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

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

4

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23 **Abstract**

24 In the present study, the physicochemical (pH, water activity, moisture content,  
25 salt concentration) classical plate counts (total viable counts, yeasts, lactic acid  
26 bacteria, *Staphylococcus aureus*, *Pseudomonas* spp., *Enterobacteriaceae*) and  
27 amplicon sequencing of naturally black dry-salted olives obtained from different retail  
28 outlets of the Greek market were investigated. According to the results, the values of  
29 the physicochemical characteristics presented great variability among the samples.  
30 Specifically, pH and water activity ( $a_w$ ) values ranged between 4.0 and 5.0, as well as  
31 between 0.58 and 0.91, respectively. Moisture content varied between 17.3 and 56.7%  
32 (g H<sub>2</sub>O/100 g of olive pulp), whereas salt concentration ranged from 5.26 to 9.07% (g  
33 NaCl/100 g of olive pulp). No lactic acid bacteria, *S. aureus*, *Pseudomonas* spp. and  
34 *Enterobacteriaceae* were detected. The mycobiota consisted of yeasts that were  
35 further characterized and identified by culture-dependent (rep-PCR, ITS-PCR, and  
36 RFLP) and amplicon target sequencing (ATS). *Pichia membranifaciens*, *Candida*  
37 *sorbosivorans*, *Citeromyces nyonsensis*, *Candida etchelsii*, *Wickerhamomyces*  
38 *subpelliculosus*, *Candida apicola*, *Wickerhamomyces anomalus*, *Torulaspora*  
39 *delbrueckii* and *Candida versatilis* were the dominant species according to ITS  
40 sequencing (culture-dependent), while ATS revealed the dominance of *C. etchelsii*,  
41 *Pichia triangularis*, *P. membranifaciens*, and *C. versatilis* among samples. The results  
42 of this study demonstrated considerable variability in quality attributes among the  
43 different commercial samples of dry-salted olives, reflecting a lack of standardization  
44 in the processing of this commercial style. However, the majority of the samples were  
45 characterized by satisfactory microbiological and hygienic quality and complied with  
46 the requirements of the trade standard for table olives of the International Olive  
47 Council (IOC) for this processing style in terms of salt concentration. In addition, the  
48 diversity of yeast species was elucidated for the first time in commercially available  
49 products, increasing our knowledge on the microbial ecology of this traditional food.  
50 Further investigation into the technological and multifunctional traits of the dominant  
51 yeast species may result in better control during dry-salting and enhance the quality  
52 and shelf-life of the final product.

53

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

## 56 1. Introduction

57 Naturally black dry-salted olives are traditionally produced in the island of  
58 Thassos in Northern Greece, using the olives of a local table olive cultivar (cv.  
59 Thassos), although any other cultivar of black or alkali treated green olives can be  
60 processed in the same way to produce dry-salted olives (Brenes et al., 2022). Olives  
61 are harvested when fully mature (the surface color of the drupes is black) or overripe  
62 and subsequently placed in concrete tanks in layers with coarse salt (Panagou et al.,  
63 2002). Under these conditions, practically no fermentation takes place and for this  
64 reason dry salting is characterized as a “curing” or “desiccation” process (Panagou,  
65 2006; Ramirez et al., 2013). Due to the high osmotic pressure exerted by the salt,  
66 water and water-soluble components, such as oleuropein, are gradually removed from  
67 the mesocarp, resulting in gradual debittering of olives that are ready for consumption  
68 after 30-40 days (Değirmencioglu et al., 2014). From the organoleptic point of view,  
69 dry-salted olives are considered to have a slight bitter and sweet taste, which is well  
70 accepted by consumers, but the high salt content needed for the preservation of the  
71 final product makes it unattractive to international markets. According to the CXS 66-  
72 1981 standard of table olives of the Codex Alimentarius (FAO, 1981) that was revised  
73 in 2013, the minimum salt content for this trade preparation of olives should be 8%  
74 (w/v). Consequently, the shelf-life of the final product is ensured by the high salt  
75 concentration and the corresponding low water activity ( $a_w$ ) of dry-salted olives. It has  
76 been reported that the  $a_w$  level of the final product ranges between 0.75 and 0.85,  
77 depending on salt concentration during the dry-salting process and therefore only salt-  
78 tolerant microorganisms, such as yeasts, could become the dominant microbiota  
79 (Panagou, 2006; Panagou et al., 2002; Papagora et al., 2013). Among the pathogenic  
80 microorganisms, the presence of *Staphylococcus aureus* should be taken into serious  
81 consideration, because of its ability to grow in low  $a_w$  levels ( $a_w < 0.86$ ), where it  
82 presents a generation time of 300 min (Adams and Moss, 2008).

83 Taxonomic identification of microbial species is a difficult process that requires  
84 the application of several genetic markers to be sequenced. Several studies related to  
85 the microbial diversity of fermented products have employed amplicon sequencing  
86 techniques (Bokulich and Mills, 2012; Cocolin et al., 2013; Ercolini et al., 2012,  
87 2013; Jampaphaeng et al., 2017; Mota-Gutierrez et al., 2019). However, new  
88 approaches to explore diversity using next generation sequencing technologies (NGS)

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

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

115

## 116 **2. Materials and Methods**

### 117 *2.1 Olive samples*

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

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

126

## 127 *2.2 Microbiological and physicochemical analyses*

128 Lactic acid bacteria (LAB), yeasts, *Enterobacteriaceae*, *Pseudomonas* spp., total  
129 mesophilic counts and *Staphylococcus aureus* were determined for each sample in  
130 duplicate. Twenty-five grams (25 g) of olive pulp were aseptically added in 225 mL  
131 sterile 1/4 Ringer's solution and homogenized in a Stomacher device (LabBlender,  
132 Seward Medical, London, UK) for 60 s at room temperature. The resulting suspension  
133 was serially diluted in the same diluent and 1.0 or 0.1 mL of the appropriate dilutions  
134 were mixed or spread on the following non-selective and selective media: (i) de Man-  
135 Rogosa-Sharpe (MRS; 401728, Biolife, Milan, Italy) for LAB, adjusted to pH 5.7 and  
136 supplemented with 0.05% (w/v) cycloheximide (AppliChem GmbH, Darmstadt,  
137 Germany), incubated at 30 °C for 48-72 h; (ii) Rose Bengal Chloramphenicol agar  
138 (RBC; LAB036, LAB M, Lancashire, UK) for yeasts/molds, supplemented with  
139 selective supplement X009 (Bury, United Kingdom), incubated at 25 °C for 48-72 h;  
140 (iii) Violet Red Bile Glucose agar (VRBGA; 4021882, Biolife) for  
141 *Enterobacteriaceae*, incubated at 37 °C for 24 h; (iv) *Pseudomonas* agar base (CFC;  
142 LAB108, LAB M) for *Pseudomonas* spp., supplemented with CFC supplement  
143 (X108), incubated at 25°C for 48-72 h; (v) Baird-Parker agar (BP; LAB285, LAB M)  
144 for *S. aureus*, supplemented with egg yolk (X075), incubated at 37 °C for 48 h, and  
145 (vi) total viable counts on Plate Count Agar (PCA; Biolife)) incubated at 25 °C for  
146 48-72 h. Each sample was microbiologically analyzed in duplicate and the results  
147 were expressed as log values of colony forming units per gram (log CFU/g) of olives  
148 ± standard deviation.

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

153 temperature using an Ultra Turrax T25 blender (IKA Labortechnik, Staufen,  
154 Germany) in 50 mL of distilled water. The pH was determined by immersing the  
155 electrode of the digital pH-meter (Orion 940, Orion Research Inc., Boston, MA, USA)  
156 in the olive paste using three independent samples of olive paste. Olive moisture was  
157 determined by oven drying five portions of the homogenized paste (ca. 5 g each) at  
158 105 °C until constant weight. The water activity ( $a_w$ ) of the olives was measured with  
159 an Aqualab 4TE apparatus (Meter Group Inc., Pullman, WA, USA). Four to five  
160 pieces of olive pulp were placed in the disposable cap of the instrument and  $a_w$  was  
161 determined in triplicate. For the determination of sodium chloride content, 10 g of  
162 olive paste were diluted in 90 mL of distilled water. The suspension was filtered  
163 through filter paper and 10 mL of the filtrate were titrated using a standardized  
164 solution of 0.05 N AgNO<sub>3</sub> with potassium chromate solution as indicator and  
165 expressed as % NaCl per 100 g olive pulp. Finally, the values of salt content in olive  
166 juice were determined according to the method described by García-Serrano et al.  
167 (2023) taking into account the humidity of the samples and expressed as % NaCl per  
168 100 mL of olive juice. This was considered necessary, as international regulatory  
169 standards for table olives express salt content per 100 mL of olive juice and not 100  
170 mL of olive pulp. Sodium chloride determinations were performed in triplicate using  
171 three independent samples of olive paste. In all physicochemical determinations,  
172 results were expressed as mean values  $\pm$  standard deviation.

173

### 174 *2.3 Yeasts characterization using culture dependent and independent molecular* 175 *techniques*

#### 176 *2.3.1 Yeasts isolation, samples preparation and DNA extraction*

177 Twenty percent (20%) of the colonies were randomly selected from Plate Count  
178 Agar (PCA) plates from each sample of dry-salted olives according to Harrigan  
179 (1998). The isolated colonies were purified by successive streaking on Yeast Mold  
180 Agar (YM) medium consisting of 0.5% Bacteriological Peptone (Biolife), 0.3% Yeast  
181 extract (LAB M, Lancashire, UK), 0.3% Malt extract (LAB M), and 1% Dextrose  
182 (Merck, Darmstadt, Germany), incubated at 25 °C for 48 h. After incubation, the  
183 colonies were subjected to visual inspection, catalase and oxidase assays, Gram-  
184 staining and microscopic observation. Pure cultures were maintained at -80 °C in YM

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

188 For culture independent analysis, 10 g of olives were placed into a filtered  
189 stomacher bag and 20 mL of sterilized Ringer's solution was added to wash the  
190 olives. After 2 min of mild malaxation, the solution was discarded to remove the  
191 non/loosely attached cells on olive epidermis. Then, 40 mL of the same diluent were  
192 added and a second mild malaxation followed to detach the biofilms from the olives.  
193 Finally, the suspension was subjected to centrifugation for 10 min ( $5000 \times g$  at  $4^\circ\text{C}$ )  
194 to collect the pellet.

195 DNA extraction was performed for the total number of the 180 purified isolates  
196 and the nine samples of olives according to Bonatsou et al. (2018) with an extra step  
197 of DNA re-suspension in elution buffer containing  $1 \mu\text{L}$  RNase followed by  
198 incubation at  $40^\circ\text{C}$  for 30 min before quantification.

199

### 200 *2.3.2 Culture dependent molecular analysis*

#### 201 *2.3.2.1 Repetitive DNA amplification (rep-PCR)*

202 Genotypic diversity was assessed in a final volume of  $25 \mu\text{L}$  containing  $1.5 \mu\text{L}$   
203 mM  $\text{MgCl}_2$ ,  $0.5 \mu\text{L}$  (GTG)<sub>5</sub> primer,  $0.2 \mu\text{L}$  Taq polymerase (KAPA Taq PCR kit,  
204 KAPA Biosystems, United States),  $0.5 \mu\text{L}$  dNTP's and  $2 \mu\text{L}$  DNA of template DNA  
205 ( $100 \text{ ng}/\mu\text{L}$ ). Amplification was carried out in a thermocycler (ProFlex PCR System,  
206 Applied Biosystems, Milan, Italy) under the following conditions: initial denaturation  
207 at  $95^\circ\text{C}$  for 5 min; 30 cycles at  $95^\circ\text{C}$  for 30 s,  $40^\circ\text{C}$  for 1 min, and  $65^\circ\text{C}$  for 8 min;  
208 and a final extension at  $65^\circ\text{C}$  for 16 min. All PCR products were separated by gel  
209 electrophoresis in 2% agarose (Biorad, Madrid, Spain) in TBE 1X at 120 V for 2 h  
210 and gels were scanned in UVIpro Platinum (Uvitec) using the software UV  
211 proplatinum 1.1 (Eppendorf, Hamburg, Germany). Analysis was performed with  
212 Bionumerics ver. 6.1 software (Applied Maths, Sint-Martens-Latem, Belgium) using  
213 the Dice coefficient and the unweighted pair group method with arithmetic mean  
214 (UPGMA) cluster analysis. Isolates with identification percentage higher than 90%  
215 were considered to belong to the same cluster.



216

#### 217 *2.3.2.2 Internal Transcribed Spacer Regions (ITS) PCR*

218 From each group derived by cluster analysis with rep-PCR, one representative  
219 isolate was selected for ITS PCR amplification, using ITS<sub>1</sub> (5'–  
220 TCCGTAGGTGAACCTGCGG–3') and ITS<sub>4</sub> (5' – TCCTCCGCTTATTGATATGC  
221 – 3') as primers (White et al., 1990). Amplification of ITS regions was performed in  
222 a final volume of 50 µL containing 3 µL MgCl<sub>2</sub>, 1 µL of each primer, 0.3 µL Taq  
223 polymerase (Applied Biosystems) 1µL dNTP's and 75 ng of template DNA.  
224 Amplification was carried out under the following conditions: initial denaturation at  
225 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min;  
226 and a final extension at 72 °C for 1 min (Esteve-Zarzoso et al., 1999) Amplicons were  
227 separated in 1.5% (w/v) agarose in TBE 1X at 100 V for 20 min. For each DNA  
228 amplicon, the RFLP method was performed for further classification, as described  
229 below.

230

#### 231 *2.3.2.3 Restriction Fragment Length Polymorphism (RFLP)*

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

245

#### 246 *2.3.3 Culture independent molecular methods*

### 247 2.3.3.1 Next Generation Sequencing (NGS)

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

259

### 260 2.4 Statistical analysis

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

270

## 271 3. Results and discussion

### 272 3.1 Microbiological analyses

273 The results of the microbiological analysis showed that in the majority of the  
274 examined samples, yeasts were the dominant microorganisms, with variations in their  
275 populations between samples (Table 1). Specifically, sample S6 showed the highest  
276 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

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

315

### 316 3.2 *Physicochemical analyses*

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

327 The physicochemical characteristics of the commercial naturally black dry-salted  
328 olives are presented in Table 2. The average pH value of the samples was 4.7 (range  
329 4.0-5.0) in agreement with the average pH value reported recently for dehydrated  
330 black olives surveyed from 9 countries (García-Serrano et al., 2023) and slightly  
331 lower than 5.0-5.2 units reported previously for black dry-salted olives cv. Thassos  
332 (Panagou, 2006; Panagou et al., 2002). It needs to be noted that in a previous market  
333 survey (Panagou et al., 2006), the pH value of dry-salted olives cv. Thassos obtained  
334 from retail outlets was 4.6, which is in line with the mean pH value reported in this  
335 work. However, in the same work, dry-salted olives cv. Conservolea presented lower  
336 pH values (3.84) indicating that the olives were subjected to fermentation prior to dry-  
337 salting (Panagou et al., 2006). It is worth noting that 5 out of 9 samples presented pH  
338 values higher than 4.6, which is considered a limit for the growth of *Clostridium*  
339 *botulinum* (Raatjes and Smelt, 1979) and thus additional hurdles should be taken into  
340 consideration to ensure the safety of the final product. The additional hurdle in this  
341 trade preparation is the high salt concentration of the olives. Indeed, the average value  
342 of salt content was 6.9 g NaCl/100 g olive pulp. It must be underlined that the IOC

343 trade standard for table olives (IOC, 2004) defines a minimum salt concentration of  
344 10% (w/v) for this type of olives. However, the trade standard indicates that the salt  
345 content should be determined in the olive juice and not in the olive pulp, which is the  
346 standard method of analysis (Değirmencioğlu et al., 2014). For this reason, the  
347 concentration of salt in the olives was expressed as % NaCl per 100 mL of olive juice  
348 taking into consideration the moisture content of the olives and based on the  
349 assumption that the salt is concentrated in the aqueous phase of the olive (García-  
350 Serrano et al., 2023). Thus, only sample S6 presented NaCl content in the olive juice  
351 less than 10% (w/v) and could not meet the specifications of the trade standard of the  
352 IOC. Another important parameter is water activity ( $a_w$ ). It has been reported that  $a_w$   
353 ranges from 0.99-0.98 in raw olive drupes to 0.75-0.92 in the final dry-salted product  
354 (Panagou, 2006; Ramírez et al., 2013). In this work, the average  $a_w$  was 0.75, which is  
355 in good agreement with previously published values for Greek dry-salted olives  
356 (García-Serrano et al., 2023; Panagou, 2006). It should also be noted that  $a_w$  ranged  
357 between 0.58 and 0.91 reflecting a lack of standardization in the dry-salting process  
358 among the different processors. Another noteworthy observation is that the highest  $a_w$   
359 value corresponded to sample S6 that presented also and lowest salt concentration and  
360 the highest yeast population (6.4 log CFU/g) along with the presence of LAB in high  
361 numbers (6.0 log CFU/g). Finally, the average moisture of the samples was 29.5%,  
362 which is in good agreement with the moisture content reported recently for packaged  
363 dehydrated Greek dry-salted olives (García-Serrano et al., 2023). However, sample S6  
364 presented a high moisture content close to 57% that could support the growth of fungi  
365 during the shelf-life of the product, unless preservatives (e.g., sorbic acid in the form  
366 of potassium sorbate) or pasteurization can be applied to inhibit the growth of yeasts  
367 and moulds (Casado et al., 2010). As a consequence of high moisture content, sample  
368 S6 presented also a high  $a_w$  value that when combined with the low salt content of the  
369 olives may jeopardize the safety of the product, a fact that was also evident from the  
370 microbiological analysis for this sample.

371

### 372 *3.3 Culture dependent and culture independent molecular identification*

#### 373 *3.3.1 Repetitive DNA amplification (rep-PCR)*

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

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

382

### 383 3.3.2 Internal Transcribed Spacer Regions (ITS) PCR and Restriction Fragment 384 Length Polymorphism

385 One representative isolate of the 36 groups from rep-PCR was selected and  
386 consequently subjected to ITS-PCR and RFLP for further clustering. Comparison of  
387 the electrophoretic profiles obtained from RFLP resulted in further classification of  
388 the isolates in a final number of 20 clusters (Table 3). One isolate of each cluster was  
389 subjected to sequencing and taxonomic assessment was performed via BLAST  
390 software leading to the final classification of the representative isolates at species  
391 level (Table 4). According to Tables 3 and 4, the total number of isolates belonged to  
392 9 species, namely *Candida versatilis*, *Wickerhamomyces subpelliculosus*, *Candida*  
393 *apicola*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii*, *Citeromyces*  
394 *nyonsensis*, *Candida etchelsii*, *Pichia membranifaciens* and *Candida sorbosivorans*. 4  
395 isolates belonging to the same cluster (cluster 3) after the final grouping could not be  
396 identified. The identification percentage of the yeast species isolated and identified for  
397 each of the nine samples with culture dependent techniques is shown in Figure 1.  
398 *Candida etchelsii* was identified in most of the samples, except S4, S6, and S9.  
399 Specifically, 100% isolation frequency was observed in sample S3, whereas  
400 coexistence with *Candida versatilis* was noticeable for samples S1 (5.0%-95.0%), S7  
401 (50.0%-50.0%), and S8 (65.0%-35.0%), followed by *Citeromyces nyonsensis* for  
402 sample S5 (73.7%-21.1%-5.3%). *Pichia membranifaciens* was dominant in samples  
403 S6 (90.0%) and S9 (60.0%), followed by *Candida sorbosivorans* with 10.0% and  
404 40.0%, respectively. *Candida etchelsii* was also identified in sample S2 (45.0%)  
405 followed by *Wickerhamomyces subpelliculosus* (30.0%) and *Candida apicola* (5.0%).  
406 *Wickerhamomyces subpelliculosus* was the dominant species in sample S4 (84.2%)  
407 followed by *Torulaspora delbrueckii* (10.5%) and *Wickerhamomyces anomalus*

408 (5.3%). *Citeromyces nyonsensis* was also identified at a low percentage close to 5%  
409 for sample S5, while 20% of the isolates (5 isolates) in sample S2 were characterized  
410 as non-identified. The results obtained are in accordance with previous studies, since  
411 all identified species have been also isolated from different fermented food matrices  
412 and/or environments with low  $a_w$  and high salt concentrations. More specifically, *C.*  
413 *etchelsii* and *C. versatilis* which are salt tolerant yeasts are commonly isolated from  
414 miso and soy fermentations and are important in flavour enhancing through the  
415 production of 4-ethyl guaiacol (4EG) and 4-ethyl phenol (4EP), which are  
416 characteristic flavours of soy sauce and miso (Feng et al. 2012; Suezawa et al., 2006).  
417 Both species have been previously isolated from bloaters of fermented green table  
418 olives during storage (Asehraou et al., 2000). *Candida apicola*, *Pichia*  
419 *membranifaciens* and *Wickerhamomyces anomalus* are well known species related to  
420 table olive fermentations (Arroyo-López et al., 2006; Bautista-Gallego et al., 2011;  
421 Bleve et al., 2014, 2015; Nisiotou et al., 2010). *Wickerhamomyces subpelliculosus* is a  
422 salt-tolerant microorganism previously isolated from fermented cucumbers (Kurtzman  
423 et al., 1998) and is considered as an alternative to baker's yeast (Zhou et al., 2017).  
424 *Citeromyces nyonensis* is a yeast previously isolated from fermented black olive  
425 brines and olive oil (Casaregola et al., 2013), while *Torulaspora delbrueckii* is a well-  
426 known yeast presenting high resistance to osmotic stress, as well as biotechnological  
427 potential due to aroma-enhancing properties and therefore is widely employed in  
428 wine, beer and bread dough fermentations (Fernandes et al., 2021). *T. delbrueckii* has  
429 been previously isolated from black olives (Kotzekidou, 1997). *Candida*  
430 *sorbosivorans* is a salt tolerant yeast previously isolated from honey (Carvalho et al.,  
431 2010) and more recently isolated during the production of Algerian Smen, a  
432 traditional fermented butter (Boussekine et al., 2022).

433

### 434 3.3.3 Amplicons target sequencing

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

440 *boidinii* and *Hortaea werneckii*. *Candida etchelsii* and *C. versatilis* were the dominant  
441 species in most samples with relative abundances ranging from 23.0% to 55.3%,  
442 followed by *W. anomalus* (13.4%), *O. boidinii* (7.9%) and *C. apicola* (6.9%) for  
443 sample S4, *O. boidinii* (5.5%) for sample S8, *A. pullulans* (11.3%) and *C. apicola*  
444 (5.5%) for sample S9. In addition, in samples S2 and S5 the dominant species were *P.*  
445 *triangularis* with relative abundances of 41.0% and 31.0%, respectively, followed by  
446 *C. etchelsii* (33.1% and 30.9%, respectively), *C. versatilis* (8.1% and 27.1%,  
447 respectively) and *Hortaea werneckii* (7.4%) for sample S2. These results are in  
448 accordance with the results obtained from culture dependent techniques in this study  
449 since the dominant identified species were common among the two techniques with  
450 slight deviations. Specifically, *P. triangularis*, that was identified only with amplicon  
451 sequencing, has been previously isolated from environments with high salt  
452 concentrations, such as natural fermented Italian sausages (Rantsiou and Cocolin,  
453 2006), soy sauce, sea water and fermented mushrooms (Elbandy et al., 2008; Romero  
454 et al., 2012), while *A. pullulans* and *O. boidinii* (or *C. boidinii*) have been previously  
455 isolated from natural black olive fermentation (Bonatsou et al., 2018; Coton et al.,  
456 2006; Nisiotou et al., 2010). Finally, *H. werneckii* is a black yeast with remarkable  
457 tolerance to salt with both pathogenic and spoilage potential that is usually isolated  
458 from hypersaline environments (Zalar et al., 2019). Despite the fact that several  
459 studies have previously studied microbial populations and physicochemical  
460 characteristics of dry-salted olives (Brenes et al., 2022; García-Serrano et al., 2023;  
461 Panagou, 2006; Ramírez et al., 2013), it is the first-time microbial diversity of this  
462 product is identified using molecular techniques.

463 In order to clarify the relationship between the physicochemical parameters and  
464 the yeast species identification after ITS PCR analysis of dry-salted naturally black  
465 olives samples, PCA was performed, and results are presented in Fig. 3. The plot of  
466 the PCs enables the investigation of correlation between the variables in the input  
467 dataset. The projection of the variables in the subspace of the first two PCs explained  
468 57.8% of the information included in the raw data and allowed the segregation of  
469 identified yeast species in different clusters. In this way, *P. membranifaciens* and *C.*  
470 *sorbosivorans* were positively correlated with the vector of moisture content and  $a_w$   
471 (Fig. 3A) and they were also associated with sample S6 (Fig. 3B) that presented the  
472 highest moisture (56.7%) and  $a_w$  (0.91) from all samples analyzed. Moreover, *C.*



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

487

#### 488 **4. Conclusion**

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

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

514

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520

#### 521 Declaration of competing interest

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

524

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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  $\pm$   
 679 standard deviation) of naturally black dry-salted olive samples from retail outlets.

Sample	Yeasts	TVC	<i>Enterobacteriaceae</i>	<i>S. aureus</i>	<i>Pseudomonas</i> <i>spp.</i>	LAB
S1	5.4 $\pm$ 0.2a	5.3 $\pm$ 0.1a	< 1.0	< 2.0	< 2.0	< 1.0
S2	3.9 $\pm$ 0.1b	4.2 $\pm$ 0.1b	< 1.0	< 2.0	< 2.0	< 1.0
S3	< 2.0c	4.4 $\pm$ 0.7b	< 1.0	< 2.0	< 2.0	< 1.0
S4	5.7 $\pm$ 0.3d	5.8 $\pm$ 0.4c	< 1.0	< 2.0	< 2.0	< 1.0
S5	3.2 $\pm$ 0.1e	3.4 $\pm$ 0.2d	< 1.0	< 2.0	< 2.0	< 1.0
S6	6.4 $\pm$ 0.1f	6.4 $\pm$ 0.1e	< 1.0	< 2.0	< 2.0	6.0 $\pm$ 0.4
S7	5.7 $\pm$ 0.2d	6.0 $\pm$ 0.1c	< 1.0	< 2.0	< 2.0	< 1.0
S8	3.4 $\pm$ 0.6e	4.9 $\pm$ 0.2f	< 1.0	< 2.0	< 2.0	< 1.0
S9	3.4 $\pm$ 0.1e	4.9 $\pm$ 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 <sup>(a)</sup>	a <sub>w</sub> <sup>(b)</sup>	Moisture (%)	NaCl (%) <sup>(c)</sup>	NaCl (%) <sup>(d)</sup>
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 <sup>(a),(b)</sup> Standard deviation values for pH and a<sub>w</sub> were < 0.01 and 0.001, respectively; <sup>(c)</sup> g NaCl/100 g of  
 685 olive pulp; <sup>(d)</sup> 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.

<b>Clusters after REP-PCR</b>	<b>Isolates within each cluster</b>	<b>Clusters after RFLP</b>	<b>Isolates within each cluster</b>
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		

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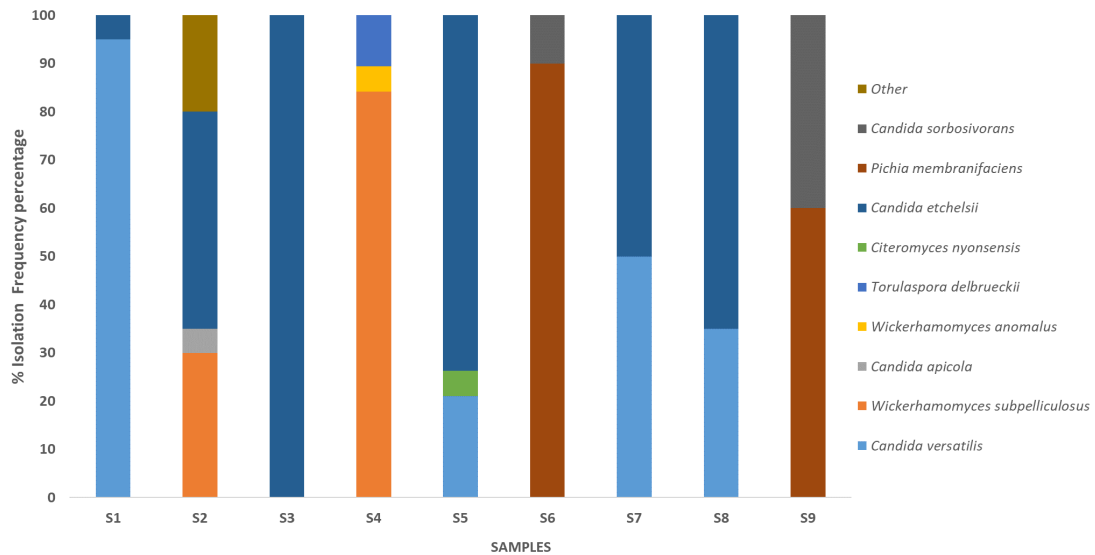
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693 **Table 4.** Species identification of yeast isolates according to the RFLP clustering (20 clusters)

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

694 \* Isolate from cluster 3 was not identified.

695 <sup>a</sup>: abundance of isolates in each cluster based on Table 3

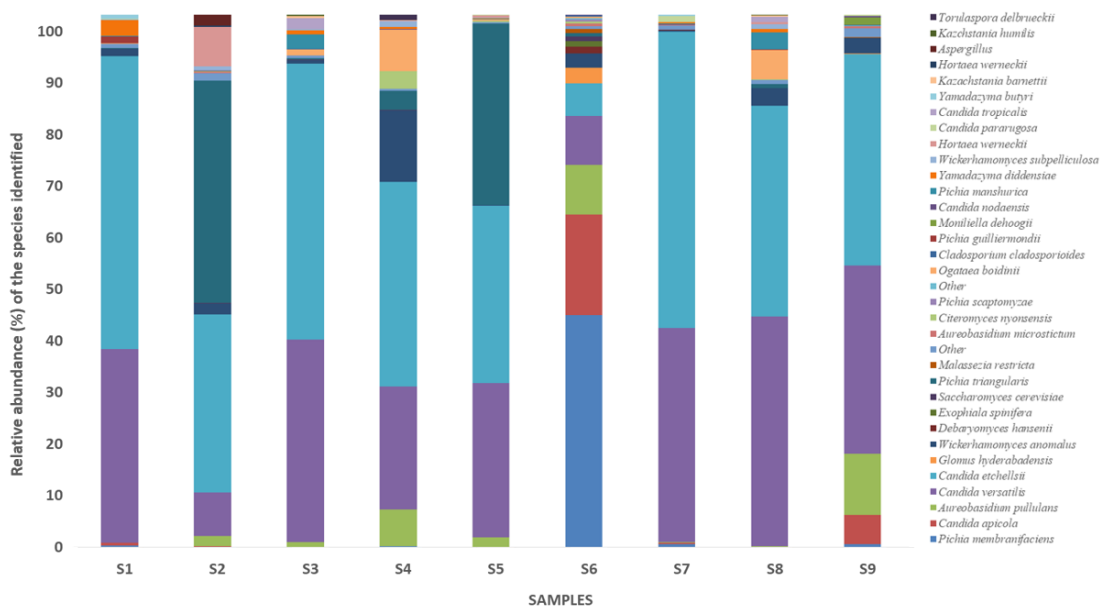


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697 **Fig. 1.** Isolation frequency (%) of the yeast species for the nine samples of dry-salted olives  
 698 with culture dependent techniques.

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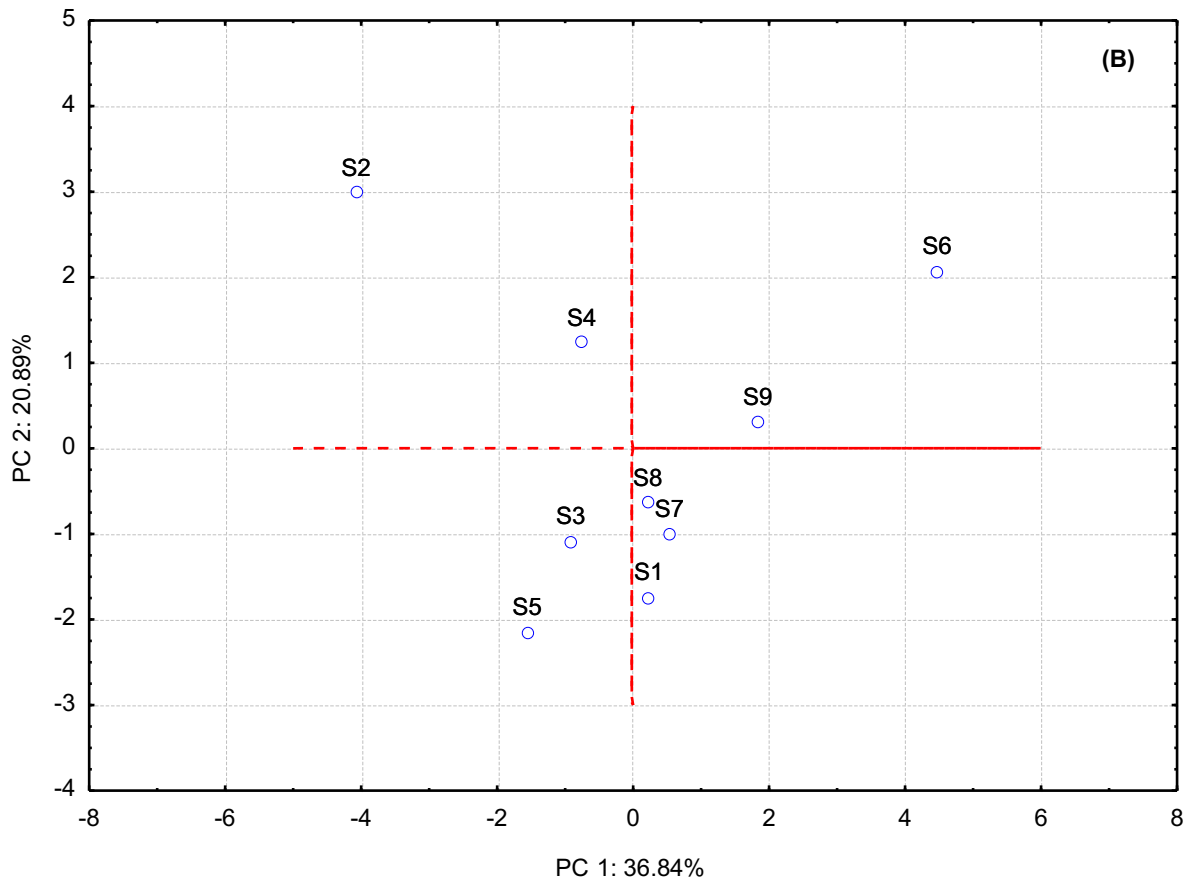
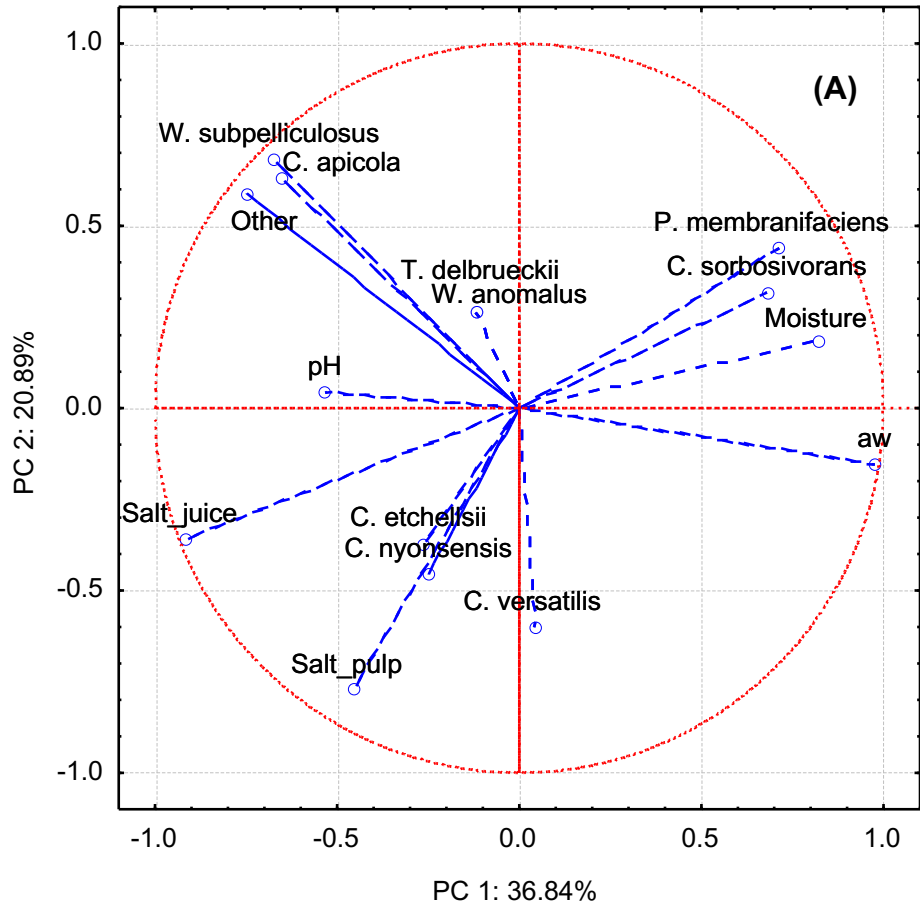
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702 **Fig. 2.** Relative abundance (%) of yeast species for the nine samples of dry-salted olives  
 703 derived by amplicon sequencing.

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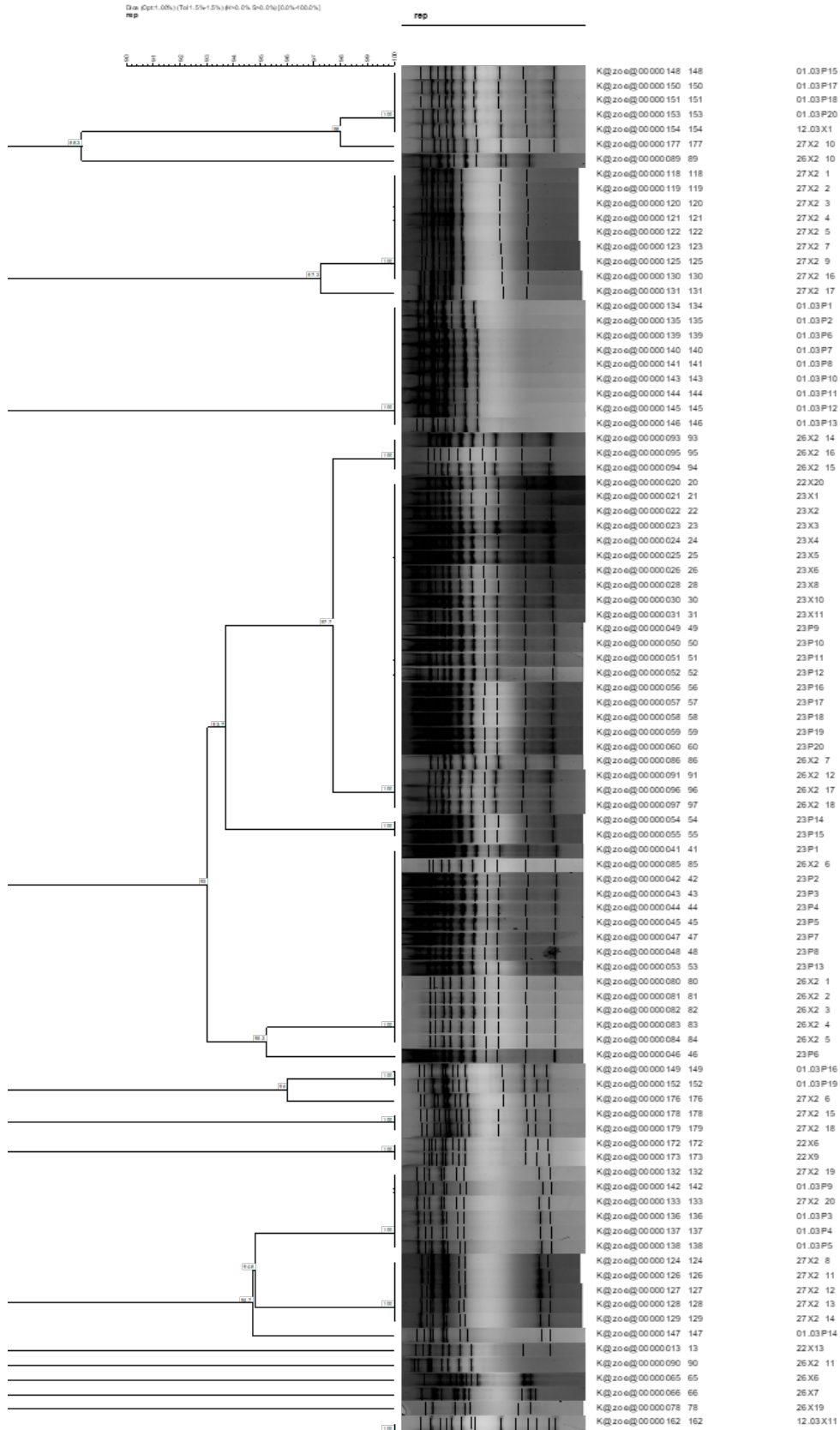
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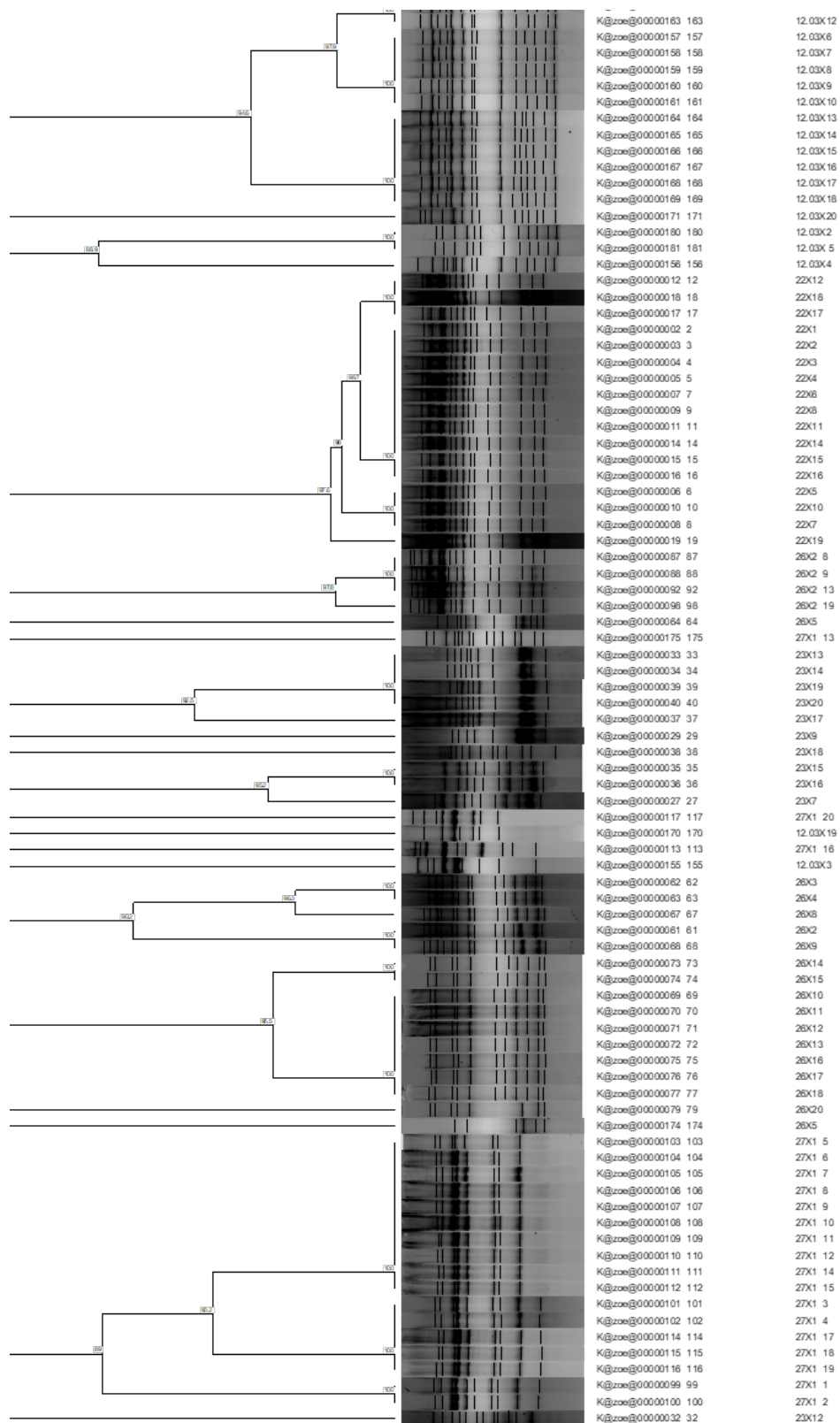
735 **Fig. 3.** PCA plot of loadings (A) (abundance of yeasts and physicochemical parameters) and  
736 scores (B) (samples of dry salted olives) of the first two principal components (PCs).

737 **Supplementary Material**



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739



742 **Supplementary Fig. 1.** Cluster analysis of yeast isolates after rep-PCR with (GTG)<sub>5</sub> primer.