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2   fermentations

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21 ABSTRACT

22 Cracked green table olives, from the Manzanilla variety, are a fermented food produced

23 and consumed in Portugal. The objective of the present work was to study the

24 microbiological characteristics and yeast population evolution during the fermentation

25 of cracked green olives. The predominant microorganisms were yeasts while lactic acid

26 bacteria were not detected and a clear decrease of the mould population was observed.

27 At the end of the fermentations, no viable counts of *Enterobacteriaceae* were found.

28 Yeast isolates were identified by the 5.8S rRNA-ITS region restriction analysis and by

29 sequencing the D1/D2 region of the 26S rRNA gene. During the initial phases of the

30 fermentations a great diversity of yeasts was observed. However, as the processes

31 evolved the biodiversity decreased with the fermentative yeasts *Citeromyces*

32 *matritensis, Zygotorulaspora mrakii* and *Saccharomyces cerevisiae* becoming the

33 dominant species. The presence of these fermentative yeasts at the end of the production

34 processes may explain the instability of the final product and is associated to a risk of

35 spoilage. The results obtained represent a first attempt towards the comprehension of

36 the microbiota of this type of "Natural olives" that constitute an important component of

37 the Mediterranean diet.

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1. Introduction

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45   Olives are the fruits of *Olea europeae* L., which offer two relevant components of

46 Mediterranean diet: olive oil and table olives. Table olives are classified according to

47 the degree of ripeness of the fresh drupes used in the production: green olives, turning

48 color olives and black olives (IOOC, 2004). The most significant industrial production

49 methods of table olives are the Spanish-type olives and the Californian style olives

50 involving both a de-bittering process with sodium hydroxide solutions (Gómez, García

51 & Navarro, 2006). However, around the Mediterranean fringe there are other methods

52 to produce table olives where the fruits are brined after harvesting, without going

53 through a NaOH treatment to debitter and left to ferment until they lose their bitterness,

54 at least partially (Hurtado, Reguant, Esteve-Zarzoso, Bordons & Rozès, 2008; Bautista-

55 Gallego, Arroyo-López, Durán-Quintana & Garrido-Fernández, 2010). In some cases,

56 the drupes undergo pre-treatments in water to fasten the debitterisation process

57 (Valenčič, Mavsar, Bučar-Miklavčič, Butinar, Čadež, Golob, Raspor, & Možina, 2010).

58 The "natural green olives" are different from the lye treated ones, mainly due to their

59 taste, color and residual bitterness (Garrido-Fernández, Fernández Díez, & Adams,

60 1997).

61   The most important microbial groups involved in olive fermentations are mainly

62 lactic acid bacteria and yeasts. When the growth of lactic acid bacteria overcomes the

63 growth of yeasts, lactic acid fermentation is favoured and a final food product with

64 lower pH value is obtained, as in Spanish style olives. However, if yeasts become the

65 dominant microorganisms, the olives produced will have higher pH values as in Greek

66 style olives (Gómez et al., 2006). The main roles of yeasts in the processing of

67 fermented olives, are associated with the production of alcohols, ethyl acetate,

68 acetaldehyde and organic acids, compounds that are relevant for the development of

69 taste and aroma and for the preservation characteristics of this fermented food

70 (Hernández, Martín, Aranda, Pérez-Nevado, & Córdoba, 2007; Arroyo-López, Querol,

71 Bautista-Gallego, & Garrido-Fernández, 2008). Nevertheless, in certain processing

72 conditions and after packing, yeasts may have a negative role as they are responsible for

73 the production of CO_2, softening of the fruits due to pectinolitic activity, clouding of

74 brines, biofilm production and, probably, production of off flavors (Arroyo-López et al.,

75 2008).

76   In Portugal there are few reports related to microbial characteristics of

77 black/green olives. They refer manly to olives produced and commercialized in the

78 North and Center of the country where varieties such as Galega, Gordal, Cordovil,

79 Negrinha do Freixo (*Olea europeae* L.) are the most frequent (Oliveira, Brito, Catulo,

80 Leitão, Gomes, Silva, Vilas-Boas, Peito, Fernandes, Gordo, & Peres, 2004, Pereira,

81 Pereira, Bento & Estevinho, 2008). In the Southern part of Portugal cracked green table

82 olives, from the Manzanila variety, not debittered with lye solutions is one of the most

83 popular methods of producing table olives. The process remains empirical as only the

84 salt content and the overall sensorial characteristics are verified. The main problem of

85 these olives is related to the instability of the packed product after fermentation.

86   In this context, the objective of the present work was to study the microbiological

87 characteristics, yeast population diversity and succession during the production of

88 Portuguese cracked green olives.

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90 **2. Material and methods**

91 *2.1. Fermentation conditions*

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93 Olives from the Manzanilla variety were harvested, by hand, in the Southern part

94 of Portugal during October of 2008, when the surface colour was green and were

95 transported to the factory, located in the Algarve. On arrival, fruits were washed and

96 selected to remove damaged fruits. After selection they were cracked by passing the

97 olives between two stainless steel plates. Then, fruits were calibrated, transferred to

98 screw-capped fermentation vessels and covered with a freshly prepared brine (6-12 g

99 NaCl/100 ml). Fermentations were carried out at room temperature, due to

100 microorganisms present on the fruits and environment, for periods of 52 days.

101 During the fermentation period, brine samples were collected from two

102 independent vessels (A and B) under sterile conditions, using 0.5 m length disinfected

103 plastic tubes. The collected samples were aseptically transferred, to sterile 50 ml tubes

104 and transported, under refrigeration, to the laboratory where the microbiological study

105 took place.

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107 *2.2. Microbiological analysis*

108

109 From each sample obtained, 1 mL of fermenting brine was treated, following the

110 decimal dilution protocol, in sterile ¼ Ringer solution. Aliquots were plated, in

111 duplicate, by surface spreading or incorporation, on media for the detection and

112 enumeration of microorganisms: a) Tryptic Soy Agar (TSA, Sharlau, Spain) (pH 5) for

113 total viable count, incubated at 25 ºC for 5 days; b) de Man, Rogosa and Sharpe agar

114 (MRS, Sharlau, Spain) (pH 5) with cycloheximide (0,05 %) overlaid with the same

115 medium without cycloheximide, for lactic acid bacteria, incubated at 25ºC for 5 days; c)

116 Malt Extract Agar (MEA, Sharlau, Spain) (pH 5) for yeasts, incubated at 25ºC for 5

117 days (this medium was used to count the yeasts and to obtain isolates for further

118   identification); d) Rose Bengal Chloramphenicol Agar (Biokar Diagnostics) for moulds,

119   incubated at 25ºC for 5 days; e) Chromocult Agar (Difco, England) for

120   *Enterobacteriaceae* and *Escherichia coli* incubated at 35ºC for 2 days.

121   Yeast colonies were selected from those grown on MEA, according to their macro

122   morphology and isolated in proportion to their frequencies. Isolates were purified by

123   successive streaking in YEPD (yeast extract, peptone, dextrose, agar) (Sharlau) or

124   Potato Dextrose Agar (Sharlau). A total of 108 colonies from the fermentations was

125   obtained and identified. The isolated strains were preserved at -80ºC with glycerol (20%

126   v/v) as the cryprotectant agent.

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128   *2.3. Yeast Identification*

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130   Yeasts isolates identification was performed by the PCR-RFLP method described

131   by Esteve-Zarzoso, Belloch, Uruburu, & Querol, (1999) in combination with sequence

132   analysis of the D1/D2 domain of ribossomal DNA (rDNA). Genomic DNA from YEPD

133   liquid cultures were extracted according to Querol, Barrio, & Ramon, (1992). PCR

134   reaction   mixtures   (75   µl)   containing   0.5   µM   primer   ITS1

135   (5'TCCGTAGGTGAACCTGCGG3')   and   0.5   µM   primer   ITS4

136   (5'TCCTCCCGCTTATTGATATGC3') (White, Bruns, Lee, & Taylor, 1990), 10 µM

137   deoxynucleotides (Promega), 1.5 mM MgCl2 and 1 unit DNA polymerase (Promega)

138   were prepared. Amplifications were performed in a thermocycler (Thermo Electron,

139   USA) under the following conditions: initial denaturation at 95ºC for 5 min; 30 cycles

140   of denaturating at 94ºC for 1 min; annealing at 55.5ºC for 2 minutes; extension at 72 ºC

141   for 2 minutes and a final extension step at 72ºC for 10 min. PCR products (10 µl) were

142   digested without further purification with the restriction endonucleases *Cfo*I, *Hae*III and

143   *Hin*fI (Roche). The PCR products and their restriction fragments were separated on 1%

144   and 3% agarose gels, respectively, with 1xTAE buffer. After electrophoresis, gels were

145   stained with etidium bromide, visualized under UV light in a G-Box Syngene-Genesis

146   10 UV Scanner (UK). Fragment sizes were estimated by comparison against a DNA

147   ladder (100 bp BioRad). Yeasts were identified to the species level by comparison of

148   the amplified products and their restriction-fragments profiles with those described by

149   Guillamón, Sabaté, Barrio, Cano, & Querol, 1998; Esteve-Zarzoso et al., 1999; Esteve-

150   Zarzoso, Zorman, Belloch & Querol, 2003; de Llanos-Frutos, Fernandez-Espinar, &

151   Querol, 2004 and Coton, Coton, Levert, Casaregola, & Sohier, 2006. A representative

152   number of isolates corresponding of each PCR-RFLP profile was selected and sequence

153   analysis of the domains D1 and D2 of the 26S rDNA region was accomplished. PCR

154   amplification of the referred region in the 26S rDNA gene was performed with the

155   primers   NL1   (5'-GCATATCAATAAGCGGAGGAAAAG-3')   and   NL4   (5'-

156   GGTCCGTGTTTCAAGACGG-3') (Kurtzman & Robnett, 1998). The amplification

157   reaction and PCR conditions were identical to those described above for the analysis of

158   the ITS 5.8 rRNA region except for the primers used (NL1 and NL4). Amplified

159   products were sequenced elsewhere (LGC Genomics, Germany) and sequences were

160   compared to those available in GenBank database at the National Center for

161   Biotechnology   Information   (NCBI)   using   BLAST

162   (http://www.nvbi.nlm.nih.gov/BLAST/).

163

164   **3. Results and discussion**

165

166   *3.1. Microbiological analysis*

167

168   The evolution of total viable counts, yeasts, moulds and *Enterobacteriaceae* in

169   cracked green olives' brines of the two independent fermentation vessels A and B is

170   represented in Fig. 1 (Fig. 1A, Fig. 1B, Fig 1C and Fig 1D). During the first 8 days, the

171   total counts and the yeast population showed an adaptation phase which was followed

172   by exponential growth reaching the stationary phase, after, approximately 17 days. At

173   the start of the processes, counts of the yeast population increased from 4.9-5.0 log 10

174   CFU/ mL of brine to maximum values of 6.0-6.5 log 10 CFU/ mL of brine, in both

175   vessels (Fig. 1A and 1B). Yeasts were present all along the fermentation period studied

176   in both fermenters (Fig. 1B) and seem to be the group of organisms responsible for the

177   main changes occurring in the table olive fermentation studied in the present work. The

178   yeast counts obtained were higher than the values obtained by Hernández et al. (2007)

179   in Manzanilla variety olives but in the range of those described in the review of Arroyo–

180   López et al. (2008) who reported yeasts to be present throughout fermentation processes

181   reaching populations that ranged from 4.0 to 6.0 log 10 CFU/mL of brine.

182   The mould population was present since the beginning of fermentations (4.4 and

183   3.9 log CFU/ ml of brine respectively in both Vessels A and B) and a decline of its

184   number was observed until day 45 of the fermentation (Fig. 1C). The presence of

185   *Penicillium* and *Aspergillus* fungi are reported to cause softening of the olives and may

186   be responsible by the production of malodorous compounds (Hutkins, 2006).

187   Additionally, the presence of moulds in olive´s brines can compromise the safety of the

188   product, especially in the case of fungi producers of mycotoxins (Zinedine & Mañes,

189   2009).

190   The results obtained showed that the lactic acid bacteria group was not detected in

191   the brines. Aponte, Ventorino, Blaiotta, Volpe, Farina, Avellone, Lanza, & Moschetti

192   (2010) also did not find lactic acid bacteria in some varieties of Sicilian olives,

193   including Manzanilla, when culture-dependent and independent methods were used. In

194   addition, this group of bacteria was not detected as well in fermentations of Slovenian

195   olives (Valenčič et al., 2010). The most relevant factors related to the absence of lactic

196   acid bacteria in cracked green table olives may be the initial NaCl concentration (6-

197   12%) and the presence of natural inhibitors compounds, such as phenolic compounds,

198   that quickly diffuse from the broken drupes into the brines. Ruiz-Barba, Rios-Sanchez,

199   Fedriani-Iriso, Olias, Rios, & Jimenez-Diaz (1990) studied the antimicrobial properties

200   of olive phenols against *Lactobacillus plantarum* in non-alkali treated olives, indicating

201   that phenolic compounds have a pronounced bactericidal effect on this microorganism,

202   through the alteration of the cellular ultrastructure on two targets: cell wall and

203   cytoplasmic membrane. For many years, oleuropein and its hydrolysis products

204   (aglycon, elenoic acid and hydroxytyrosol) have been pointed out as responsible for the

205   growth inhibition of lactic acid bacteria (Ruiz-Barba, Brenes, Jiménez, García, &

206   Garrido, 1993). Recently, Medina, Romero, Castro & Brenes (2008) reported that the

207   main lactic acid bacteria inhibitors in brines are the dialdehydic form of

208   decarboxymethyl elenolic acid free (EDA) or linked to hydroxytyrosol (HyEDA). As

209   referred by Medina, García, Romero, de Castro, & Brenes (2009) HyEDA may be

210   formed during olive brining as a consequence of the enzymatic hydrolysis of oleuropein

211   by β-glucosidase which is present in brines.

212   In the case of the *Enterobacteriaceae,* this population was detected in the beginning

213   of both fermentations at levels ranging from 2.6 to 3.5 log 10 CFU/ mL of brine during

214   the first 30 days. Thereafter, a decline of those bacteria was observed and no viable

215   counts (<10 CFU/ml) were found at the end of both fermentations studied (Fig. 1D).

216   Typical *Escherichia coli* colonies were not detected throughout the entire study. A

217   waning in the *Enterobacteriaceae* population followed by its disappearance has been

218   also described in various olives' fermentation processes (Tassou, Panagou, &

219   Katsaboxakis, 2002; Bautista-Gallego, et al., 2010). High numbers of

220   *Enterobacteriaceae* represent a risk of deterioration due to the production of off flavors

221   and gas pocket spoilage in the olives' surface (Garrido-Fernández et al., 1997).

222

223   *3.2. Yeast identification*

224

225   The study of the microbial populations evolution presented previously showed that

226   the yeasts was a quite relevant microbial group in the processing of cracked green table

227   olives which led to the identification of that microbiota. A total of 108 yeast isolates

228   was obtained from the two independent olive fermentations studied. Their identification

229   was performed by the ITS1-5.8 rRNA-ITS2 region PCR-RFLP, using the restriction

230   enzymes *Cfo*I, *Hae*III and *Hinf*I, in combination with the sequence analysis of the D/D2

231   26S rRNA region. The ITS region of the yeast isolates originated PCR products with

232   size ranging from 450 bp to 850 bp. The yeast isolates gave origin to nine ITS-RFLP

233   different restriction fragment length polymorphism profiles which were compared to

234   patterns published previously (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999;

235   Esteve-Zarzoso et al., 2003; de Llanos-Frutos et al., 2004; Coton et al., 2006). This

236   comparison allowed the assignment of the following species: *Aureobasidium pullulans*

237   (yeast like species), *Candida boidinii, Candida diddensiae, Candida oleophila,*

238   *Citeromyces matritensis, Rhodotorula mucilaginosa, Saccharomyces cerevisiae and*

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239 *Zygotorulaspora mrakii* (previously *Zygosaccharomyces mrakii* and reassigned to a new

240 genus by Kurtzman (2003) (Table 1). From the nine RFLP profiles obtained only one

241 (Profile V) did not match any of the already published pattern. In addition to PCR-

242 RFLP identification method, 22 representative strains of the ITS-RFLP profiles had

243 their 26S rRNA gene' D1/D2 region sequenced and the obtained sequences were

244 compared to the NCBI GeneBank data base using BLAST. Table 2 resumes the results

245 of the percentages of sequence similarity in one selected strain (as an example) of each

246 RFLP group sequenced, after BLAST analysis, as well as the GeneBank *accession*

247 *numbers* of the species used in the comparisons. All the isolates corresponding to the

248 different RFLP profiles, showed a sequence similarity of the D1/D2 region higher than

249 98%. These results allowed the confirmation of the identifications performed through

250 restriction analysis in eight of the nine profiles obtained (I, II, III, IV, VI, VII, VIII and

251 IX) and the identification of the isolates characterized by RFLP-Profile V as *Candida*

252 *quercitrusa*.

253　　　　As fermentations evolved, a succession of microbial species took place (Table

254 3). The earlier fermentation samples, corresponding to the first four days, contained a

255 mixture of species including *A. pullulans* (yeast like fungus), *C. boidinii, C. diddensiae,*

256 *C. oleophila*, *C. quercitrusa* and *R. mucilaginosa.* Although these species were isolated

257 in low percentages (<6.4%) (Table 1) they might have had an influence in the

258 fermentations conditions in the processing of olives. As fermentations progressed, the

259 majority of those species were not detected with the exception of *C. diddensiae* which

260 was isolated during 2 weeks (Table 3). The presence of *C. diddensiae* was also referred

261 by Arroyo-López, Durán-Quintana, Ruiz-Barba, Querol, & Garrido-Fernández (2006) in

262 green table olives (Aloreña cultivar) and by Hurtado et al. (2008) in *Arbequina* table

263 olives. The decreasing of oxygen concentration in the fermentation vessels, the levels of

11

264 phenolic compounds or the accumulation of fermentation products in the brines may

265 explain why those earlier species were not detected after the first days. For example, *A.*

266 *pullulans* is an oxidative ubiquitous yeast like species found on fruits surfaces and

267 phyllosphere (Zalar, Gostinčar, Hoog, Uršič, Sudhadham, & Gunde-Cimerman, 2008).

268 It was recently referred in olives by Nisiotou, Chorianopoulos, Nychas, & Panagou

269 (2010) and Valenčič et al. (2010) but has been commonly isolated from the surface of

270 grapes and during the initial phases of wine production, being inhibited after the first

271 fermentation days (Sabate, Cano, Esteve-Zarzoso, & Guillamón, 2002). The

272 basidiomicetous yeasts of the genus *Rhodotorula* have been identified in the early

273 stages of olive fermentation since the beginning of this kind of studies (Vaughn,

274 Jakubczyk, MacMillan, Higgins, Davé, & Crampton, 1969; Arroyo-López et al., 2006,

275 Nisiotou et al., 2010). An important aspect of this pink species' role in olive's

276 production environment is their involvement in the softening of olives due to the

277 production of polygalacturonases (Vaughn, et al., 1969). The yeast *C. boidinii* was

278 isolated in a relative frequency of 2.8% in the early phase of the fermentation, although

279 it has been detected in high percentages either in black olives of the Hojiblanca

280 (Arroyo-López et al., 2006) and Conservolea varieties (Nisiotou et al., 2010) as well as

281 in green *Arbequina* olives (Hurtado et al., 2008). The species *C. oleophila* was also

282 found in brines from Slonenian Istria (Valenčič et al., 2010). On the other hand, *C.*

283 *quercitrusa,* although in low percentage, was described for the first time in an olive

284 environment. This specie has mainly been associated to wine ecosystems (Chavan,

285 Mane, Kulkarni, Shaikh, Ghormade, Nerkar, Shouche, & Deshphande, 2009). The

286 greatest number of yeast isolates from cracked green table olives' brines corresponded

287 to *Cit. matritensis* (9,2%), *S. cerevisiae* (11,9%) and *Z. mrakii* (59,6%) (Table 2). These

288 species were isolated some days after fermentation had initiated and lasted until the end

289 of the process with the exception of *Cit. matritensis* which is a fermentative yeast able

290 to grow in the presence of high concentrations of NaCl. *Cit matritensis* was reported in

291 French black olives by Coton et al. (2006). *Z. mrakii* is a fermentative yeast (Kurtzman,

292 2003) that has never been described in table olives while *S. cerevisiae* is also

293 fermentative and its presence in olives' brines has been recorded in black or green

294 olives (Marquina, Peres, Caldas, Marques, Peinado & Spencer-Martins, 1992; Arroyo

295 López et al., 2006; Rodríguez–Gómez et al., 2010). Both of these species have been

296 associated to vigorous production of gas which may explain the instability of the packed

297 olives characterized by brine spills resulting in a very short shelf life. The genus

298 *Zygosaccharomyces* to which *Zygotorulaspora mrakii* previously belonged to is known

299 for including the major spoiler microorganisms in fruit juices, sauces, soft drinks,

300 alcoholic beverages among others. In fact, the presence of species of that genus is

301 normally an indicator of future spoilage despite their capacity of eventually confer to

302 fermented food useful organoleptic qualities (Esteve-Zarzoso et al., 2003; Loureiro &

303 Malfeito-Ferreira, 2003).

304 In the wine industry, *S. cerevisiae* is responsible for the production of wine but is

305 considered a spoilage yeast in bottled wines due to its capacity to cause refermentation

306 when sugars are available and ethanol is present (Loureiro & Malfeito-Ferreira, 2003).

307 In the case of olive production, *S. cerevisiae* has also been attributed positive roles and

308 negatives activities as discussed by Arroyo-López et al. (2008). In fermented food, the

309 line between beneficial fermenting activity and spoilage activity of yeasts is difficult to

310 draw depending on the phase of the processing/storage the yeast activity takes place. In

311 view of these facts, the production process of cracked green olives should include an

312 operation/strategy to reduce the yeast number and limit their growth before storage and

313 packaging in order to improve its stability and shelf life.

314   On the other hand, it is interesting to note that species commonly related to olive

315   production previously described in Portugal, Morocco and Spain, belonging to the

316   genera *Pichia*, *Debaryomyces* or *Kluyveromyces* were not isolated during the present

317   study (Marquina et al., 1992; Hernández et al., 2007; Rodríguez–Gómez et al., 2010).

318   Yeasts biodiversity reported in distinct olive types may explain the different

319   characteristics in terms of chemical, microbiological and sensorial quality of table

320   olives. It would be interesting to study the relation between the microbiota present

321   during the processing and the development of the final product´s organoleptic profile.

322

323   **4. Conclusions**

324

325   Cracked green table olives from the Manzanilla variety are characterized by a fresh

326   natural green colour, a rich aroma and typical bitter taste. These olives are included in

327   the category of "natural olives" which undergo a spontaneous fermentation carried out

328   by fruit and environmental microbiota, with yeasts found to be the dominant group. The

329   identification methods used (PCR-RFLP and 26S rDNA partial sequencing analysis)

330   appeared to be adequate to characterize the yeast biota, revealing that the most

331   representative species present at the end of the fermentation processes were *Z. mrakii*

332   and *S. cerevisiae* which active fermenting metabolism compromises the stability of the

333   final product during the storage. The olives' fermentations studied guaranteed the

334   absence of *E. coli* and other *Enterobacteriaceae* in the table olives as well as a reduction

335   in the mould viable counts. However, an optimization of the production process to

336   improve the stability and consequently, the shelf life of cracked green table olives in

337   necessary. The knowledge gathered during the course of the described work could

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338 enable the selection of native strains to be used as starter cultures to improve the quality

339 of the product and prevent spoilage during the storage period. Using controlled and

340 defined inocula to initiate the fermentation may be a way to obtain more reliable and

341 better quality food products. The production of good quality food products creates

342 economic gains and avoids financial losses.

343 **ACKNOWLEDGEMENTS**

347

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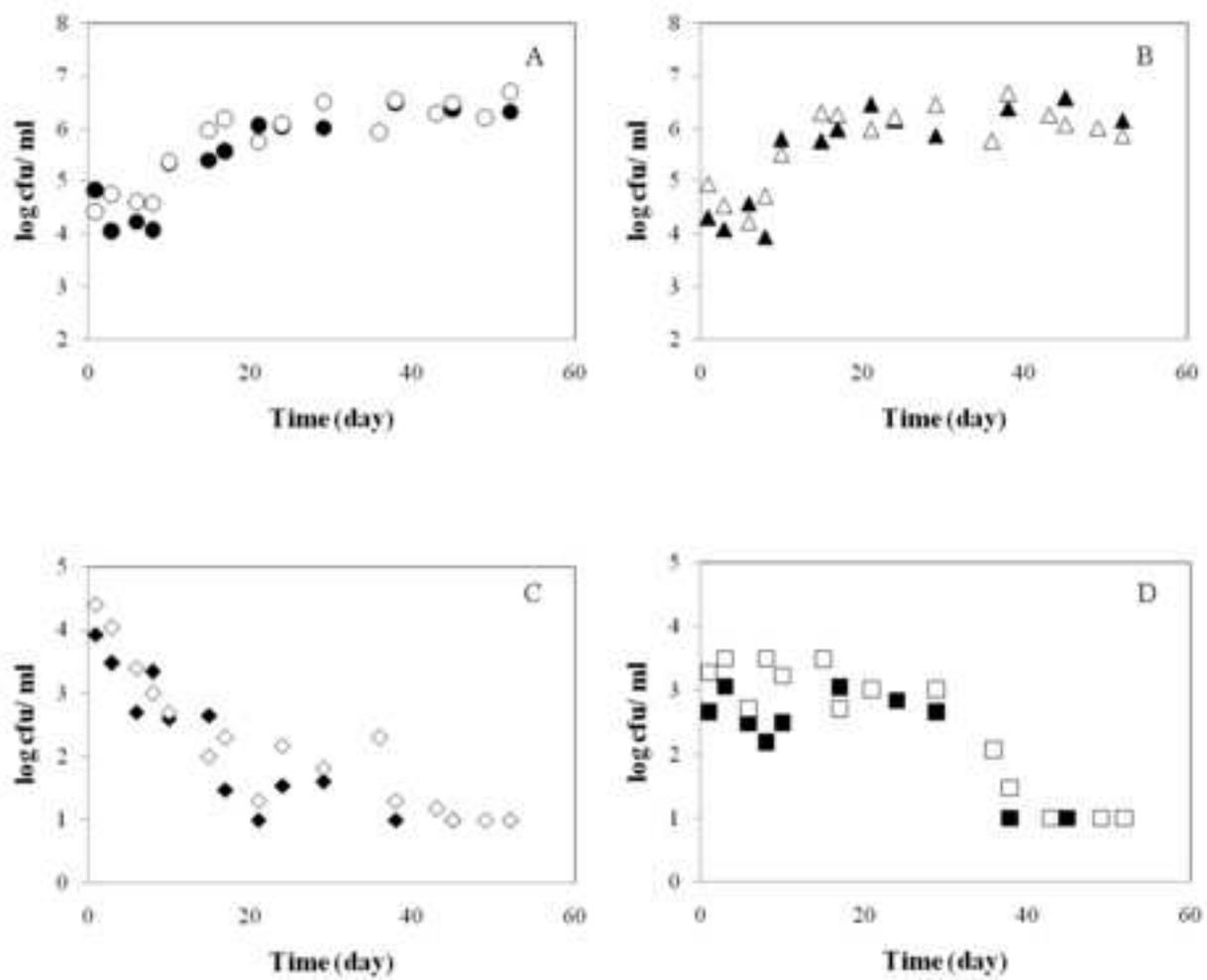
1   Figure Legends

2

3   Fig. 1 Evolution of microbiota during the fermentation of cracked green table olives.

4   Total microbiota (Fig 1A), yeasts (Fig 1B), moulds (Fig 1C) and *Enterobacteriaceae*

5   (Fig 1D) in fermentation Vessel A (open symbols) and Vessel B (closed symbols).

Table 1

Table 1 Identification of yeast isolates obtained from the fermentations of cracked green table olives through PCR-RFLP method (RFLP profiles, PCR products, restriction fragments and identified species (* Non identified by RFLP).

RFLP Profile Nº	PCR Product (bp)	Restriction Fragments (bp)			Identified species	Isolation (%)
		CfoI	*HaeIII*	*HinfI*		
I	590	190+180+100	450+150	280+180+130	*Aureobasidium pullulans*	4.6
II	750	350+310+90	700	390+190+160	*Candida boidinii*	2.8
III	630	290+170+130	420+130+90	310+310	*Candida diddensiae*	6.4
IV	630	290+290	420+140+80	310+310	*Candida oleophila*	0.9
V	600	280+205	400+120+90	300+190+110	*	1.8
VI	700	320+200+100+80	420+200+100	400+320	*Cyteromyces matritensis*	9.2
VII	640	320+240+80	430+210	340+220+80	*Rhodotorula mucilaginosa*	1.8
VIII	850	380+320+150	330+230+170+120	380+360+110	*Saccharomyces cerevisiae*	11.9
IX	660	300+300	400+100+80	320+190+120	*Zygotorulaspora mrakii*	59.6

Table 2

Table 2 Results of the comparison of the isolate sequences with those present in GeneBank from NCBI database (ATCC- American Type Culture Collection, USA; CBS-Centraalbureau voor Schimmelcultures, Nederland; NRRL- Agricultural Research Culture Collection, USA; [a] Relation of similarity of number of nucleotides in Domain D1/D2 between isolates and GeneBank accession strains).

Profile	Fragment sequenced (bp)	GeneBank accession number – Species and strain designation	Similarity [a] (%)
I	530	FJ150916 A*ureobasidium pullulans* var. *pullulans* Strain CBS 146.30	530/530 100%
II	494	FJ914947 *Candida boidinii* Srain ATCC 90438	493/494 99.7%
III	443	U45750 *Candida diddensiae* Strain NRRL Y-7589	443/443 100%
IV	507	AF178050 *Candida oleophila* Strain CBS 6106	507/507 100%
V	533	DQ655691 *Candida quercitrusa* Strain NRRL Y-27941	533/533 100%
VI	502	EF550346 *Citeromyces matritensis* Strain NRRL Y-2407	497/502 99%
VII	493	AF335986 *Rhodotorula mucilaginosa* Strain ATCC 32763	492/493 99.7%
VIII	535	AY048154 *Saccharomyces cerevisiae* Strain NRRL Y-12632	535/535 100%
IX	534	U72159 *Zygosaccharomyces mrakii* Strain NRRL Y-12654	533/534 99.8%

Table 3

Table 3 Succession of yeasts during the fermentation of cracked green table olives.

Species	Fermentation days										
	05	10	15	20	25	30	35	40	45	50	55
Aureobasidium pullulans	▬										
Candida boidinii	▬▬▬										
Candida diddensiae	▬▬▬										
Candida oleophila	▬										
Candida quercitrusa	▬										
Citeromyces matritensis			▬▬▬▬▬▬▬								
Rhodotorula mucilaginosa	▬										
Saccharomyces cerevisiae						▬▬▬▬▬					
Zygotorulaspora mrakii		▬▬▬▬▬▬▬▬▬									