Exploring yeast diversity of dry-salted naturally black olives from Greek retail outlets with culture dependent and independent molecular methods

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Zoe Gounari[a], Stamatoula Bonatsou[a], Ilario Ferrocino[b], Luca Cocolin[b], Olga S. Papadopoulou[c], Efstathios Z. Panagou[a,*]

[a] Agricultural University of Athens, Department of Food Science and Human Nutrition, School of Food and Nutritional Sciences, Laboratory of Microbiology and Biotechnology of Foods, Iera Odos 75, Athens 11855, Greece

[b] University of Turin, Department of Agricultural, Forestry and Food Sciences, Largo Paolo Braccini 2, 10095 Grugliasco, Torino, Italy

[c] Institute of Technology of Agricultural Products, Hellenic Agricultural Organization DIMITRA, S. Venizelou 1, Lycovrissi 14123, Attiki, Greece

Corresponding author: E.Z. Panagou, E-mail: stathispanagou@aua.gr

Abstract

In the present study, the physicochemical (pH, water activity, moisture content, salt concentration) classical plate counts (total viable counts, yeasts, lactic acid bacteria, *Staphylococcus aureus*, *Pseudomonas* spp., *Enterobacteriaceae*) and amplicon sequencing of naturally black dry-salted olives obtained from different retail outlets of the Greek market were investigated. According to the results, the values of the physicochemical characteristics presented great variability among the samples. Specifically, pH and water activity (a_w) values ranged between 4.0 and 5.0, as well as between 0.58 and 0.91, respectively. Moisture content varied between 17.3 and 56.7% (g H_2O/100 g of olive pulp), whereas salt concentration ranged from 5.26 to 9.07% (g NaCl/100 g of olive pulp). No lactic acid bacteria, *S. aureus*, *Pseudomonas* spp. and *Enterobacteriaceae* were detected. The mycobiota consisted of yeasts that were further characterized and identified by culture-dependent (rep-PCR, ITS-PCR, and RFLP) and amplicon target sequencing (ATS). *Pichia membranifaciens*, *Candida sorbosivorans*, *Citeromyces nyonsensis*, *Candida etchelsii*, *Wickerhamomyces subpelliculosus*, *Candida apicola*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii* and *Candida versatilis* were the dominant species according to ITS sequencing (culture-dependent), while ATS revealed the dominance of *C. etchelsii*, *Pichia triangularis*, *P. membranifaciens,* and *C. versatilis* among samples. The results of this study demonstrated considerable variability in quality attributes among the different commercial samples of dry-salted olives, reflecting a lack of standardization in the processing of this commercial style. However, the majority of the samples were characterized by satisfactory microbiological and hygienic quality and complied with the requirements of the trade standard for table olives of the International Olive Council (IOC) for this processing style in terms of salt concentration. In addition, the diversity of yeast species was elucidated for the first time in commercially available products, increasing our knowledge on the microbial ecology of this traditional food. Further investigation into the technological and multifunctional traits of the dominant yeast species may result in better control during dry-salting and enhance the quality and shelf-life of the final product.

Keywords: black olives, dry-salted olives, yeasts, rep-PCR, RFLP, amplicon target sequencing

1. Introduction

Naturally black dry-salted olives are traditionally produced in the island of Thassos in Northern Greece, using the olives of a local table olive cultivar (cv. Thassos), although any other cultivar of black or alkali treated green olives can be processed in the same way to produce dry-salted olives (Brenes et al., 2022). Olives are harvested when fully mature (the surface color of the drupes is black) or overripe and subsequently placed in concrete tanks in layers with coarse salt (Panagou et al., 2002). Under these conditions, practically no fermentation takes place and for this reason dry salting is characterized as a ‘‘curing’’ or ‘‘desiccation’’ process (Panagou, 2006; Ramirez et al., 2013). Due to the high osmotic pressure exerted by the salt, water and water-soluble components, such as oleuropein, are gradually removed from the mesocarp, resulting in gradual debittering of olives that are ready for consumption after 30-40 days (Değirmencioğlu et al., 2014). From the organoleptic point of view, dry-salted olives are considered to have a slight bitter and sweet taste, which is well accepted by consumers, but the high salt content needed for the preservation of the final product makes it unattractive to international markets. According to the CXS 66-1981 standard of table olives of the Codex Alimentarius (FAO, 1981) that was revised in 2013, the minimum salt content for this trade preparation of olives should be 8% (w/v). Consequently, the shelf-life of the final product is ensured by the high salt concentration and the corresponding low water activity (a_w) of dry-salted olives. It has been reported that the a_w level of the final product ranges between 0.75 and 0.85, depending on salt concentration during the dry-salting process and therefore only salt-tolerant microorganisms, such as yeasts, could become the dominant microbiota (Panagou, 2006; Panagou et al., 2002; Papagora et al., 2013). Among the pathogenic microorganisms, the presence of *Staphylococcus aureus* should be taken into serious consideration, because of its ability to grow in low a_w levels (a_w<0.86), where it presents a generation time of 300 min (Adams and Moss, 2008).

Taxonomic identification of microbial species is a difficult process that requires the application of several genetic markers to be sequenced. Several studies related to the microbial diversity of fermented products have employed amplicon sequencing techniques (Bokulich and Mills, 2012; Cocolin et al., 2013; Ercolini et al., 2012, 2013; Jampaphaeng et al., 2017; Mota-Gutierrez et al., 2019). However, new approaches to explore diversity using next generation sequencing technologies (NGS)

have revealed the importance of the reliability of PCR primers targeting a specific genetic marker (Bokulich et al., 2013; Mota-Gutierrez et al., 2019). Restriction endonucleases are enzymes that are able to recognize a specific sequence motif of the DNA known as restriction site. This taxonomic method can create a restriction map through the enzymatic digestion of DNA into smaller fragments, which are then separated by electrophoresis based on the composition of DNA and the % G+C content. One of the most common methods based on the enzymatic reaction of these restriction enzymes is Restriction Fragment Length Polymorphism (RFLP) used in the classification of both bacteria and yeasts (Nadin-Davis et al., 2007). The identification occurs after the digestion of the regions of the unidentified sample compared to reference strains and the discriminant ability is increasing with the implementation of multiple enzymes. The gene targets for the sequencing of 26S rRNA gene using the Sanger method are the variables ITS1 and ITS2. However, because of the limitations of these regions for yeast identification, D1/D2 of 26S rRNA gene in the large subunit of eukaryotic ribosomes is commonly used (Kurtzman and Robnett, 1997). It needs to be noted that Sanger method could be insufficient due to the complexity of bacterial diversity and therefore Next Generation Sequencing (NGS) or "massively parallel sequencing" can overcome these limitations, not only by promoting millions of parallel sequencing reactions, but also permitting the sequence of uncultured and unpurified samples. To avoid bias, it is important to compare different targeting regions and molecular techniques to explore the microbial populations on a food matrix.

The objectives of this work were (i) to characterize the physicochemical and microbiological profile of commercial naturally black dry-salted olives, and (ii) to elucidate the yeast diversity of the olives through culture dependent and independent molecular approaches.

2. Materials and Methods

2.1 Olive samples

Nine different commercially available samples of naturally black dry-salted olives cv. Thassos were studied. Each sample was obtained from a different supermarket in the wider area of Athens. Olives were displayed in bulk in retail

outlets and none of them was subjected to thermal treatment (pasteurization) or preserved by the use of authorized preservatives (information provided by suppliers). Instead, their preservation was based exclusively on the physicochemical characteristics attained during the dry-salting process, namely salt concentration. The average size of each sample was ca. 500 g.

2.2 Microbiological and physicochemical analyses

Lactic acid bacteria (LAB), yeasts, *Enterobacteriaceae*, *Pseudomonas* spp., total mesophilic counts and *Staphylococcus aureus* were determined for each sample in duplicate. Twenty-five grams (25 g) of olive pulp were aseptically added in 225 mL sterile 1⁄4 Ringer's solution and homogenized in a Stomacher device (LabBlender, Seward Medical, London, UK) for 60 s at room temperature. The resulting suspension was serially diluted in the same diluent and 1.0 or 0.1 mL of the appropriate dilutions were mixed or spread on the following non-selective and selective media: (i) de Man-Rogosa-Sharpe (MRS; 401728, Biolife, Milan, Italy) for LAB, adjusted to pH 5.7 and supplemented with 0.05% (w/v) cycloheximide (AppliChem GmbH, Darmstadt, Germany), incubated at 30 °C for 48-72 h; (ii) Rose Bengal Chloramphenicol agar (RBC; LAB036, LAB M, Lancashire, UK) for yeasts/molds, supplemented with selective supplement X009 (Bury, United Kingdom), incubated at 25 °C for 48-72 h; (iii) Violet Red Bile Glucose agar (VRBGA; 4021882, Biolife) for *Enterobacteriaceae*, incubated at 37 °C for 24 h; (iv) Pseudomonas agar base (CFC; LAB108, LAB M)) for *Pseudomonas* spp., supplemented with CFC supplement (X108), incubated at 25°C for 48-72 h; (v) Baird-Parker agar (BP; LAB285, LAB M)) for *S. aureus,* supplemented with egg yolk (X075), incubated at 37 °C for 48 h, and (vi) total viable counts on Plate Count Agar (PCA; Biolife)) incubated at 25 °C for 48-72 h. Each sample was microbiologically analyzed in duplicate and the results were expressed as log values of colony forming units per gram (log CFU/g) of olives ± standard deviation.

Determinations of pH, water activity (a_w), salt and moisture content in dry-salted olive pulp were performed as detailed elsewhere (Garrido-Fernández et al., 1997; Panagou et al., 2002; Argyri et al., 2015). Specifically, the pH of the olives was determined in a sample (50 g) of olive mesocarp that was subjected to homogenization at room

temperature using an Ultra Turrax T25 blender (IKA Labortecknik, Staufen, Germany) in 50 mL of distilled water. The pH was determined by immersing the electrode of the digital pH-meter (Orion 940, Orion Research Inc., Boston, MA, USA) in the olive paste using three independent samples of olive paste. Olive moisture was determined by oven drying five portions of the homogenized paste (ca. 5 g each) at 105 °C until constant weight. The water activity (a_w) of the olives was measured with an Aqualab 4TE apparatus (Meter Group Inc., Pullman, WA, USA). Four to five pieces of olive pulp were placed in the disposable cap of the instrument and a_w was determined in triplicate. For the determination of sodium chloride content, 10 g of olive paste were diluted in 90 mL of distilled water. The suspension was filtered through filter paper and 10 mL of the filtrate were titrated using a standardized solution of 0.05 N $AgNO_3$ with potassium chromate solution as indicator and expressed as % NaCl per 100 g olive pulp. Finally, the values of salt content in olive juice were determined according to the method described by García-Serrano et al. (2023) taking into account the humidity of the samples and expressed as % NaCl per 100 mL of olive juice. This was considered necessary, as international regulatory standards for table olives express salt content per 100 mL of olive juice and not 100 mL of olive pulp. Sodium chloride determinations were performed in triplicate using three independent samples of olive paste. In all physicochemical determinations, results were expressed as mean values ± standard deviation.

2.3 Yeasts characterization using culture dependent and independent molecular techniques

2.3.1 Yeasts isolation, samples preparation and DNA extraction

Twenty percent (20%) of the colonies were randomly selected from Plate Count Agar (PCA) plates from each sample of dry-salted olives according to Harrigan (1998). The isolated colonies were purified by successive streaking on Yeast Mold Agar (YM) medium consisting of 0.5% Bacteriological Peptone (Biolife), 0.3% Yeast extract (LAB M, Lancashire, UK), 0.3% Malt extract (LAB M), and 1% Dextrose (Merck, Darmstadt, Germany), incubated at 25 ^{o}C for 48 h. After incubation, the colonies were subjected to visual inspection, catalase and oxidase assays, Gram-staining and microscopic observation. Pure cultures were maintained at -80 °C in YM

broth medium supplemented with 20% glycerol. A total of 180 colonies (20 colonies selected per sample × 9 samples) were subjected to culture dependent molecular analysis. Yeast isolates were numerically coded from 1 to 180.

For culture independent analysis, 10 g of olives were placed into a filtered stomacher bag and 20 mL of sterilized Ringer's solution was added to wash the olives. After 2 min of mild malaxation, the solution was discarded to remove the non/loosely attached cells on olive epidermis. Then, 40 mL of the same diluent were added and a second mild malaxation followed to detach the biofilms from the olives. Finally, the suspension was subjected to centrifugation for 10 min (5000 × *g* at 4 ºC) to collect the pellet.

DNA extraction was performed for the total number of the 180 purified isolates and the nine samples of olives according to Bonatsou et al. (2018) with an extra step of DNA re-suspension in elution buffer containing 1 µL RNase followed by incubation at 40 ºC for 30 min before quantification.

2.3.2 Culture dependent molecular analysis

2.3.2.1 Repetitive DNA amplification (rep-PCR)

Genotypic diversity was assessed in a final volume of 25 µL containing 1.5 µL mM $MgCl_2$, 0.5 µL $(GTG)_5$ primer, 0.2 µLTaq polymerase (KAPA Taq PCR kit, KAPA Biosystems, United States), 0.5 µL dNTP's and 2 µL DNA of template DNA (100 ng/µL). Amplification was carried out in a thermocycler (ProFlex PCR System, Applied Biosystems, Milan, Italy) under the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 40 °C for 1 min, and 65 °C for 8 min; and a final extension at 65 °C for 16 min. All PCR products were separated by gel electrophoresis in 2% agarose (Biorad, Madrid, Spain) in TBE 1X at 120 V for 2 h and gels were scanned in UVIpro Platinum (Uvitec) using the software UV proplatinum 1.1 (Eppendorf, Hamburg, Germany). 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) cluster analysis. Isolates with identification percentage higher than 90% were considered to belong to the same cluster.

2.3.2.2 Internal Transcribed Spacer Regions (SSU) PCR

From each group derived by cluster analysis with rep-PCR, one representative isolate was selected for ITS PCR amplification, using ITS_1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS_4 (5' – TCCTCCGCTTATTGATATGC – 3') as primers (White et al., 1990). Amplification of ITS regions was performed in a final volume of 50 µL containing 3 µL $MgCl_2$, 1 µL of each primer, 0.3 µL Taq polymerase (Applied Biosystems) 1µL dNTP's and 75 ng of template DNA. Amplification was carried out under the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 1 min (Esteve-Zarzoso et al., 1999) Amplicons were separated in 1.5% (w/v) agarose in TBE 1X at 100 V for 20 min. For each DNA amplicon, the RFLP method was performed for further classification, as described below.

2.3.2.3 Restriction Fragment Length Polymorphism (RFLP)

RFLP was performed according to Esteve-Zarzoso et al. (1999) using three restrictive endonucleases namely, Hinf I, Hae III, and Cfo I. For each enzymatic reaction with a total volume of 15 µL, 10 µL from each amplicon of ITS-PCR and 0.5 µL of the enzymes were used, followed by incubation at 37 °C for 90 min in a thermocycler ProFlex PCR System (Applied Biosystems) and electrophoresis in 1.5% agarose gel in TBE 1X at 120 V for 80 min. The analysis and comparison of the electrophoretic profiles of the isolates after ITS-amplification and RFLP, resulted in classification of the isolates to further groups. One representative isolate from each group was selected for sequencing after 26S RNA amplification. Amplicons were sent for sequencing to GATC-Biotech (Cologne, Germany). To determine the closest known relatives of the 26S rRNA gene sequences obtained, searches were performed in public data libraries (GenBank) with the BLAST search program (http://www.ncbi.nlm.nih.gov/blast/).

2.3.3 Culture independent molecular methods

2.3.3.1 Next Generation Sequencing (NGS)

For the implementation of NGS, amplification and sequencing of the D1/D2 domain of 26S rRNA gene was performed according to Mota-Gutierrez et al. (2019), using primers LS2 and NL4MS Amplicons were then purified and tagged by using the nextera XT index following the illumina metagenomic pipeline. Sequencing was then performed on a MiSeq platform (2X250bp). After sequencing raw.fastq files were first joined by FLASH software and then quality filtered using QIIME 1.9 according to the pipeline of Mota-Gutierrez et al. (2019). Taxonomic assignment was performed against SILVA database. BlastN tool (http://www.ncbi.nlm.nih.gov/blast/) was used to confirm the taxonomy assignment. OTU tables (clustered at 97% of similarity) were rarefied at the lowest number of sequences per sample and filtered for OTUs occurring at 1% of the relative abundance in at least 2 samples.

2.4 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the Statgraphics Centurion XVII ver. 17.2.00 (Statgraphics Technologies Inc., The Plains, Virginia). The Tuckey's HSD (Honest Significant Difference) test was employed to compare mean values and significant differences were determined at $p < 0.05$. In addition, Principal Components Analysis (PCA) was employed as an exploratory unsupervised approach to investigate any association between the abundance (%) of yeast species defined by culture dependent molecular analysis and the physicochemical characteristics of the nine samples of dry-salted olives. PCA was performed with Statistica software version 7.0 (Statsoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1 Microbiological analyses

The results of the microbiological analysis showed that in the majority of the examined samples, yeasts were the dominant microorganisms, with variations in their populations between samples (Table 1). Specifically, sample S6 showed the highest population level with counts up to 6.4 log CFU/g, while samples S1, S4 and S7 had

277 population levels of approximately 5.4-5.7 log CFU/g. Yeast population from samples
278 S2, S5, S8 and S9 was found close to 3.4-3.9 log CFU/g, depending on the sample. In
279 contrast, in sample S3 yeasts were below the detection limit of the enumeration
280 method (< 2.0 log CFU/g). It needs to be noted that total viable counts (TVC)
281 represented the dominant microbiota in each sample and the counts were comparable
282 with the yeast counts, indicating the dominance of yeasts in the dry-salting process in
283 8 out of the 9 samples. However, for sample S3, where no yeasts were enumerated,
284 total viable counts were close to 4.4 log CFU/g. No *Enterobacteriaceae, S. aureus*
285 and *Pseudomonas* spp. were detected in any of the 9 examined dry-salted olive
286 samples. Finally, no LAB could be detected in any sample, with the exception of
287 sample S6, where a high population of 6.0 log CFU/g was detected. The results
288 obtained in this work were in agreement with a previously published work (Panagou,
289 2006), where the impact of dry-salting process was assessed on the physicochemical
290 and microbiological profile of cv. Thassos naturally dry-salted black olives under
291 different packaging conditions followed by storage at 4 and 20 °C. Before dry salting
292 the identified microorganisms were LAB, yeasts, *Enterobacteriaceae* and
293 *Pseudomonas* spp., whereas at the end of the process only yeasts could be
294 enumerated, due to the low a_w (0.761) combined with the high salt concentration
295 (7.4%). In another work (Mantzouridou and Tsimidou, 2011) performed on hot air-
296 dried black olives of the same cultivar, the initial microbiota of the raw material
297 consisted of LAB and yeasts, but in the end of the process the dominant microbiota
298 was yeasts. In the same work, no undesirable microorganisms were detected
299 (*Enterobacteriaceae, Bacillus* spp., *Clostridium* spp., *Staphylococcus aureus*) on
300 olives during storage at 4 and 20 °C, under different packaging conditions for 180
301 days of storage. These authors also reported that the low a_w (0.893), the naturally
302 occurring phenolic compounds and the free fatty acids were inhibitory for the growth
303 of the above microorganisms. Finally, the results obtained in this work were in line
304 with a recently published work (García-Serrano et al., 2023), assessing the
305 microbiological and physicochemical profile of 20 commercially available samples of
306 dehydrated olives using different processing methods from different countries. The
307 authors reported high yeast populations in 4 of these samples, with counts ranging
308 from 3.5 log CFU/g to 4.1 CFU/g. In addition, no LAB and *Enterobacteriaceae* were
309 enumerated on any sample of dehydrated olives with the exception of one sample,
310 where the latter microorganisms were detected in 3.4 log CFU/g that could be

attributed to the high pH (6.10) and a_w (0.91) of the specific sample. In total, all of the aforementioned works, as well as the current work, highlighted the high presence of yeasts in such products, a fact that can be attributed to their physicochemical characteristics attained during the dry-salting process.

3.2 Physicochemical analyses

Naturally black dry-salted olives are a ready-to-eat food containing at least 10% (w/v) NaCl, when these products are preserved by the specific chemical characteristics attained during the dry-salting process, according to the specifications laid out in the trade standard applying to table olives of the International Olive Council (IOC, 2004). On the contrary, no limits are defined for the maximum pH and the minimum acidity expressed in % lactic acid (w/v) of this product, yet a GMP (Good Manufacturing Practice) must be employed throughout the dry-salting process. However, this high salt concentration has been reconsidered in the revised standard for table olives of the Codex Alimentarius (FAO, 1981), providing a reduced minimum NaCl concentration of 8% (w/v) in the final product.

The physicochemical characteristics of the commercial naturally black dry-salted olives are presented in Table 2. The average pH value of the samples was 4.7 (range 4.0-5.0) in agreement with the average pH value reported recently for dehydrated black olives surveyed from 9 countries (García-Serrano et al., 2023) and slightly lower than 5.0-5.2 units reported previously for black dry-salted olives cv. Thassos (Panagou, 2006; Panagou et al., 2002). It needs to be noted that in a previous market survey (Panagou et al., 2006), the pH value of dry-salted olives cv. Thassos obtained from retail outlets was 4.6, which is in line with the mean pH value reported in this work. However, in the same work, dry-salted olives cv. Conservolea presented lower pH values (3.84) indicating that the olives were subjected to fermentation prior to dry-salting (Panagou et al., 2006). It is worth noting that 5 out 9 samples presented pH values higher than 4.6, which is considered a limit for the growth of *Clostridium botulinum* (Raatjes and Smelt, 1979) and thus additional hurdles should be taken into consideration to ensure the safety of the final product. The additional hurdle in this trade preparation is the high salt concentration of the olives. Indeed, the average value of salt content was 6.9 g NaCl/100 g olive pulp. It must be underlined that the IOC

trade standard for table olives (IOC, 2004) defines a minimum salt concentration of 10% (w/v) for this type of olives. However, the trade standard indicates that the salt content should be determined in the olive juice and not in the olive pulp, which is the standard method of analysis (Değirmencioğlu et al., 2014). For this reason, the concentration of salt in the olives was expressed as % NaCl per 100 mL of olive juice taking into consideration the moisture content of the olives and based on the assumption that the salt is concentrated in the aqueous phase of the olive (García-Serrano et al., 2023). Thus, only sample S6 presented NaCl content in the olive juice less than 10% (w/v) and could not meet the specifications of the trade standard of the IOC. Another important parameter is water activity (a_w). It has been reported that a_w ranges from 0.99-0.98 in raw olive drupes to 0.75-0.92 in the final dry-salted product (Panagou, 2006; Ramírez et al., 2013). In this work, the average a_w was 0.75, which is in good agreement with previously published values for Greek dry-salted olives (García-Serrano et al., 2023; Panagou, 2006). It should also be noted that a_w ranged between 0.58 and 0.91 reflecting a lack of standardization in the dry-salting process among the different processors. Another noteworthy observation is that the highest a_w value corresponded to sample S6 that presented also and lowest salt concentration and the highest yeast population (6.4 log CFU/g) along with the presence of LAB in high numbers (6.0 log CFU/g). Finally, the average moisture of the samples was 29.5%, which is in good agreement with the moisture content reported recently for packaged dehydrated Greek dry-salted olives (García-Serrano et al., 2023). However, sample S6 presented a high moisture content close to 57% that could support the growth of fungi during the shelf-life of the product, unless preservatives (e.g., sorbic acid in the form of potassium sorbate) or pasteurization can be applied to inhibit the growth of yeasts and moulds (Casado et al., 2010). As a consequence of high moisture content, sample S6 presented also a high a_w value that when combined with the low salt content of the olives may jeopardize the safety of the product, a fact that was also evident from the microbiological analysis for this sample.

3.3 Culture dependent and culture independent molecular identification

3.3.1 Repetitive DNA amplification (rep-PCR)

After rep-PCR, cluster analysis of the profiles of the 178 yeast isolates was performed using Bionumerics software (Supplementary Fig. 1). The dendrogram

obtained after cumulative image analysis of Rep-PCR patterns resulted in 36 different clusters. The prevalence of the different isolates within each of the 36 clusters is summarized in Table 3. Most isolates (43) were included in Cluster 5 and were recovered from samples S1, S2, S3, S5, and S8 (data not shown). The rest of the isolates were distributed among the remaining clusters without showing any specific trend.

3.3.2 Internal Transcribed Spacer Regions (SSU) PCR and Restriction Fragment Length Polymorphism

One representative isolate of the 36 groups from rep-PCR was selected and consequently subjected to ITS-PCR and RFLP for further clustering. Comparison of the electrophoretic profiles obtained from RFLP resulted in further classification of the isolates in a final number of 20 clusters (Table 3). One isolate of each cluster was subjected to sequencing and taxonomic assessment was performed via BLAST software leading to the final classification of the representative isolates at species level (Table 4). According to Tables 3 and 4, the total number of isolates belonged to 9 species, namely *Candida versatilis*, *Wickerhamomyces subpelliculosus*, *Candida apicola*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii*, *Citeromyces nyonsensis*, *Candida etchelsii*, *Pichia membranifaciens* and *Candida sorbosivorans*. 4 isolates belonging to the same cluster (cluster 3) after the final grouping could not be identified. The identification percentage of the yeast species isolated and identified for each of the nine samples with culture dependent techniques is shown in Figure 1. *Candida etchelsii* was identified in most of the samples, except S4, S6, and S9. Specifically, 100% isolation frequency was observed in sample S3, whereas coexistence with *Candida versatilis* was noticeable for samples S1 (5.0%-95.0%), S7 (50.0%-50.0%), and S8 (65.0%-35.0%), followed by *Citeromyces nyonsensis* for sample S5 (73.7%-21.1%-5.3%). *Pichia membranifaciens* was dominant in samples S6 (90.0%) and S9 (60.0%), followed by *Candida sorbosivorans* with 10.0% and 40.0%, respectively. *Candida etchelsii* was also identified in sample S2 (45.0%) followed by *Wickerhamomyces subpelliculosus* (30.0%) and *Candida apicola* (5.0%). *Wickerhamomyces subpelliculosus* was the dominant species in sample S4 (84.2%) followed by *Torulaspora delbrueckii* (10.5%) and *Wickerhamomyces anomalus*

(5.3%). *Citeromyces nyonsensis* was also identified at a low percentage close to 5% for sample S5, while 20% of the isolates (5 isolates) in sample S2 were characterized as non-identified. The results obtained are in accordance with previous studies, since all identified species have been also isolated from different fermented food matrices and/or environments with low a_w and high salt concentrations. More specifically, *C. etchelsii* and *C. versatilis* which are salt tolerant yeasts are commonly isolated from miso and soy fermentations and are important in flavour enhancing through the production of 4-ethyl guaiacol (4EG) and 4-ethyl phenol (4EP), which are characteristic flavours of soy sauce and miso (Feng et al. 2012; Suezawa et al., 2006). Both species have been previously isolated from bloaters of fermented green table olives during storage (Asehraou et al., 2000). *Candida apicola*, *Pichia membranifaciens* and *Wickerhamomyces anomalus* are well known species related to table olive fermentations (Arroyo-López et al., 2006; Bautista-Gallego et al., 2011; Bleve et al., 2014, 2015; Nisiotou et al., 2010). *Wickerhamomyces subpelliculosus* is a salt-tolerant microorganism previously isolated from fermented cucumbers (Kurtzman et al., 1998) and is considered as an alternative to baker's yeast (Zhou et al., 2017). *Citeromyces nyonensis* is a yeast previously isolated from fermented black olive brines and olive oil (Casaregola et al., 2013), while *Torulaspora delbrueckii* is a well-known yeast presenting high resistance to osmotic stress, as well as biotechnological potential due to aroma-enhancing properties and therefore is widely employed in wine, beer and bread dough fermentations (Fernandes et al., 2021). *T. delbrueckii* has been previously isolated from black olives (Kotzekidou, 1997). *Candida sorbosivorans* is a salt tolerant yeast previously isolated from honey (Carvalho et al., 2010) and more recently isolated during the production of Algerian Smen, a traditional fermented butter (Boussekine et al., 2022).

3.3.3 Amplicons target sequencing

The yeast species identified with amplicons sequencing are illustrated in Figure 2. Due to the high number of yeasts identified, only OTUs with a relative abundance higher than 5% are displayed. For the nine samples of dry-salted olives the dominant yeast species were *Candida etchelsii*, *C. versatilis, P. membranifaciens, C. apicola, P. triangularis*, *Wickerhamomyces anomalus, Aureobasidium pullulans*, *Ogataea*

boidinii and *Hortaea werneckii*. *Candida etchelsii* and *C. versatilis* were the dominant species in most samples with relative abundances ranging from 23.0% to 55.3%, followed by *W. anomalus* (13.4%), *O. boidinii* (7.9%) and *C. apicola* (6.9%) for sample S4, *O. boidinii* (5.5%) for sample S8, *A. pullulans* (11.3%) and *C. apicola* (5.5%) for sample S9. In addition, in samples S2 and S5 the dominant species were *P. triangularis* with relative abundances of 41.0% and 31.0%, respectively, followed by *C. etchelsii* (33.1% and 30.9%, respectively), *C. versatilis* (8.1% and 27.1%, respectively) and *Hortaea werneckii* (7.4%) for sample S2. These results are in accordance with the results obtained from culture dependent techniques in this study since the dominant identified species were common among the two techniques with slight deviations. Specifically, *P. triangularis,* that was identified only with amplicon sequencing, has been previously isolated from environments with high salt concentrations, such as natural fermented Italian sausages (Rantsiou and Cocolin, 2006), soy sauce, sea water and fermented mushrooms (Elbandy et al., 2008; Romero et al., 2012), while *A. pullulans* and *O. boidinii* (or *C. boidinii*) have been previously isolated from natural black olive fermentation (Bonatsou et al., 2018; Coton et al., 2006; Nisiotou et al., 2010). Finally, *H. werneckii* is a black yeast with remarkable tolerance to salt with both pathogenic and spoilage potential that is usually isolated from hypersaline environments (Zalar et al., 2019). Despite the fact that several studies have previously studied microbial populations and physicochemical characteristics of dry-salted olives (Brenes et al., 2022; García-Serrano et al., 2023; Panagou, 2006; Ramírez et al., 2013), it is the first-time microbial diversity of this product is identified using molecular techniques.

In order to clarify the relationship between the physicochemical parameters and the yeast species identification after ITS PCR analysis of dry-salted naturally black olives samples, PCA was performed, and results are presented in Fig. 3. The plot of the PCs enables the investigation of correlation between the variables in the input dataset. The projection of the variables in the subspace of the first two PCs explained 57.8% of the information included in the raw data and allowed the segregation of identified yeast species in different clusters. In this way, *P. membranifaciens* and *C. sorbosivorans* were positively correlated with the vector of moisture content and a_w (Fig. 3A) and they were also associated with sample S6 (Fig. 3B) that presented the highest moisture (56.7%) and a_w (0.91) from all samples analyzed. Moreover, *C.*

*etchelsi*i and *C. nyonsensis* were associated with the vectors of salt level in the pulp and olive juice and they were related to samples S3 and S5 that presented the highest levels of salt (Table 3). *C. versatilis* was in the same quadrant as a_w and it was correlated with samples S1, S7 and S8 that presented a_w values of 0.77 and 0.79. These samples were located close to each other demonstrating that there is a positive correlation between them and a negative correlation with samples S2 and S4 located on the diagonally opposite quadrant. Finally, sample S4 was associated with *T. delbrueckii* and *W. anomalus*, whereas sample S2 was correlated with *W. subpelliculosus* and *C. apicola*. Both samples were positioned in diagonally opposed quadrants with the vector for a_w, meaning that they had the lowest a_w values. This is more evident for sample S2 that presented the lowest a_w (0.58) among all samples analyzed. Concerning the distribution of samples in the plot of loadings (Fig. 3B), PC1 could be associated with the parameter of a_w, since there is a transition from lower (a_w<0.72, left side) to higher (a_w>0.77, right side) a_w values.

4. Conclusion

Nine samples of naturally black dry-salted olives from the Greek retail market were analyzed for the determination of their physicochemical characteristics and microbial diversity, using both culture dependent and independent molecular techniques. The values of the physicochemical characteristics showed great variability reflecting the lack of standardization of the dry-salting process. Moreover, the diversity of yeasts was elucidated in final products obtained from the market. *Pichia membranifaciens*, *Candida sorbosivorans*, *Citeromyces nyonsensis*, *Candida etchelsii*, *Wickerhamomyces subpelliculosus*, *Candida apicola*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii* and *Candida versatilis* were the dominant yeast species identified with ITS sequencing, while NGS revealed higher yeast diversity including *Candida etchelsii, Candida versatilis, Pichia membranifaciens, Candida apicola, Pichia triangularis, Wickerhamomyces anomalus, Pichia membranifaciens, Aureobasidium pullulans, Ogataea boidinii and Hortaea werneckii.* The genotypic profile obtained from culture dependent techniques is similar to the profile obtained by NGS, indicating that despite the discrimination power of NGS, validation with the traditional sequencing methods is required for the classification of the microbial

diversity. Finally, this study provides a comparison among culture dependent and independent molecular techniques for the identification of the microbial diversity of dry-salted olives. The obtained results are important, since they enhance our knowledge on the microbial ecology of naturally black dry-salted olives and specifically contribute to a better understanding of the microbial diversity present due to the different physicochemical parameters among samples. Further research into the evaluation of the technological characteristics and multifunctional potential of the dominant yeast species may result in better control of the dry-salting process, improve the nutritional value and enhance the quality and shelf-life of the final product.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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677 **Table 1.** Population of yeasts, Total Viable Counts (TVC), *Enterobacteriaceae,*
678 *Staphylococcus aureus, Pseudomonas* spp. and Lactic Acid Bacteria (LAB) (log CFU/g ±
679 standard deviation) of naturally black dry-salted olive samples from retail outlets.

Sample	Yeasts	TVC	*Enterobacteriaceae*	*S. aureus*	*Pseudomonas spp.*	LAB
S1	5.4 ± 0.2a	5.3 ± 0.1a	< 1.0	< 2.0	< 2.0	< 1.0
S2	3.9 ± 0.1b	4.2 ± 0.1b	< 1.0	< 2.0	< 2.0	< 1.0
S3	< 2.0c	4.4 ± 0.7b	< 1.0	< 2.0	< 2.0	< 1.0
S4	5.7 ± 0.3d	5.8 ± 0.4c	< 1.0	< 2.0	< 2.0	< 1.0
S5	3.2 ± 0.1e	3.4 ± 0.2d	< 1.0	< 2.0	< 2.0	< 1.0
S6	6.4 ± 0.1f	6.4 ± 0.1e	< 1.0	< 2.0	< 2.0	6.0 ± 0.4
S7	5.7 ± 0.2d	6.0 ± 0.1c	< 1.0	< 2.0	< 2.0	< 1.0
S8	3.4 ± 0.6e	4.9 ± 0.2f	< 1.0	< 2.0	< 2.0	< 1.0
S9	3.4 ± 0.1e	4.9 ± 0.1f	< 1.0	< 2.0	< 2.0	< 1.0

680 For each microbial group, values followed by a different lowercase letter in the same column define
681 statistically significant differences according to Tuckey's test ($p < 0.05$).

682 **Table 2.** Physicochemical characteristics of naturally black dry-salted olive samples from
683 retail outlets.

Sample	pH[(a)]	a_w[(b)]	Moisture (%)	NaCl (%)[(c)]	NaCl (%)[(d)]
S1	4.4a	0.79a	28.21±2.13a	8.22±0.13a	18.69±0.31a
S2	4.9b	0.58b	17.33±1.89c	6.18±0.1b	20.92±0.34b
S3	5.0c	0.70c	26.83±1.38ab	7.90±0.14c	18.67±0.33a
S4	4.8b	0.70c	27.77±1.01a	7.03±0.26d	16.18±0.60c
S5	5.0c	0.72d	28.05±2.16a	9.15±0.16e	20.81±0.37b
S6	4.6e	0.91e	56.65±2.16d	5.26±0.14f	7.33±0.20e
S7	4.1f	0.77f	27.69±1.77a	6.43±0.12g	14.84±0.28d
S8	4.9b	0.77f	27.12±1.82ab	6.44±0.01g	15.08±0.05cd
S9	4.0g	0.83g	24.67±2.35b	5.61±0.01h	14.18±0.04d

684 [(a),(b)] Standard deviation values for pH and a_w were < 0.01 and 0.001, respectively; [(c)] g NaCl/100 g of
685 olive pulp; [(d)] g NaCl/100 mL juice
686 For each physicochemical parameter, values followed by a different lowercase letter define statistically
687 significant difference according to Tuckey's test ($p < 0.05$)

688 **Table 3.** Clustering of yeast isolates from dry-salted olives according to repetitive extragenic
689 palindromic DNA sequence-based PCR (REP-PCR) and subsequent clustering of
690 representative isolates using RFLP.

Clusters after REP-PCR	Isolates within each cluster	Clusters after RFLP	Isolates within each cluster
1	6	1	3
2	1	2	3
3	9	3	2
4	9	4	1
5	43	5	1
6	3	6	2
7	2	7	3
8	1	8	2
9	12	9	3
10	1	10	2
11	1	11	1
12	1	12	2
13	1	13	2
14	1	14	1
15	7	15	1
16	7	16	1
17	2	17	3
18	1	18	1
19	17	19	1
20	4	20	1
21	1		
22	1		
23	5		
24	1		
25	1		
26	3		
27	1		
28	1		
29	1		
30	1		
31	5		
32	9		
33	1		
34	10		
35	7		
36	1		

691 .

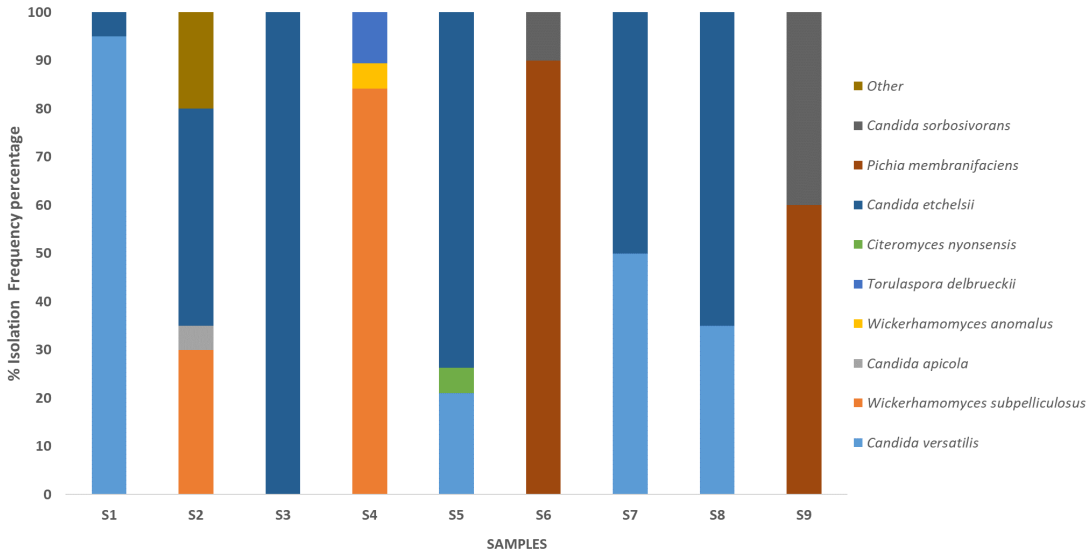
692

693 **Table 4.** Species identification of yeast isolates according to the RFLP clustering (20 clusters)

Cluster number	Species (ITS PCR)	Number of isolates [a]
1 and 17	*Candida versatilis*	40
2	*Wickerhamomyces subpelliculosus*	7
4	*Candida apicola*	1
5 and 7	*Wickerhamomyces anomalus*	16
6	*Torulaspora delbrueckii*	2
8	*Citeromyces nyonsensis*	1
9, 13, 16, 18, and 19	*Candida etchelsii*	78
10 and 11	*Pichia membranifaciens*	18
12, 14, 15, and 20	*Candida sorbosivorans*	11
3*	–	–

694 * Isolate from cluster 3 was not identified.

695 [a]: abundance of isolates in each cluster based on Table 3

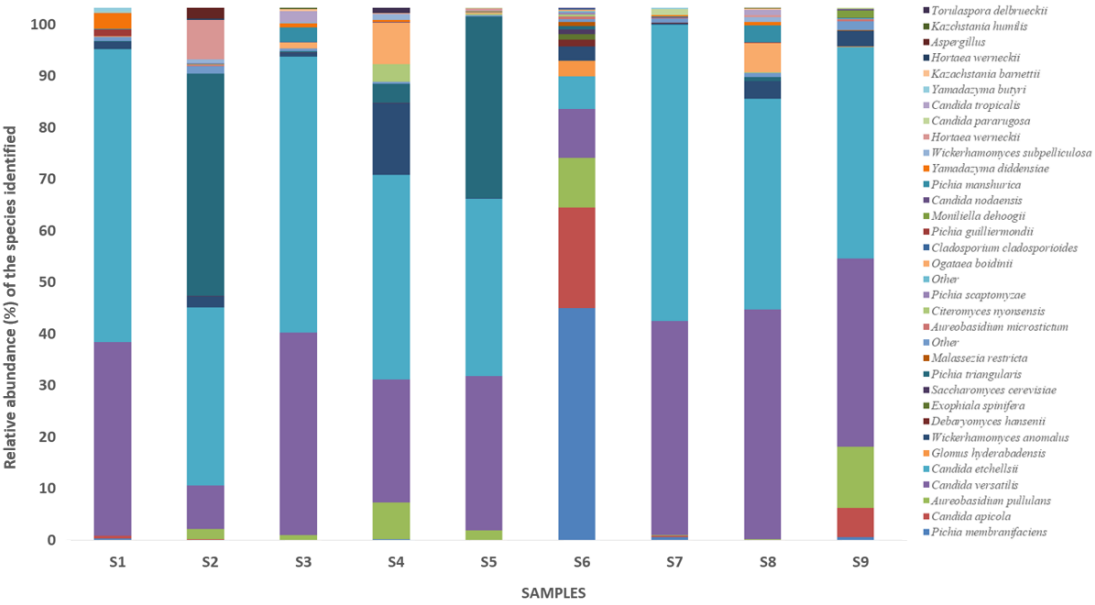


696

697 **Fig. 1.** Isolation frequency (%) of the yeast species for the nine samples of dry-salted olives
698 with culture dependent techniques.

699

700



701

702 **Fig. 2.** Relative abundance (%) of yeast species for the nine samples of dry-salted olives
703 derived by amplicon sequencing.

704

705

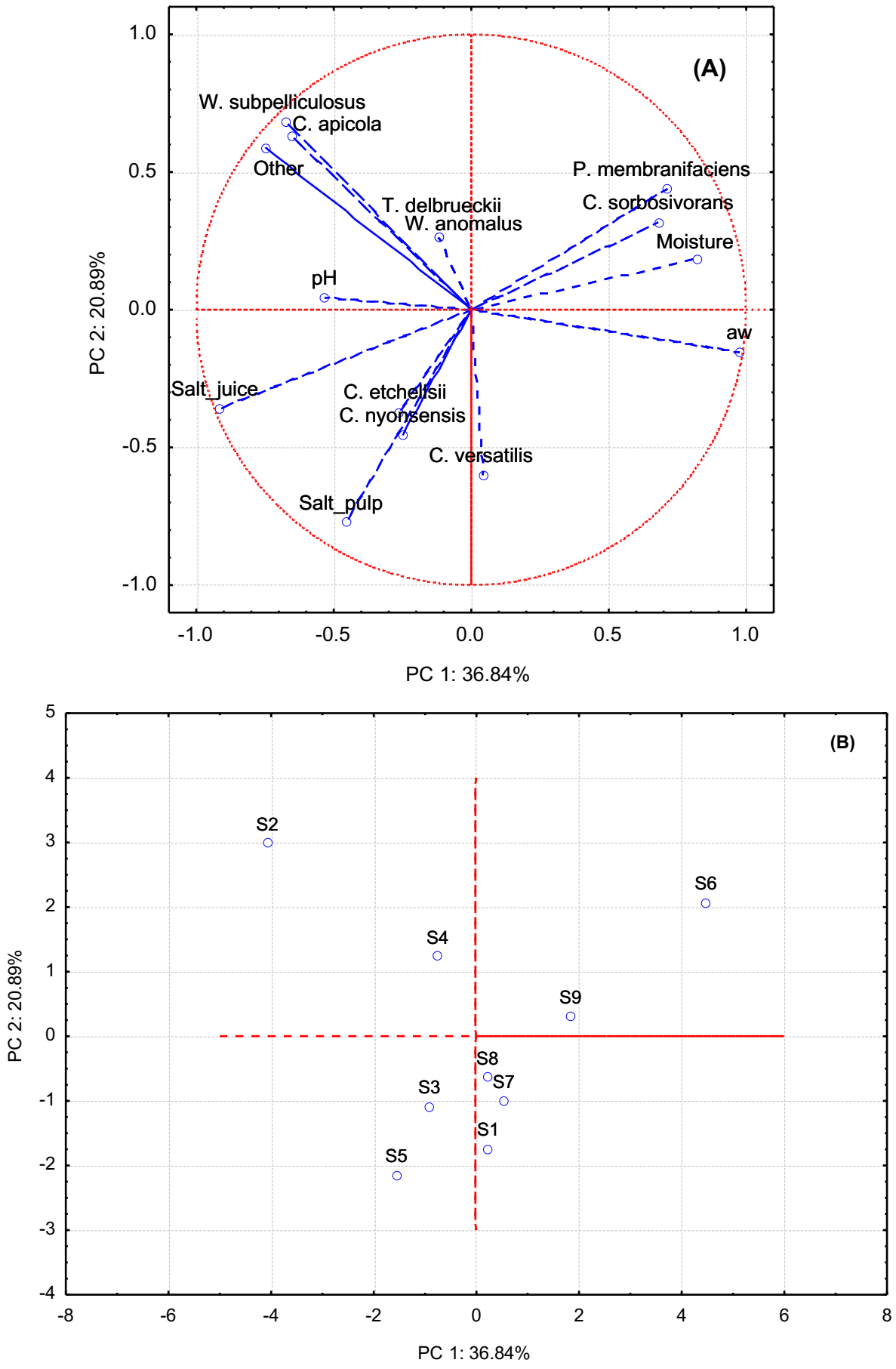
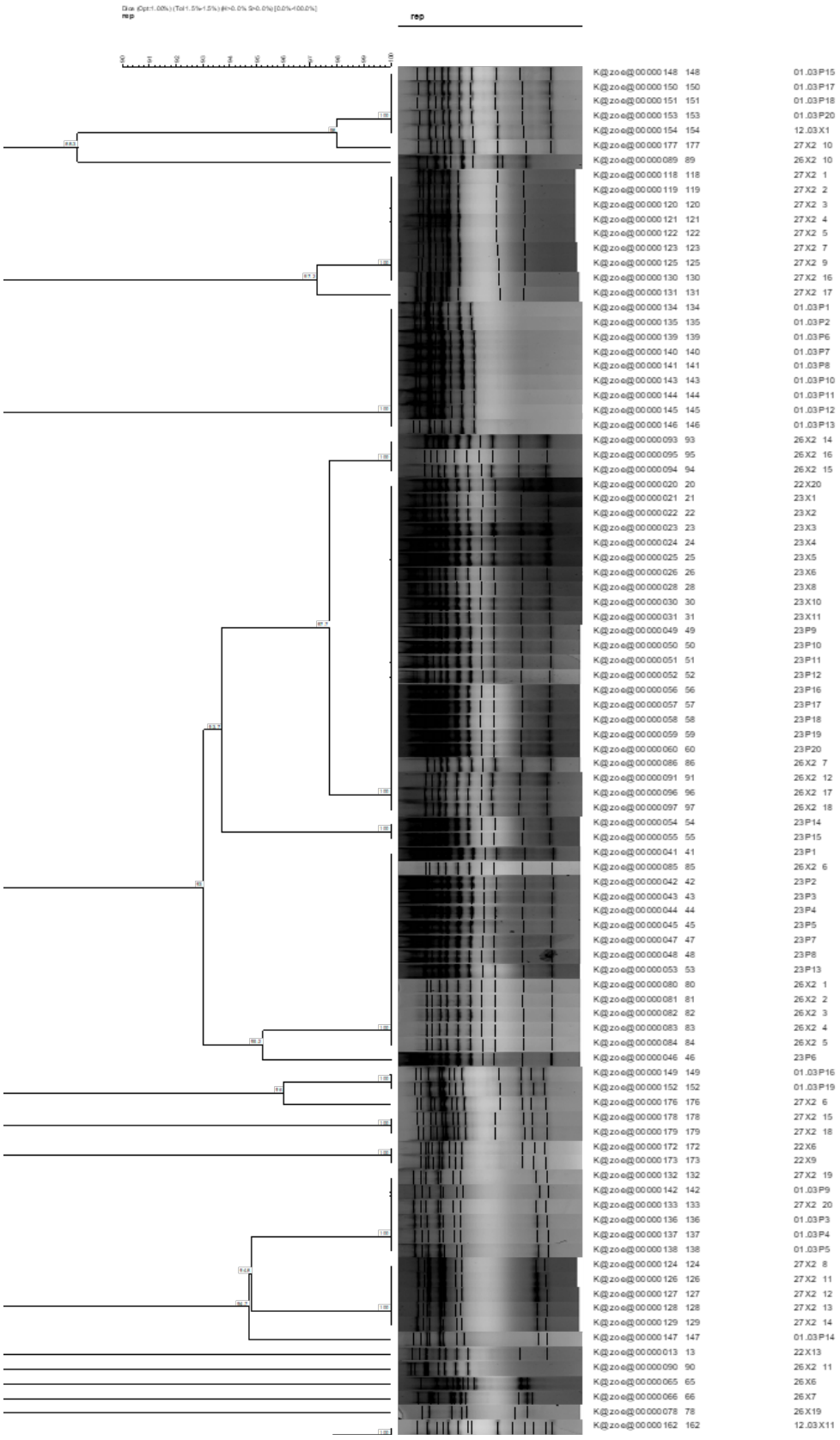


Fig. 3. PCA plot of loadings (A) (abundance of yeasts and physicochemical parameters) and scores (B) (samples of dry salted olives) of the first two principal components (PCs).

737 **Supplementary Material**



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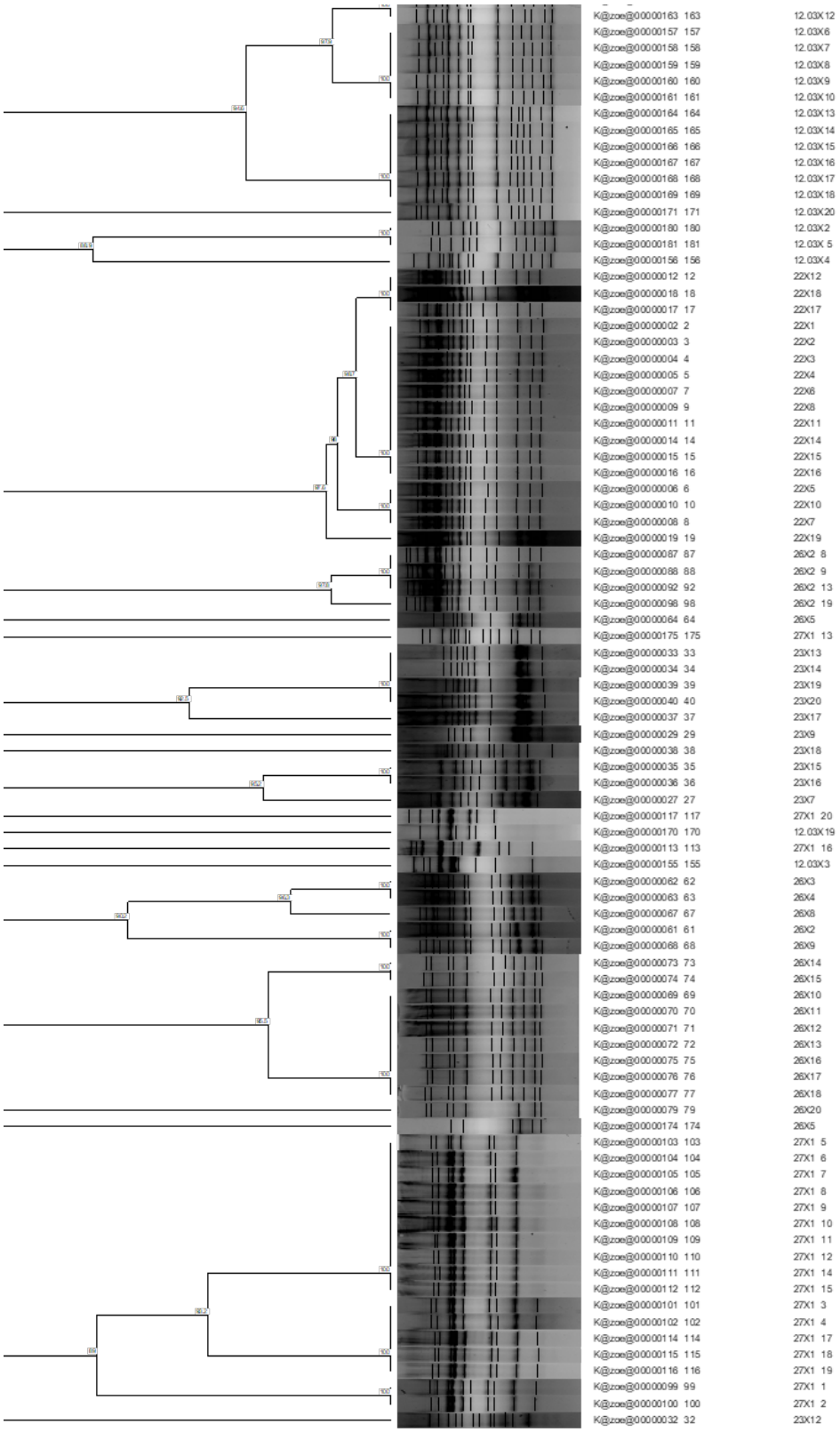

741

742 **Supplementary Fig. 1.** Cluster analysis of yeast isolates after rep-PCR with $(GTG)_5$ primer.