative isolates from each group with a unique rep-PCR profile was identified by D1/D2 of 26S rRNA gene amplification with primer pairs NL1-NL4 (Kurtzman and Robnett, 1997). PCR products were visualized on a 1 % agarose gel with TAE 1× Buffer, visualized as previously described and sent to sequencing (GENEWIZ Germany GmbH part of Azenta Life Sciences, Leipzig, Germany). The obtained sequences were compared with the ones found in GenBank, using the Nucleotide BLAST search program (http://www.ncbi.nlm.nih.gov/blast/).

#### 2.3.3. Genomic DNA extraction and next generation sequencing (NGS)

One mL of the 1:10 olive and brine dilutions were centrifuged at 13,000 ×*g* for 5 min. Genomic DNA (gDNA) extraction was performed on the resulting pellets with the MasterPure complete DNA and RNA purification kit (Epicentre, Madison, WI), following the manufacturer's instructions. Amplification of the D1 domain of the 26S rRNA gene was performed with primer pair NL4R (5′-GGT CCG TGT TTC AAG ACG G-3′) and LS2-MF (5′-GAG TCG AGT TGT TTG GGA AT-3′), as described by Mota-Gutierrez et al. (2019). PCR products were purified and tagged with Nextera XT Index Kit (Illumina, San Diego, USA), following the manufacturer's instructions and libraries were sequenced on the MiSeq platform (2 × 250 bp) (Illumina, San Diego, USA).

### 2.4. Bioinformatics

For data analysis, adapters and primers were trimmed with *cutadapter* and DADA2 algorithm was used to remove chimeric sequence and to join the reads. Taxonomy was assigned by comparison with the SILVA database for 26S rRNA gene. Sequences were double-checked with the BlastN tool (http://www.ncbi.nlm.nih.gov/blast/) and ASVs tables were built. When the taxonomy assignment did not reach species level, a higher taxonomic group was used.

### 2.5. Statistical analysis

Microbial counts and pH were analyzed by one way analysis of variance (ANOVA) using IBM SPSS Statistics software version 28.0.1.1, with Duncan's post-hoc test to compare means and standard deviations with a $P < 0.05$. Microbiota assessment statistics were carried out with R (www.r-project.org) where alpha and beta diversity indices were calculated with the diversity function of QIIME2. The Shannon index H′ was analyzed using Kruskal-Wallis test to assess differences between types of sample (brines and olives). Bray-Curtis distance matrix was used to build the PCoA as a function of type and time.

### 2.6. Volatile organic compound analysis

Headspace VOCs analysis was carried out using a GC/MS Shimadzu QP-2010 Plus quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with a SPME autosampler (PAL System, CombiPAL, Switzerland). Twenty-mL HS-SPME vials was filled with 2 mL of olive dilution (1:10 v/v) or undiluted brine sample with 0.5 μg of 5-nonanol (internal standard) for the semiquantification analysis. A 1 cm–50/30 mm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Milan, Italy) was employed and conditioned according to the manufacture instructions After sample equilibration (5 min at 45 °C), volatile compounds were extracted by exposing the fiber to the vial headspace for 30 min at 45 °C with stirring (250 rpm) (Rees et al., 2016). The fiber was immediately desorbed into GC/MS injector at 260 °C for 2 min in splitless mode. A fused silica Stabilwax-MS capillary column (30 m × 0.25 mm × 0.25 mm film thickness; Restek, Bellafonte, PA) was used to perform the separation. Helium was chosen as a carrier gas with a linear velocity of 37.1 cm/s. The oven temperature was kept at 45 °C and held for 2 min, then it was raised (2 °C/min) to 120 °C, and to 210 °C at 18 °C/min for 5 min. The temperature of ion source and the transfer line were set at 230 °C. The mass acquisition range was 33–450 amu with a scan velocity equal to 1666 amu/s, using 70 eV IE energy. Acquisition and peak integration were carried out in TIC (*Total Ion Current*). The identification of VOCs was performed by comparing their mass spectra with those are reported in the NIST05 (National Institute of Standards and Technology). Semi-quantitative data (mg/kg) were obtained by measuring the characteristic *m/z* peak area of each identified compounds in relation to the *m/z* characteristic ion of the added internal standard.

## 3. Results

### 3.1. Microbial counts and pH measurements

Changes in pH and microbial loads are shown in Table 1. Olive pH at the beginning of fermentation (T0) was 6.25 and 6.30 in batch 1 and 2, respectively. In both batches, olive samples showed a similar decreasing trend until the end of fermentation (T180), however the lowest value (4.75) was reached in batch 2 after 90 days (Table 1, $P < 0.05$). Brine samples shared similar decreases throughout the fermentation process, with the most significant differences found from T0 (5.99) to T180 (4.09) in batch 1 and from T0 (7.34) to T7 (4.01) in batch 2. In batch 2 from T7 on, pH started to increase with significant differences after the first month (4.66), which were kept constant until the end of the process. *Enterobacteriaceae* were only detected in olives and brines belonging to batch 2 in the early stages of fermentation (T0 in olives and T3 in olives and brines), whereas yeasts and TVC were found in all fermentation stages throughout time, except for olives at T7. TVC were higher in brines compared to olives, where they kept a more stable level. Brines at T120 showed the highest values (5.89 and 6.81 CFU/mL in batch 1 and 2, respectively), although towards the end of fermentation B_2_T180 underwent a significant decrease (3.90 CFU/mL, $P < 0.05$). In olives, yeast counts throughout time fluctuated, decreasing until T7 and then significantly increasing until T120 in both batches where they stabilized around 3.75–4.34 Log CFU/g (Table 1, $P < 0.05$). At the end of fermentation, yeasts decreased in both batches by approximately 0.7 log cycles. In contrast, yeast counts in brine samples increased over time, showing the most significant differences from T0 (3.94 Log CFU/mL) to T90 (7.27 Log CFU/mL) in batch 1 and from T0 (3.53 Log CFU/mL) to T120 (7.70 Log CFU/mL) in batch 2. At T180, both samples showed a significant decrease of approximately 2 log cycles. Finally, no lactic acid bacteria were detected on plate in any sample, even those subjected to the enrichment step.

**Table 1**
Changes in pH and microbial counts of Taggiasca table olives and brines. *Enterobacteriaceae*, yeasts and total viable counts (TVC) are expressed as means of two replicates with the respective standard deviation.

| Sample code | Time (days) | Type | Batch | pH | Microbial counts (Log CFU/g/mL)[a] | | |
|---|---|---|---|---|---|---|---|
| | | | | | *Enterobacteriaceae* | Yeasts | TVC |
| O_1_T0 | 0 | Olive | 1 | 6.25 ± 0.09d | n.d. | 7.15 ± 0.00e | 3.42 ± 0.00a |
| O_1_T3 | 3 | Olive | 1 | 6.27 ± 0.00d | n.d. | 3.71 ± 0.02d | 4.99 ± 0.36a |
| O_1_T7 | 7 | Olive | 1 | 6.45 ± 0.00e | n.d. | n.d. | 4.94 ± 0.00a |
| O_1_T28 | 28 | Olive | 1 | 6,97 ± 0.00f | n.d. | 2.78 ± 0.00c | 3.73 ± 0.11a |
| O_1_T60 | 60 | Olive | 1 | 5.70 ± 0.00c | n.d. | 2.48 ± 0.00b | 3.11 ± 0.00a |
| O_1_T90 | 90 | Olive | 1 | 5.51 ± 0.00b | n.d. | 3.82 ± 0.03d | 4.00 ± 0.00a |
| O_1_120 | 120 | Olive | 1 | 5.09 ± 0.00a | n.d. | 3.75 ± 0.15d | 4.03 ± 0.01a |
| O_1_T180 | 180 | Olive | 1 | 5.12 ± 0.00a | n.d. | 2.98 ± 0.02c | 2.99 ± 0.00a |
| B_1_T0 | 0 | Brine | 1 | 5.99 ± 0.78c | n.d. | 3.94 ± 0.00a | 4.25 ± 0.00a |
| B_1_T7 | 7 | Brine | 1 | 4.97 ± 0.00a-c | n.d. | 4.45 ± 0.38ab | 4.33 ± 0.22a |
| B_1_T28 | 28 | Brine | 1 | 4.80 ± 0.00ab | n.d. | 4.86 ± 0.37ab | 4.37 ± 0.10a |
| B_1_T60 | 60 | Brine | 1 | 5.23 ± 0.00bc | n.d. | 4.29 ± 0.03ab | 5.21 ± 0.01ab |
| B_1_T90 | 90 | Brine | 1 | 4.91 ± 0.00ab | n.d. | 7.27 ± 0.03c | 6.18 ± 0.06b |
| B_1_120 | 120 | Brine | 1 | 5.01 ± 0.00a-c | n.d. | 7.18 ± 0.25c | 5.89 ± 0.02b |
| B_1_T180 | 180 | Brine | 1 | 4.09 ± 0.00a | n.d. | 5.69 ± 0.15bc | 5.56 ± 0.03b |
| O_2_T0 | 0 | Olive | 2 | 6.30 ± 0.04 h | 2.65 ± 0.05 | 3.58 ± 0.06b | 3.96 ± 0.05c |
| O_2_T3 | 3 | Olive | 2 | 5.91 ± 0.00 g | 1.8 ± 0.00 | 4.03 ± 0.25b | 4.00 ± 0.00c |
| O_2_T7 | 7 | Olive | 2 | 5.32 ± 0.00d | n.d. | n.d. | n.d. |
| O_2_T28 | 28 | Olive | 2 | 5.50 ± 0.00e | n.d. | 1.89 ± 0.00ab | 3.43 ± 0.11bc |
| O_2_T60 | 60 | Olive | 2 | 5.25 ± 0.00c | n.d. | 3.62 ± 0.11b | 3.79 ± 0.01c |
| O_2_T90 | 90 | Olive | 2 | 4.75 ± 0.00a | n.d. | 2.06 ± 0.00ab | 1.54 ± 0.00ab |
| O_2_T120 | 120 | Olive | 2 | 5.12 ± 0.00b | n.d. | 4.32 ± 0.08b | 3.94 ± 0.14c |
| O_2_T180 | 180 | Olive | 2 | 5.74 ± 0.21f | n.d. | 3.68 ± 0.08b | 3.90 ± 0.16c |
| B_2_T0 | 0 | Brine | 2 | 7.34 ± 0.15e | n.d. | 3.53 ± 0.05a | 5.21 ± 0.16bc |
| B_2_T3 | 3 | Brine | 2 | 6.46 ± 0.00d | 2.85 ± 0.08 | 3.85 ± 0.00a | 4.84 ± 0.01ab |
| B_2_T7 | 7 | Brine | 2 | 4.01 ± 0.00a | n.d. | 5.26 ± 0.23bc | 5.12 ± 0.16bc |
| B_2_T28 | 28 | Brine | 2 | 4.66 ± 0.00c | n.d. | 5.13 ± 0.03b | 6.14 ± 0.00 cd |
| B_2_T60 | 60 | Brine | 2 | 4.61 ± 0.00bc | n.d. | 5.28 ± 0.14bc | 6.02 ± 0.04 cd |
| B_2_T90 | 90 | Brine | 2 | 4.61 ± 0.00bc | n.d. | 6.09 ± 0.01c | 6.12 ± 0.03 cd |
| B_2_T120 | 120 | Brine | 2 | 4.47 ± 0.00b | n.d. | 7.70 ± 0.05d | 6.81 ± 0.01d |
| B_2_T180 | 180 | Brine | 2 | 4.58 ± 0.00bc | n.d. | 5.31 ± 0.05bc | 3.90 ± 0.02ab |

n.d.: not detected.
Values with different lower-case letters in the same column indicate significant differences among fermentation times ($P < 0.05$) according to Duncan's post-hoc test.

### 3.2. Molecular identification: culture-dependent and independent results

#### 3.2.1. Culture-dependent: rep-PCR fingerprinting

After clustering based on similarity of rep-profiles, 373 yeast isolates were grouped into 46 different clusters. One or two representatives for each cluster were selected for sequencing and taxonomic assignment was performed using GenBank library with the BLAST search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Classification of the isolates at species level together with their prevalence is reported in Table S1. Fig. 1 reports the relative abundances of yeasts isolated from olives and brines belonging to the two batches. In batch 1, the main species throughout the fermentation process were *W. anomalus* (46.2 %), *C. diddensiae* (35.2 %) and *A. pullulans* (6.7 %), whereas in batch 2 the dominant ones were *C. diddensiae* (33.7 %), *A. pullulans* (26.4 %) and *P. membranifaciens* (24.5 %). *W anomalus* in batch 1 was isolated in all samples except at T0 and its presence gradually increased over time, reaching a peak at T90 in olives (93.33 %) and at T60 in brines (100 %). *C. diddensiae* followed an opposite trend in batch 1, where its percentage in both types of samples dropped after the first month of fermentation (T28). In batch 2, *C. diddensiae* and *P. membranifaciens* coexisted from T28 on with variations among samples, whereas the very early stages of fermentation of both brines and olives were dominated by *A. pullulans* (83,3 % and 80,0 %, respectively). Interestingly, at the end of fermentation (T180) both olives and brines showed the presence of *Pichia manshurica*, representing 35,7 % and 42,9 % of the total isolates, respectively. Clusters generated via rep-PCR fingerprinting showed the persistence of *C. diddensiae* throughout time in olive and brines belonging to batch 1 (from T0 to T28 and T60, respectively), as well as in brines of batch 2 from T7 on (data not shown).

#### 3.2.2. Mycobiota of olives and brines during Taggiasca olives fermentation

Amplicon sequencing (Fig. 2) showed a prevalence of *Aureobasidium* spp. (45.6 %), *Citeromyces nyonsensis* (16.3 %) and *W. anomalus* (13.1 %), overall. In batch 1, the main ASVs found were *A. pullulans* (35.2 %) and *W. anomalus* (42.9 %) in olives and brines, respectively. In batch 2, the main representative ASVs were *A. pullulans* (30.8 %) and *C. nyonsensis* (67.3 %) for olives and brines, respectively. Interestingly, *Aureobasidium* spp. in batch 1 persisted in all samples, except for olives at T180, with opposite trends for olives and brines. *A. pullulans* ranged from 7.6 % to 71.3 % in olives, where it increased until T60 and then started decreasing, while in brines it decreased from T0 (20.9 %) until the end of fermentation (0.2 %). A similar trend was found for batch 2. *W. anomalus* was predominant in brines of batch 1 where it was found mostly abundant from the first month of fermentation until the end (T180), where it represented 46.7 % of the relative frequency in brines and 58.5 % in olives. Noticeably, brines of batch 2 were dominated by *C. nyonsensis* from T28 (87.3 % to 100 %). In olives, final stages of fermentation (T120 and T180) were characterized by the presence of *Debaryomyces hansenii* (from 5.5 % to 8.6 % in batch 1 and from 1.4 % to 2.5 % in batch 2) and *Pichia* spp. (from 0.4 % to 6.5 % in batch 1 and from 2.6 % to 0.9 % in batch 2). In the first week of fermentation, brines showed the highest variability in terms of populations with *Cladosporium* slightly increasing in the first days from 11.3 to 14.1 % in batch 1 and from 7.8 to 14.0 % in batch 2. Comparing the olive's mycobiota composition at the lowest taxonomic level across time, only *A. pullulans* was associated with the initial stages of fermentation (T0 to T90), while *Alternaria*, *Cystobasidium minutum*, *D. hansenii*, *Pichia manshurica*, *P. membranifaciens*, *Yamadazyma atlantica* and *Yamadazyma diddensiae* were associated with T120 and T180 (Kruskal-Wallis, $P < 0,05$). In brine samples throughout fermentation, only *Alternaria*, *A. pullulans*, *Cladosporium* and *S. cerevisiae* were characteristic of the initial stages of

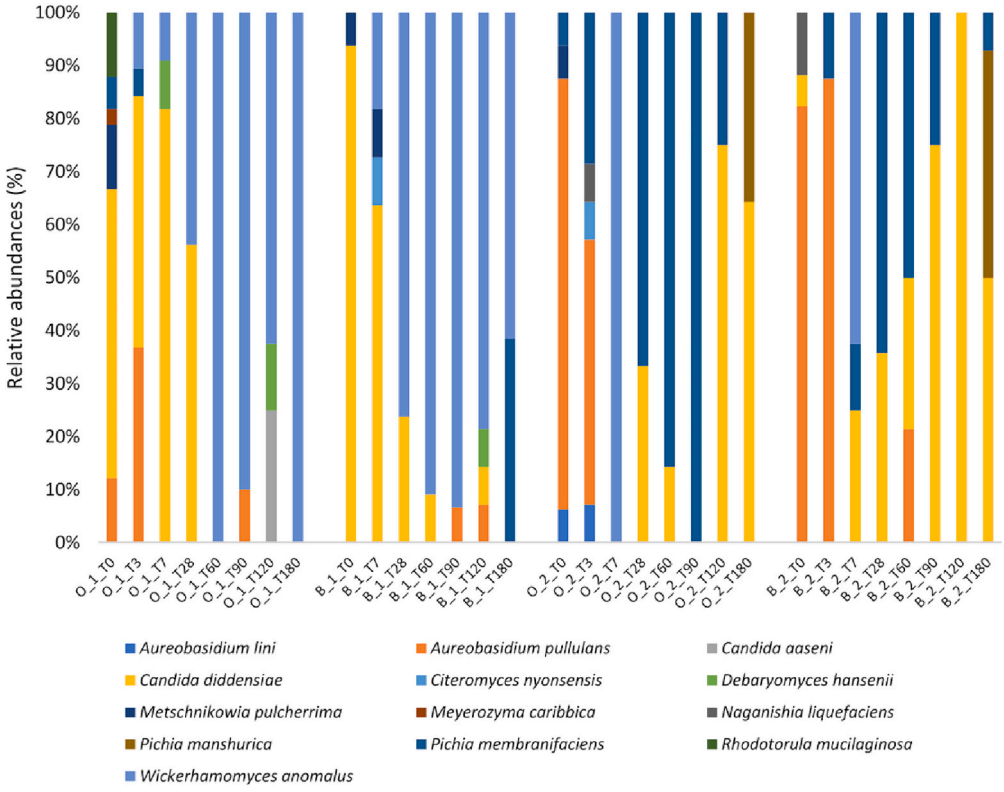


**Fig. 1.** Number of strains isolated along the fermentation process and expressed as relative abundances (%) of yeast species isolated from olives and brines of batch 1 and batch 2. Olive samples (O); Brine samples (B); first number (batch); last number (sampling time expressed in days).

fermentation (Kruskal-Wallis, $P < 0.05$) (Fig. S1.). Concerning alpha-diversity, the Shannon index showed no difference in the samples as a function of the type (olives or brines) or batch (1 or 2) (Kruskal-Wallis, $P > 0.05$). Only samples belonging to T7 of both fermentations displayed the highest Shannon diversity when compared with those of T60 (Kruskal-Wallis, $P < 0.05$). Bray-Curtis distance matrix was used to produce a PCoA plots as a function of sampling time and type (Fig. 3). Significant differences were observed as a function of type and batch (PERMANOVA, $P < 0.05$). For fermentation stages, samples from T180 displayed significant differences when compared to T0, T3 and T7 (PERMANOVA, $P < 0.05$).

### 3.3. Volatile organic compounds dynamics

Volatile compounds detected by GC/MS analysis resulted in the detection of 54 compounds in brines and 60 compounds in olives. All volatiles were grouped into eight chemical classes, namely alcohols, hydrocarbons, esters, ethers, terpenes, aldehydes, ketons and carboxylic acids. VOCs were correlated with olive or brine samples via hierarchical clustering (Fig. 4). Although no clear separation was shown between batches, groups clustered better as a function of the fermentation time (Fig. 4A and B). Olives belonging to the later stages of fermentation (T120 and T180), were mainly characterized by hydrocarbons (hexadecane, pentamethyl-heptane, benzene, 1,3-bis(1,1-dimethylethyl), dimethyl-1-heptene, dimethyl-2-undecene), alcohols (2-hexyl-1-decanol, 2-methoxy phenol, 1-nonanol) and ketons (2-octanone). Olives in the early stages of fermentation were characterized by hydrocarbons (decane, dodecane, dimethyl-hexane) as well as aldehydes (nonanal), and medium chain fatty acids (MCFA) (nonanoic acid). Brines at the end of fermentation were mainly associated with alcohols (2-methyl-1-propanol, 2-methyl-2-propanol, 2-methoxy phenol), esters (nonanoic acid-methylester) and acetic acid, while in the initial stages differences were observed between batch 1 and batch 2 at T0 and T3. Alcohols (benzylacohol, phenylethylalcohol, ethanol), MCFA (nonanoic acid), terpenes (D-limonene) and ketons (5-nonanone) were positively correlated with brines at T0 and T3, while in batch 2 they showed a strong negative correlation.

Comparing VOCs composition in olive samples throughout time, dodecane and 2,4-dimethylhexane were associated with the initial stages of fermentations (T0-T90), whereas 2-hexyl-1-decanol and benzene, 1,3-bis(1,1-dimethylethyl) were associated with the later stages (T120, T180) (Kruskal-Wallis, $P < 0,05$). In brines, nonanal, hexanal and trichlorometane were characteristic of the initial stages of fermentation, while acetic acid, 2-methyl-1-propanol, 2-methyl-2-propanol and 2-methoxy phenol, characterized the final fermentation stage (Kruskal-Wallis, $P < 0.05$) (Fig. S2.).

Correlation between microbiota and VOCs was performed to better understand the potential relationships between yeast and metabolites (Fig. 5). Among the main ASVs fraction in olives, *A. pullulans* showed positive correlation with tridecane and negative correlation with 4,6-dimethyl dodecane while *A. protae* was negatively correlated with 7 VOCs ($P < 0.05$). *W. anomalus*, mainly present in batch 1, was positively correlated with 1-heptanol and negatively with *n*-hexylmethylamine. In brines, all three fungal species (*A. pullulans*, *A. protae*, *W. anomalus*) showed negative correlations with the same VOCs, namely 1-heptanol and 2-octanol. *C. nyonsensis* accounted for 67.33 % of the ASVs in brines of batch 2, and positively correlated with benzaldehyde. Regarding less frequent ASVs, *C. minutum* was correlated with 15 VOCs in olives and 3 in brines, 8 of which were alcohols, while *Vishniacozyma carnescens* was positively associated with hexanal and trichloromethane in brines, both characteristic of early fermentation stages ($P < 0.05$). Finally, microorganisms associated to the final stages of fermentations (*C. minutum*, *Pichia* spp., and *Y. diddensiae*) ($P < 0.05$) positively correlated with 1-heptanol and 3-methyl-benzaldehyde.

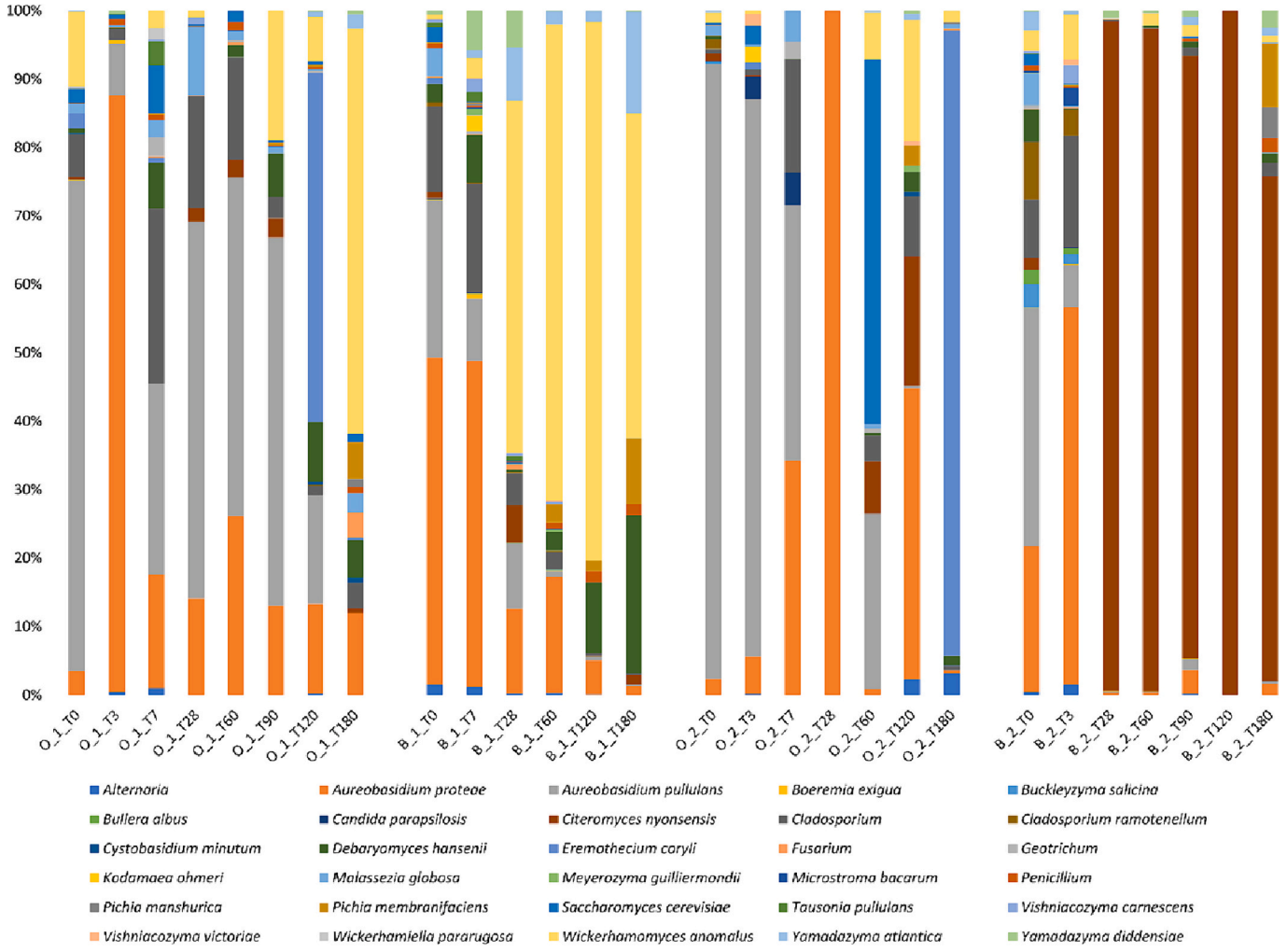


**Fig. 2.** Relative frequency (%) of ASVs detected by 26S rRNA gene sequencing in batch 1 and 2. Olive samples (O); Brine samples (B); first number (batch); final number (sampling time expressed in days).

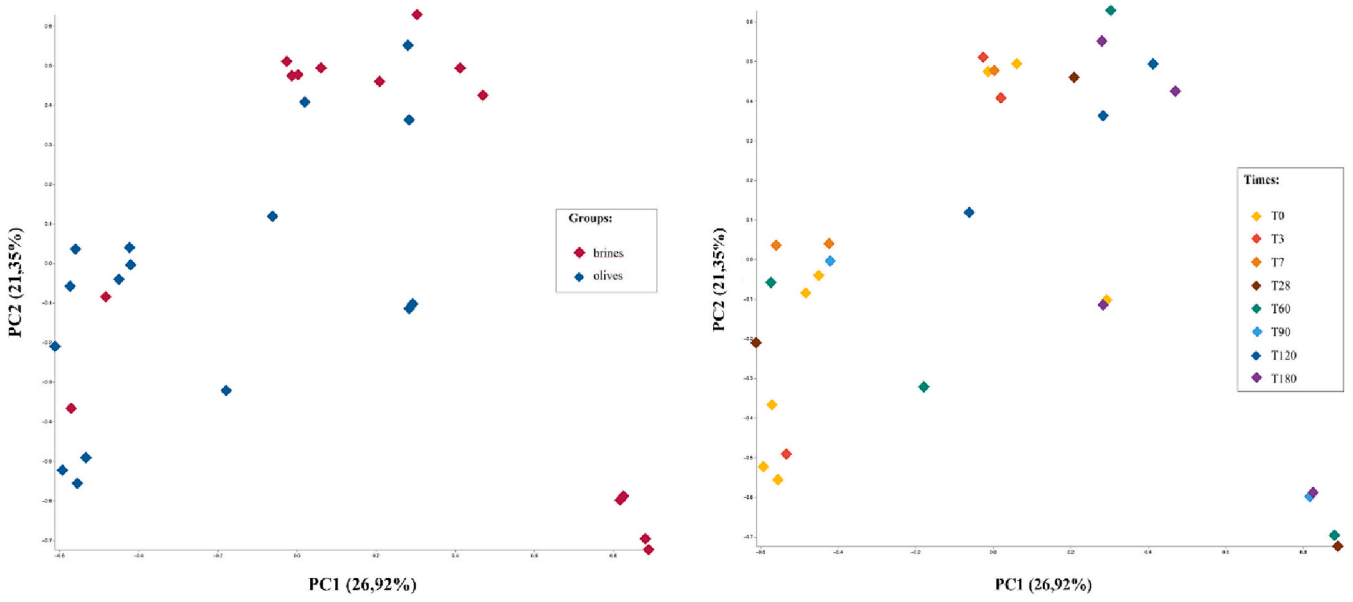


**Fig. 3.** Principal coordinate Analysis (PCoA) plots based on Bray-Curtis distance matrix as a function of A) type and B) fermentation time expressed in days. Samples type or sampling days are coded according to different colors.

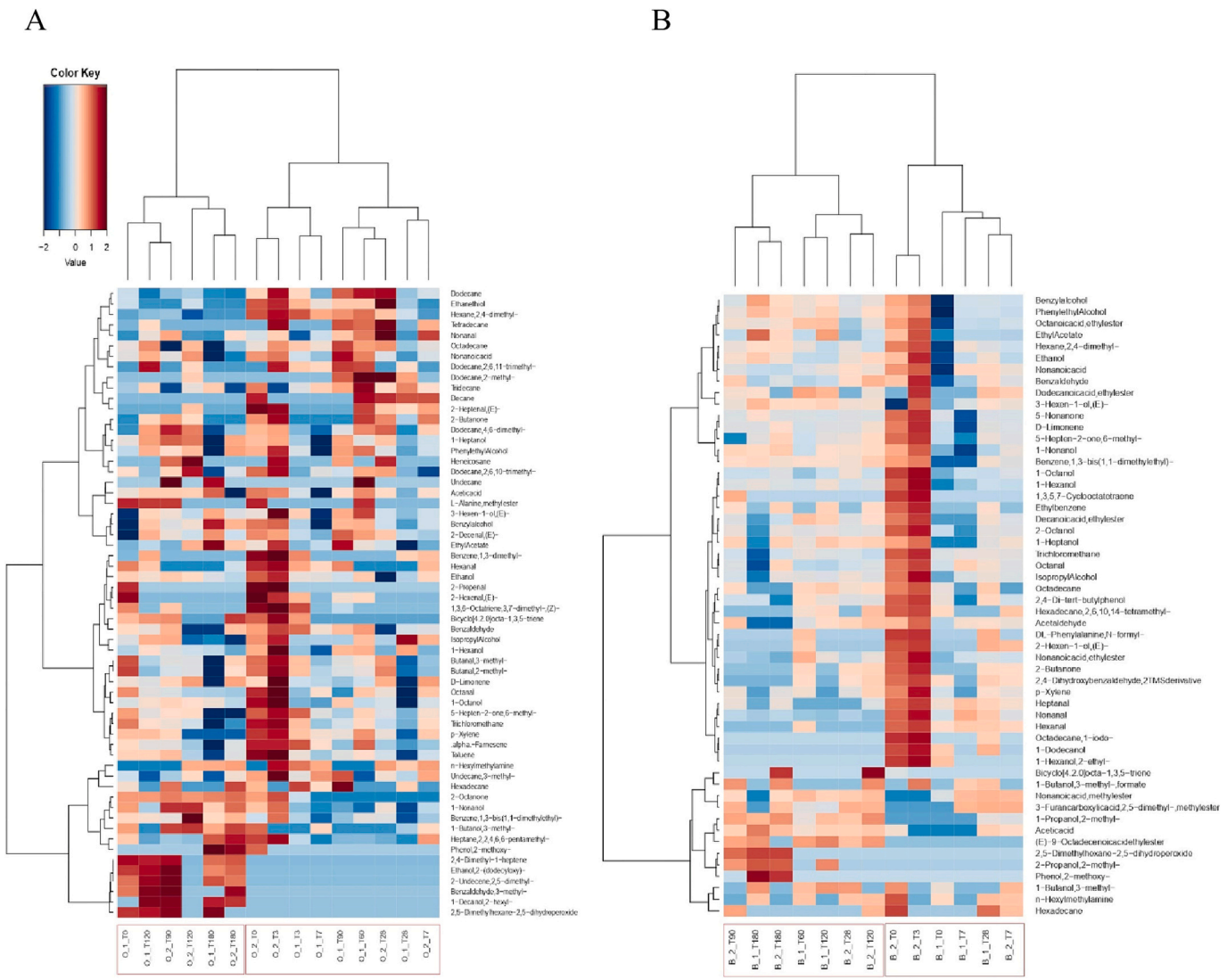


**Fig. 4.** Heatmap displaying the relationship between olive (A) and brine (B) samples with VOCs (μg/kg). Rows and columns are clustered according to Ward linkage hierarchical clustering. The intensity of the color shown in the color key indicates the degree of correlation between the samples and the VOCs, measured by Spearman's correlation.

## 4. Discussion

Spontaneously fermented olives are a complex ecosystem, characterized by autochthonous microbes in combination with environmental microorganisms (Botta and Cocolin, 2012). Taggiasca olives fermentation is an example of a spontaneous fermentation where high salt concentrations are applied (>10 % w/v), which typically favors the growth of yeasts over LAB (Ciafardini and Zullo, 2019). Higher yeasts counts can be commonly found when immersing the olives into the brine at the beginning of fermentation (Pereira et al., 2015), which could explain the initial load shown in our olives of batch 1. Filamentous fungi are often found in natural olives, although their function is still not fully elucidated (Bavaro et al., 2017). On the other hand, the role of yeasts has been deeply investigated. They contribute to the growth of LAB by producing vitamins and purines as well as the degradation of inhibitory compounds towards LAB growth, making them an essential part of the olive microbial consortia (Anagnostopoulos and Tsaltas, 2022). Recently, they were also associated to the formation of biofilms on the olive surface, as well as inside the fruits, as fermentation progresses (López-García et al., 2023). Another major role attributed to yeasts is the production of volatile compounds (VOCs), which affects the final organoleptic characteristics of the product (Anagnostopoulos and Tsaltas, 2022; Arroyo-López et al., 2008). In this study, rep-PCR fingerprinting was used as a dereplication tool, to cluster yeasts into groups sharing similar band patterns. Persistence of some microorganisms' biotypes *i* throughout the two fermentations, such as *C. diddensiae*'s in our study, might suggest the possibility to use such molecular technique as a tool to monitor microbial dynamics in the future. This finding is in accordance with (Greppi et al., 2013), which stated the role of rep-PCR in biotyping *Candida* spp. From culture-dependent results, yeasts were found to be the dominant population. Our findings were comparable to those on Arbequina natural olives by Hurtado et al. (2008) and, more recently, to those of Ciafardini et al. (2021) where uninoculated Taggiasca brines showed the dominance of yeasts. Although pH drops are typically associated with LAB-driven fermentations (Arroyo-López et al., 2008), in the present study LAB were not detected by viable counts. This finding is in accordance with Montaño et al. (2021) that studied naturally fermented Manzanilla olives. To note that the correlation data confirmed the positive association of acetic acid with olives and brines in later fermentation stages (T120 and T180) when the pH decrease was mostly significant ($P < 0.05$). To this regard, the microbiota correlation results confirmed a positive correlation of *V. carnescens* and *Tausonia pullulans* and acetic acid, which could be attributed to the accumulation of such compound as a co-product upon acetate

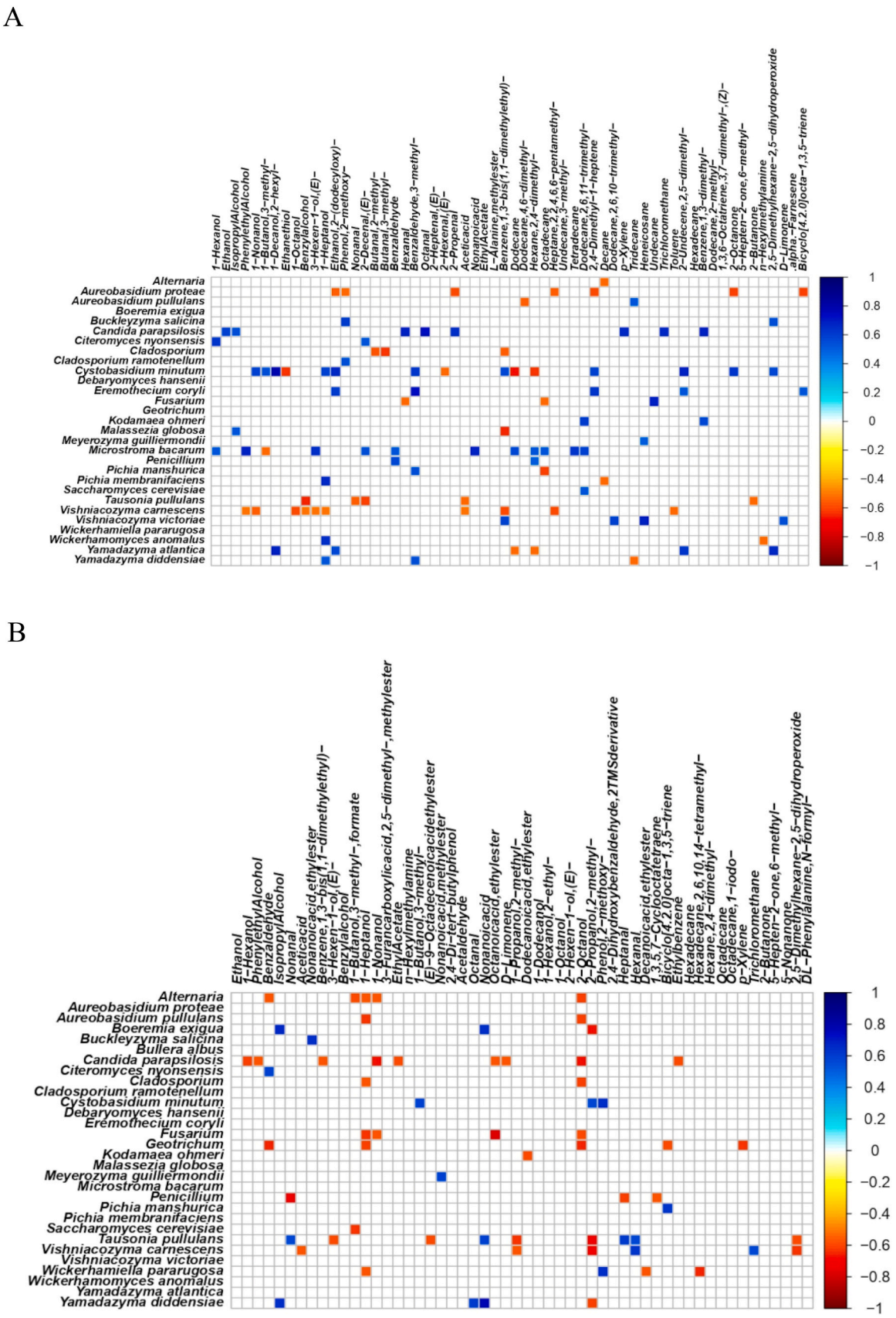


**Fig. 5.** Spearman correlation between microbiota and VOCs (μg/kg) in olives (A) and brines (B). The intensity of the color represents the degree of correlation. Only significant correlations are shown ($P < 0.05$).

metabolism, which can also be used as carbon source (Guaragnella and Bettiga, 2021). Spoilage microorganisms can occur, with *Enterobacteriaceae* being the main contaminating source at the beginning of fermentation, although generally do not persist throughout the process as shown in previous studies (Nychas et al., 2002; Panagou et al., 2003). This was confirmed here since they were detected in olives and brines of batch 2 until T3. Nevertheless, low presence of other spoilage microorganisms such as *Staphylococcus* was reported in a previous study by Kazou et al. (2020) on natural black olives and, although no other food-related pathogens (e.g., *Listeria*, *Clostridium*) were found, it is always important to apply thorough cleaning of the equipment and proper hygiene conditions at the producing plant. Furthermore, conditions such as high salt contents (>8 % w/v) and proper storage are parameters that should guarantee a long-term preservation of the product (Lucena-Padrós et al., 2014). In this study 10 % (w/v) NaCl was maintained throughout the entire fermentation and the use of bags during the processing limited the oxygen entrance, which typically leads to biofilm formation and consequent defects in the quality of the product (Ciafardini and Zullo, 2020). Finally, pasteurization of the final product, such as in this work, prevents undesirable secondary fermentation led by *Propionibacterium* spp. to take place (Lucena-Padrós et al., 2014).

Yeasts populations described through culture-dependent methods mainly consisted of *C. diddensiae*, *W. anomalus*, *P. membranifaciens* and *A. pullulans* with different distribution among samples. Those species were isolated from spontaneous and Spanish-style fermented table olives deriving from different cultivars and countries (Bevilacqua et al., 2012; Bleve et al., 2014; Gounari et al., 2023). A recent study on Taggiasca cv. also confirmed the presence of *A. pullulans* and *W. anomalus* in olives and brines from different years (Cecchi et al., 2023). *Candida* spp., together with *W. anomalus* and *Pichia* spp. are common species during olives fermentation (Arroyo-López et al., 2006; Bevilacqua et al., 2012; Nisiotou et al., 2010), with *P. membranifaciens* also being associated with strain-specific antagonistic activities towards spoilage microorganisms (Perpetuini et al., 2020). Similarly to our results, other studies also reported an increase in *Pichia* spp. towards the final stages of fermentation (Bonatsou et al., 2018; Penland et al., 2020). More specifically, Penland et al. (2020) observed a shift in the microbial composition for both matrices, with *P. membranifaciens* dominating from day 183 on, while Bonatsou et al. (2018) reported similar data in brines for *P. manshurica* from day 187 on. This is in accordance with our finding, where *P. manshurica* emerged at T180, suggesting a slower adaptation of this specie to the substrate within the native microbiota. Relevance of *W. anomalus* in olives fermentation was shown in two studies where different strains tested positively for their enzymatic activities (esterase, catalase and ß-glucosidases) and the production of antioxidant compounds (Hernández et al., 2007, 2008), making this species interesting for further screenings as potential starter culture. It is worth noticing that recently, potential applications of *Aureobasidium* spp. in biotechnology were investigated (Wang et al., 2022). Besides being isolated from fermented olives (Bonatsou et al., 2018; Cecchi et al., 2023; Nisiotou et al., 2010), these dimorphic fungi are widespread across environments (saltern pods, deep sea and glacial ice) showing resilience and halotolerance (Wang et al., 2022). However, given their noticeable xylanolytic activity (Deshpande et al., 1992; Yegin, 2017), these populations should be monitored throughout the fermentation to avoid an excessive softening of the pulp (Bevilacqua et al., 2012).

Amplicon-based sequencing showed a prevalence of *Aureobasidium* spp., *C. nyonsensis* and *W. anomalus* overall. Interestingly, prevalence of *A. pullulans* and *W. anomalus* was also confirmed via traditional culture methods. Given the dominance of *W. anomalus*, this species could be one of the major contributors to the debittering of the olives. In accordance with our hypothesis, Cecchi et al. (2023) confirmed the ability of some *W. anomalus* strains to degrade oleuropein and grow under high NaCl concentrations. Regarding the main ASVs fractions, *C. nyonsensis* was previously isolated from brines (Casaregola et al., 2013; Gounari et al., 2023; Ruiz-Moyano et al., 2019). About the minor ASV fraction, *Cladosporium* was found, in accordance with Michailidou et al. (2021) in Greek-style fermented olives. *Meyerozyma* and *Yamadazyma* were also present in another high salt content (10 % NaCl w/v) fermented food, lean ham, where they represented the dominant genera (Zhang et al., 2023).

Aroma compounds in fermented table olives are influenced by many factors, such as cultivar, maturation of the fruits and type of processing. The flavor of the final product is partly determined by the volatile profile which represents a quality parameter for producers and consumers (Sabatini and Marsilio, 2008). It is well-known that enzymatic activities of native microorganisms impact the final quality of the product. Yeasts enzymatic activities mainly involve a lipolytic and ß-glucosidase activity (Bevilacqua et al., 2012). The first is related to the presence of esterase and lipases that generate VOCs (ethanol, higher alcohols, esters) from the catabolism of free fatty acids (Hernández et al., 2007), whereas the latter coincides with the degradation of oleuropein into non-bitter markers such as hydroxytyrosol (Penland et al., 2020). In olives, the predominant chemical classes were hydrocarbons, followed by alcohols and aldehydes, while in brine samples the main representatives were hydrocarbons, alcohols, and esters. This result is in accordance with Bleve et al. (2014), that confirmed the presence of a high heterogeneity of VOCs classes in table olives. Furthermore, in Leccino olives, a high content of aldehydes and hydrocarbons was registered, confirming our result. However, such concentrations seemed to be dependent on the variety. According to our correlation data, most esters were positively correlated in early stages of fermentation, contrarily to what previously reported (Penland et al., 2020; Ruiz-Barba et al., 2023). Among alcohols that showed a statistical difference from early to later stages of fermentation, 2-hexyl-1-decanol was also detected in highest amounts in olive samples in the study of Ahmad et al. (2023). Interestingly, the pH decrease in brines corresponded to a rapid increase in organic acids, especially acetic and nonanoic acids. Finally, aldehydes are important secondary metabolites produced from the amino acids catabolism or the oxidation of unsaturated fatty acids and provide a typical herbaceous flavor in fermented vegetables (Bleve et al., 2014; Kaminarides et al., 2007). In our study, 2-hexenal was detected in olives in the first days of fermentation (T0 and T3) and showed strong positive correlation with olives from both batches. Such compound is considered a biomarker found in olive fruits produced from the lipid degradation and is commonly associated with desirable organoleptic characteristics (Muzzalupo et al., 2012).

Fungal species are common to black olives spontaneous fermentation, and in a recent study they were also associated with the Taggiasca variety (Cecchi et al., 2023). In our work, we confirmed the presence of *Alternaria*, *Aureobasidium*, and *Cladosporium* spp. by amplicon-based sequencing and their association to early stages of fermentation. This result could suggest that they are endophytic of the Taggiasca cultivar, in accordance to Cecchi et al. (2023) where they found the persistence of such microorganisms in the olives of three different farms after being washed. Alcohols are main flavor compounds associated with fermented vegetables because of yeasts metabolism and their presence in fermented table olives has been assessed in many studies (Maicas, 2020; Sabatini et al., 2009; Sabatini and Marsilio, 2008). Our data suggests that as the fermentation progresses, alcohols increased and were related to later stages of fermentation, as confirmed by Ruiz-Barba et al. (2023), where they found a gradual increase in alcohols in the fermentation of natural olives. Furthermore, early-stages fungi *Alternaria*, *Aureobasidium*, and *Cladosporium* negatively correlated with 2 alcohols (1-heptanol, 2-octanol), strengthening our finding. Interestingly, *Y. diddensiae* was significantly correlated with nonanoic acid in the brines of batch 1. This result was also consistent with the acidification that took place in the same sampling times, suggesting that this microorganism might be associated with this evidence. Its presence has previously not been reported in fermented olives, but *Yamadazyma* spp. were associated with mono-variety olive oils (Ciafardini et al., 2013; Mari et al., 2016). Another possible explanation for this phenomenon was provided by the

results of Penland et al. (2020), where a decrease in pH was associated to the increase in organic acids in brines over time. Furthermore, they found strong positive correlations between citric and malic acids with *W. anomalus* and *C. nyonsensis*, which were the most dominant species, much like in our brine samples. Finally, the increase in organic acids and $CO_2$ levels as end products of yeasts metabolism could also explain the acidification of naturally fermented olives as recently found by Ruiz-Barba et al. (2023), and confirmed by our results, where acetic acid was correlated with later stages of fermentation in brines.

## 5. Conclusion

Fermented olives are a key component of the Mediterranean diet and have a relevant economic value for the countries of the Mediterranean basin. Taggiasca olives are typical of the Liguria region, where they represent a high-quality, traditional product. Unraveling their microbial composition and sensory properties represents a great effort for the scientific community, but also from the consumers' point of view. With culture-dependent methods, it was possible to identify 13 yeast species, whereas culture-independent molecular analysis provided a deeper knowledge into the yeasts and fungal diversity. In terms of microbiota (o mycobiota) composition, significant differences were found as a function of batch, type and stages of fermentation, whereas VOCs dynamics were different when comparing early to later stages of the fermentation. Considering our results, we can conclude that the necessity of coupling culture-dependent and independent methods is important to explore the microbial diversity of a food matrix with such mixed populations. Besides, the relevance of our study is proved by the deep understanding of the ecology that characterizes such appreciated variety as well as its organoleptic profile, although fungal diversity and yeasts enzymatic activities should be investigated more deeply in future studies. Finally, this study could help guide the selection of potential autochthonous starters for a more controlled fermentation that preserves the sensory quality of this traditional product.

## CRediT authorship contribution statement

**Chiara Traina:** Data curation, Formal analysis, Writing – original draft. **Ilario Ferrocino:** Data curation, Methodology, Software, Supervision, Writing – review & editing. **Ambra Bonciolini:** Data curation, Formal analysis, Writing – review & editing. **Vladimiro Cardenia:** Data curation, Writing – review & editing. **Xinping Lin:** Data curation, Formal analysis, Investigation. **Kalliopi Rantsiou:** Conceptualization, Funding acquisition, Writing – review & editing. **Luca Cocolin:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare no conflict of interest.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110688.

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