

# Evaluation and identification of poly-microbial biofilms on natural green Gordal table olives

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**Running title:** Biofilm formation on natural green olives

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1 **Abstract**

2 This work examines the formation of poly-microbial communities adhered to the  
3 epidermis of natural green Gordal olives and the application of different methodologies for  
4 recovery and counting of the microorganisms embed in olive biofilms. The fermentation  
5 process was physicochemical and microbiologically monitored for 90 d, at which,  
6 formation of true biofilms on the skin of fermented fruits was confirmed by scanning  
7 electron microscopy. Then, samples of olives were taken and treated with sonication,  
8 enzymes, mechanic homogenization with stomacher and ultrasonic bath for biofilm  
9 disaggregation. The use of the stomacher for 1 min was the most effective treatment to  
10 release the lactic acid bacteria ( $6.6 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$ ), whereas sonication for 5 min was the  
11 most efficient method for quantification of yeasts (up to  $3.5 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$ ). Molecular  
12 identification of isolates obtained from natural Gordal olive biofilms revealed that  
13 *Lactobacillus pentosus* was the only species found among lactic acid bacteria, while *Pichia*  
14 *membranifaciens* was the dominant yeast species, with higher counts obtained for the  
15 bacteria.

16 **Keywords:** Biofilm analysis · natural fermented olives · lactic acid bacteria · table olives ·  
17 yeasts

## 18 **Introduction**

19 Spain generates almost a quarter of the worldwide table olive production, which  
20 nowadays exceeds 2.5 million tons per year (IOC 2013). Among the diverse processing  
21 methods, alkali-treated green olives (Spanish style), ripe olives by alkaline oxidation  
22 (Californian style) and directly brined olives (natural olives) are the most common  
23 (Garrido-Fernández et al. 1997). However, only directly brined olives are produced  
24 without alkaline treatment. Thereby, the fresh fruits, after a wash to remove dirty and  
25 impurities, are placed in a 7-10% NaCl solution where the addition of different organic  
26 acids (citric, acetic or lactic acid) to decrease the initial pH is a common practice. In this  
27 way, the olive sweetening is achieved by diffusion of the bitter glucoside oleuropein from  
28 fruits into the cover brines, where it is finally hydrolysed.

29 Lactic acid bacteria (LAB) are the most important microorganisms responsible for  
30 the fermentation of NaOH treated table olives and other fermented vegetables (Hurtado et  
31 al. 2012; Pérez-Díaz et al. 2013). By sugars consumption and subsequent production of  
32 lactic acid and other antimicrobial metabolites, the LAB population contributes to the safe  
33 preservation of olives by formation of lactic acid, reduction of pH and production of  
34 bacteriocins. In directly brined natural olives, both LAB and yeasts may usually coexist  
35 along the entire process although, sometimes, yeasts can play a more relevant role in the  
36 fermentation due to partial inhibition of LAB by the presence of phenolic compounds  
37 (Aponte et al. 2010; Balatsouras 1990; Brenes 2004; Garrido-Fernández et al. 1997;  
38 Sánchez et al. 2000; Tassou et al. 2002). Thereby, regardless of olive processing, both  
39 groups of microorganisms determine the quality, safety and flavour of the final products.

40 For many years, the microbiological study of table olive fermentations has been  
41 exclusively focused on the isolation, identification and characterization of microorganisms  
42 present in brines. However, recent studies carried out with table olives have shown the  
43 presence of polymicrobial communities adhered to both biotic (olive skin) and abiotic  
44 (glass slides) surfaces during the fermentation process (Arroyo-López et al. 2012a;  
45 Domínguez-Manzano et al. 2012; Nychas et al. 2002). As observed by scanning electron  
46 microscopy (SEM), these polymicrobial communities consisted of different yeast and  
47 bacteria species embedded in a matrix which keeps them in close proximity. Detachment of  
48 microorganisms from olive skin to determine the number of cells and further molecular  
49 identification using a protocol consisting of an enzymatic method and RAPD analysis,  
50 revealed the presence of *Pichia galeiformis*, *Candida sorbosa* and *Geotrichum candidum* for  
51 the yeast species, and *Lactobacillus pentosus* for the LAB population (Arroyo-López et al.  
52 2012a; Domínguez-Manzano et al. 2012). After detachment, both yeasts and bacteria  
53 species yielded high population levels ( $>7 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$ ), thus showing that the olives could  
54 be a good carrier of microorganisms. However, this methodology implies a wide variation  
55 in the number of microbial cells recovered, which also depends on the group of  
56 microorganisms. In fact, the cocktail of enzyme detaches completely the LAB population  
57 after 6 h of incubation whereas the release of yeasts requires up to 16 h treatment  
58 (personal communication). Greek researches have also evaluated mechanic disaggregation  
59 with stomacher for detachment of *Lactobacillus pentosus* and *Pichia membranifaciens*  
60 species from ripe black (darkened by oxidation) olives with good results, obtaining  $>7$   
61  $\log_{10} \text{ cfu}\cdot\text{g}^{-1}$  (Grounta and Panagou 2014). However, comparison of results is difficult  
62 because the use of different methodologies. Thus, bearing in mind the transcendence of  
63 further studies on olive biofilms, the standardization of a rapid and accurate procedure to  
64 recover microbes from these fruits is needed.

65 In this work, we study the fermentation process and the formation of true biofilms  
66 on natural green Gordal table olives. For the quantification of the microbial populations on  
67 olives, several methods for detachment, recovery and counting of microorganisms attached  
68 to fruits have been assessed. Furthermore, the biodiversity of the most important LAB and  
69 yeast species present until now unexplored biofilms formed in this type of table olive  
70 preparation was investigated by molecular methods.

## 71 **Material and methods**

### 72 Olive fermentations

73 Olive fruits from Gordal variety were obtained during the 2013/2014 season at the  
74 green ripening stage from the olive processing plant Ntra. Sra. de las Virtudes S.C.A. (La  
75 Puebla de Cazalla, Seville, Spain), and transported to our laboratory where they were  
76 classified by size, washed and directly brined in polyethylene fermentation vessels. The  
77 process was achieved as industry, by immersing 20 kg of fruits into 13 l of brine (10%  
78 NaCl, 0.5 % acetic acid and 0.1% citric acid). The fermentation was let to evolve  
79 spontaneously. The study was carried out in two independent fermentation vessels and  
80 monitored during 90 d.

### 81 Analysis of the fermentation brines

82 Physicochemical control of the fermentation was achieved through periodical  
83 analyses of brine (0, 10, 20, 40, 60 and 90 d) for determination of pH, NaCl concentration  
84 (% wt·vol<sup>-1</sup>), titratable acidity, expressed as g lactic acid per 100 ml of brine, and combined  
85 acidity (undissociated organic salts, expressed as Eq·l<sup>-1</sup>) (Garrido-Fernández et al. 1997).

86 To study the evolution of the different microbial populations, brine samples were  
87 taken at different times throughout fermentation (0, 3, 6, 10, 20, 30, 60, 90 d) and diluted, if  
88 necessary, in a sterile saline solution (0.9% NaCl). Then, they were plated using a Spiral  
89 System (model dwScientific, Don Whitley Scientific Limited, England) on appropriate  
90 media. *Enterobacteriaceae* were counted on Crystal Violet Neutral-Red Bile Glucose (VRBD)  
91 agar (Merck, Darmstadt, Germany), LAB were proliferated on de Man, Rogosa and Sharpe  
92 (MRS) agar (Oxoid, Basingstoke, Hampshire, England) supplemented with 0.02% sodium  
93 azide (Sigma, St. Luis, USA), and yeasts were grown on yeast-malt-peptone-glucose  
94 medium (YM) agar (Difco, Becton and Dickinson Company, Sparks, MD, USA) supplemented  
95 with oxytetracycline and gentamicin sulphate (0.005%) as selective agents. The plates  
96 were incubated at 30°C for 48-72 h and counted using a CounterMat v.3.10 (IUL, Barcelona,  
97 Spain) image analysis system. Brine counts were expressed as  $\log_{10}$  cfu·ml<sup>-1</sup>.

98 The plot of the  $\log_{10}$  cfu·ml<sup>-1</sup> versus time for microorganisms produced a sigmoid-  
99 shape curve that was fitted using the reparameterized Gompertz equation proposed by  
100 Zwietering et al. (1990), which has the following expression:

$$101 \quad y = N_{max} * \exp(-\exp\{(\mu_{max} * e * (\lambda - x)) / N_{max} + 1\})$$

102 where  $y$  is the microbial concentration ( $\log_{10}$  cfu·ml<sup>-1</sup>) at time  $t$ ,  $x$  is the time (days),  $N_{max}$  is  
103 the maximum population reached ( $\log_{10}$  cfu·ml<sup>-1</sup>),  $\mu_{max}$  is the maximum growth rate (d<sup>-1</sup>)  
104 and  $\lambda$  is the lag phase (d). These parameters were obtained by a nonlinear regression  
105 procedure, minimizing the sum of squares of the difference between the experimental data  
106 and the fitted model, i.e. loss function (observed - predicted). This task was accomplished  
107 using the nonlinear module of the Statistica 7.1 software package (StatSoft Inc, Tulsa, OK,  
108 USA) and its Quasi-Newton option. Fit adequacy was checked by the proportion of total  
109 variance explained by the model ( $R^2$ ).

110 In situ observation of olive epidermis

111           The presence of biofilms on the epidermis of fruits at the end of fermentation (90 d)  
112 was confirmed by using SEM techniques. For this purpose, olives were treated following  
113 the methodology described by Krouwilleypitski et al. (2009) with slight modifications.  
114 First, fruits were rinsed twice for 15 min in a PBS buffer solution (8.0 g·l<sup>-1</sup> NaCl, 0.2 g·l<sup>-1</sup> KCl,  
115 1.44 g·l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g·l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 adjusted with HCl 1M) for removing non-  
116 adhering cells, and then fixed in 2.5 % glutaraldehyde (Sigma-Aldrich, St. Louis, USA) in  
117 PBS for 2.5 h. Later, the olives were dehydrated through a graded ethanol series (50%,  
118 70%, 80%, 90%, 95% and 100%, 5 min in each one). Finally, fruits were treated for 20 min  
119 in 2-methyl-2-propanol. For SEM observation, 2 mm<sup>2</sup> slices of the skin of olives were taken  
120 and placed on glass slides and coated with gold in a Scancoat Six SEM sputter coater  
121 (Edwards, Crawley, England). Pictures were taken with a JEOL JSM- 6460LV SEM model  
122 (JEOL USA, Inc., Peabody, MA) in the Technology and Innovation Research Center at  
123 University of Seville (CITIUS, Seville, Spain).

124 Assessment of the efficacy of different methodologies for the detachment/recovery of  
125 microorganisms from biofilms and fruits

126           All methods described below were applied to 2 olives removed under sterile  
127 conditions from the fermentation vessels at the end of the fermentation process (90 d),  
128 except the ultrasonic bath which used 5 fruits. For removing microbial non-adhered cells,  
129 fruits were previously washed for 30 min in sterile distilled water, weighed (to further  
130 refer plate counts of microorganisms as log<sub>10</sub> cfu·g<sup>-1</sup>) and spread (after application of  
131 different treatments) onto the different culture media specific for *Enterobacteriaceae*,  
132 yeasts and LAB. Values (means and standard deviations) were obtained from 6  
133 measurements per level (n=6), with three technical replicates per independent duplicate.

134 *Enzymatic method*

135           The protocol developed by Böckelmann et al. (2003) was slight adapted to the  
136 specific characteristics of table olives. Three different types of enzymes (lipase,  $\beta$ -  
137 galactosidase and  $\alpha$ -glucosidase) were purchased (Sigma-Aldrich, St. Louis, USA) and  
138 mixed in the laboratory to obtain an enzymatic cocktail with the following concentrations:  
139 lipase ( $0.74 \text{ mg}\cdot\text{ml}^{-1}$ ),  $\beta$ -galactosidase ( $0.64 \text{ mg}\cdot\text{l}^{-1}$ ), and  $\alpha$ -glucosidase ( $1.05 \text{ }\mu\text{L}\cdot\text{ml}^{-1}$ ).  $\alpha$ -  
140 glucosidase and  $\beta$ -galactosidase were chosen for the cleavage of the  $\alpha$ -D-glucoside residues  
141 and  $\beta$ -galactosidic bonds of exopolysaccharides, respectively, while lipase was added to the  
142 enzyme mixture as lipids represent a considerable part of this component from biofilms  
143 (Böckelmann et al. 2003). It was used at full (standard), half (1/2), double ( $\times 2$ ) and four  
144 ( $\times 4$ ) times concentrations taking as references previous works carried out in table olives  
145 (Arroyo-López et al. 2012a; Domínguez-Manzano et al. 2012). The fruits were incubated at  
146  $30 \text{ }^\circ\text{C}$  for 1 h in 50 ml of PBS buffer containing the different enzyme preparations. The  
147 resultant suspension was centrifuged at  $9,000 \times g$  for 10 min at  $4 \text{ }^\circ\text{C}$ , the pellet was re-  
148 suspended in 2 ml of PBS buffer and finally spread.

149 *Sonication method*

150           In this case, fruits were immersed into 50 ml of a sterile saline solution (0.9% NaCl),  
151 and then sonicated using an ultrasonic liquid processor model Microson™ XL 2000  
152 (QSonica LLC., Newtown, CT, USA) which works at a wave frequency of 22.5 kHz. The  
153 processing tip of the sonicator was dipped 1 cm in the liquid. The olives were sonicated for  
154 0.08, 0.016, 0.5, 1, 2, 5, 10, 15, 20 and 30 min at an ultrasound power of 6W (50 % of the  
155 total intensity). Suspension of the appropriate dilutions were spread plated.

156 *Stomacher method*



157 Fruits were pitted, weighed and immediately transferred into a stomacher bag  
158 containing 75 ml of a sterile saline solution (0.9% NaCl). Then, pulp was homogenized for  
159 1, 5, 10, 15 and 20 min at maximum speed (300 rpm) in a stomacher model Seward 400  
160 (Seward Medical, Ltd., West Sussex, England). Suspension of the appropriate dilutions were  
161 then spread plated.

#### 162 *Ultrasonic bath method*

163 Fruits were immersed into 35 ml of a sterile saline solution (0.9% NaCl) and treated  
164 with an ultrasound bath model Ultrasons 3000513 (J.P. Selecta, S.A., Barcelona, Spain),  
165 which works at a power of 360 W. The olives were treated for 1, 5, 10, 15, 20 and 40 min.  
166 Samples of the resulting suspensions were taken, diluted in saline solution if needed, and  
167 then spread plated. During the entire process, the water in the bath was kept constant at 30  
168 °C by adding ice.

#### 169 Molecular characterization and identification of microorganisms

170 For characterization of yeast isolates, a RAPD-PCR analysis with M13 primer was  
171 followed according to the protocol described by Tofalo et al. (2009), while in the case of  
172 lactobacilli, a rep-PCR analysis was performed using GTG<sub>5</sub> primer (Gevers et al. 2001). PCR  
173 products were electrophoresed in a 2% agarose gel, stained with ethidium bromide (20  
174 min) and visualized under ultraviolet light. The resulting fingerprints were digitally  
175 captured and analysed with the Bio-Numerics 6.6 software package (Applied Maths,  
176 Kortrijk, Belgium). The similarity among digitalized profiles was calculated using the  
177 Pearson product-moment correlation coefficient. The dendrogram was generated by means  
178 of the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering  
179 algorithm. The reproducibility and sensitivity of the method was previously evaluated

180 using, as internal control, 7 LAB and 8 yeast strains belonging to species *Lactobacillus*  
181 *pentosus*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Saccharomyces cerevisiae*,  
182 *Wickerhamomyces anomalus*, *Candida boidinii* and *Pichia galeiformis* obtained from the  
183 Table Olives Microorganisms Collection from Instituto de la Grasa (CSIC, Spain) (data not  
184 shown). Reproducibility of the technique, in the worst case, was determined in 85.1% and  
185 80.5% for LAB and yeasts, respectively.

186 Then, molecular identification of representative genotypes was performed using  
187 multiplex PCR of *recA* gene (Torriani et al., 2001) and RFLP analysis of *dnaK* gene (Huang et  
188 al. 2010) in the case of LAB, or RFLP analysis of 5.8S ITS region (Esteve-Zarzoso et al. 1999)  
189 in the case of yeasts. The yeast profiles generated were then compared with existing  
190 databases ([www.yeast-id.org](http://www.yeast-id.org), University of Valencia and CSIC, Spain).

## 191 Statistical analysis

192 An analysis of variance was performed by means of the one-way ANOVA module of  
193 Statistica 7.1 software to check for significant differences among different levels and  
194 microbial recovery methods. For this purpose, a post-hoc comparison was applied by  
195 means of the Scheffé test.

## 196 Results and discussion

### 197 Evolution and control of fermentation

198 Titratable acidity and pH are critical parameters to monitor completion of a safe  
199 olive fermentation and control the growth of spoilage and pathogenic microorganisms  
200 during fermentation (Garrido-Fernández et al. 1997; Perricore et al. 2010). In this  
201 experiment cover brine pH increased rapidly from an initial value of 2.5 to 3.5 after olive

202 brining (Figure 1a), due to the diffusion of the organic acids into the flesh. The equilibrium  
203 between the olive flesh and cover brine was reached on day 9, after which the pH value  
204 oscillated around 3.5 units until the end of the fermentation. On the contrary, titratable  
205 acidity decreased during the first 18 days from 0.95 to 0.80 g lactic per 100 ml due to, as in  
206 the case of pH, the absorption of organic acids by the pulp. However, a progressive increase  
207 was observed after the 30<sup>th</sup> day, possibly due to the production of lactic acid by the LAB  
208 population, which reached a final value of approximately 1.1 g lactic per 100 ml brine in the  
209 processed product (Figure 1b). Combined acidity increased throughout the fermentation  
210 from initial 0.000 to final 0.035 Eq·l<sup>-1</sup>, while salt concentration decreased from the initial  
211 6.0 to a final 4.5% NaCl, showing the major drop during the first 10 days (data not shown).  
212 These changes in pH and salt, together with combined and titratable acidities obtained, are  
213 typical of directly brined table olive fermentations (Garrido-Fernández et al. 1997).  
214 Furthermore, the pH value far below the limit established for green natural olives (<4.3) in  
215 the Table Olive Standard, and the titratable acidity value above 1.0 g lactic per 100 ml brine  
216 are important aspects to ensure a safe product (Garrido-Fernández et al. 1997; IOC 2004).  
217 Hence, these natural green Gordal olives followed an adequate fermentation process from  
218 the physicochemical point of view.

219           Regarding evolution of microbial populations in brines, *Enterobacteriaceae* were not  
220 detected along the 90 d of the fermentation process. Low pH levels have showed to exert a  
221 considerable inhibitory effect on this microbial group (Garrido-Fernández et al. 1997). On  
222 the contrary, LAB and yeast populations in brine showed the typical growth for this type of  
223 processes. Their evolutions could be well fitted with the reparameterized Gompertz  
224 equation for growth (Zwietering et al. 1990), with a R<sup>2</sup> (quality of the adjustment) of 0.987  
225 for LAB and 0.865 for yeasts (Figure 2). The fitted parameters obtained for LAB population  
226 (Figure 2a) showed a lag phase ( $\lambda$ ) of 3.649±0.778 d, a maximum growth rate ( $\mu_{max}$ ) of

227 0.669±0.106 (d<sup>-1</sup>) and a maximum population size ( $N_{max}$ ) of 6.727±0.239 (log<sub>10</sub> cfu·ml<sup>-1</sup>). In  
228 the case of yeasts (Figure 2b), the values obtained were:  $\lambda$ =0.227±3.483 d,  $\mu_{max}$ =  
229 0.228±0.079 d<sup>-1</sup>, and  $N_{max}$ =5.066±0.687 log<sub>10</sub> cfu·ml<sup>-1</sup>. Therefore, the process was clearly  
230 dominated by LAB, with higher growth rate than yeast (0.669 vs 0.228 d<sup>-1</sup>) and also  
231 maximum population levels (6.73 vs 5.1 log<sub>10</sub> cfu·ml<sup>-1</sup>) in brines, which were obtained  
232 approximately at the 30<sup>th</sup> day of fermentation (Figure 2) and remained stable until the end  
233 of the process. The counts and behaviour obtained for both microbial groups throughout  
234 the fermentation process can also be considered suitable for this type of table olive  
235 elaboration (Arroyo-López et al. 2012b; Nychas et al. 2002).

## 236 SEM

237 Nychas et al. (2002) reported for the first time using SEM techniques the presence of  
238 both LAB and yeast populations colonizing the epidermis of fermented Greek black olives.  
239 However, these authors did not report the presence of a matrix surrounding  
240 microorganisms (true biofilms). Years later, the formation of true mixed biofilms (with  
241 exopolysaccharide matrix) between LAB and yeasts during Spanish-style green table olive  
242 fermentations was reported for different types of olive varieties by Arroyo-López et al.  
243 (2012a) and Domínguez-Manzano et al. (2012). Recently, Grounta and Panagou (2014) also  
244 have showed by SEM the formation of biofilms on Greek black oxidized olives. In this work,  
245 we describe for the first time the formation of microbial biofilms on the epidermis of  
246 Gordal fruits processed as green directly brined “natural” olives.

247 At the end of the fermentation, both LAB and yeasts appear to be strongly adhered  
248 to the epidermis of olives and embedded in a matrix, which is a clear evidence of the  
249 presence of true biofilms in this type of table olive elaboration (Figure 3). SEM pictures also

250 show some microbial cells apparently ready for leaving the biofilms, or just trying to find  
251 physical space to form a thicker layer.

252 Comparison of different methods for quantification and recovery of biofilms

253 Usually, once the biofilm has been formed, the microorganisms are strongly adhered  
254 to the epidermis of the olives and are not released with a simple washing procedure  
255 (Arroyo-López et al. 2012a; Domínguez-Manzano et al. 2012). Furthermore, the efficiency  
256 of the procedures for the biofilm recovery has been scarcely tested. Olives from this  
257 experiment have been used to compare different procedures for detachment and  
258 quantification of microorganisms forming biofilms. The efficacy of each treatment was  
259 measured by statistical analysis of the microorganism mean counts released after its  
260 application.

261 *Sonication method*

262 When a biofilm is sonicated, microorganisms are detached by a mechanism named  
263 cavitation. This term refers to the generation, growth and collapse of microbubbles in the  
264 sonicated liquid. The changes in pressure can lead to the biofilm disaggregation (Piyasena  
265 et al. 2003). In addition of temperature and viscosity of the liquid, frequency and amplitude  
266 of the ultrasonic waves influence the degree of cavitation and therefore the effectiveness of  
267 the treatment (Mason et al. 1996; Sala 1995). A previous work has reported bactericide  
268 and bacteriostatic effects by gradually increasing time and intensity of sonication  
269 (Tsukamoto et al. 2004). In this work, sonication was fixed at medium intensity (6W),  
270 varying sonication times to determine the more effective time to disaggregate the biofilm  
271 and removing the microorganisms without producing lysis or cell inactivation.

272 The effect of different times of sonication on the recovery of LAB and yeast  
273 populations from the Gordal olive biofilms showed that the LAB counts released from the  
274 biofilm were higher than those of yeasts, and that both group of microorganisms increased  
275 their detachment as the time of sonication increased up to 15 min (Figure 4). Thereby,  
276 there was a significant difference in LAB population between the lowest time of sonication  
277 (0.083 min) and the longer treatment (30 min), which released  $\sim 4 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$  and  $\sim 6$   
278  $\log_{10} \text{ cfu}\cdot\text{g}^{-1}$ , respectively. Sonication for periods above 5 min led to similar counts (Table  
279 1). On the contrary, there were no significant differences among the yeast populations from  
280 the diverse treatment levels (period of times), and the counts ranged from  $\sim 2 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$   
281 (0.166 min) to  $\sim 4 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$  (10 min).

#### 282 *Enzymatic method*

283 Detachment of biofilms in table olives by using a cocktail of enzymes has been  
284 previously reported in the literature (Arroyo-López et al., 2012a; Domínguez-Manzano et  
285 al. 2012). Usually, an incubation time of 12 h is applied. However, in this work we have  
286 used lower incubation time (1 h) to avoid exceeding the generation time of LAB and yeasts,  
287 which according to the literature, in optimal conditions, is approximately 1.1 h for many  
288 species of LAB, and 2 h for the growth-faster yeast species (Brizuela et al. 2001; Nagpal and  
289 Kaur 2011; Willey et al. 2011). In this way, duplication of the microorganisms that are  
290 released is prevented and time is reduced. Böckelmann et al. (2003) used an incubation  
291 time of 90 min for detachment of biofilms from soils using the same cocktail of enzymes. No  
292 bacterial growth was observed during treatment for this period of time.

293 After application of the enzymatic method, LAB population levels obtained from  
294 biofilms were considerably higher (approx.  $5 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$ ) than yeasts (about  $1.5 \log_{10}$   
295  $\text{cfu}\cdot\text{g}^{-1}$ ), with no statistical significant differences between different levels of the enzyme

296 cocktail within the same microbial group (see Table 1 and Figure 4). Therefore, according  
297 to the data presented in this study, the enzyme cocktail used in the literature could be  
298 reduced to a half concentration without a loss of effectiveness in the detachment of  
299 biofilms from the olives (Arroyo-López et al. 2012a; Dominguez-Manzano et al. 2012). Due  
300 to the heterogeneity of the extracellular polysaccharides, a mixture of enzymes activities is  
301 usually necessary for destabilization of biofilms (Arroyo-López et al. 2012a; Dominguez-  
302 Manzano et al. 2012). These enzymes have targets for the lipids,  $\alpha$ -D-glucoside residues  
303 and  $\beta$ -galactosidic bonds present in the exopolysaccharide matrix (Böckelmann et al.  
304 2003).

#### 305 *Stomacher method*

306 Release of microorganisms from biofilms using a stomacher apparatus is basically a  
307 physical method where the entire structure of the olives, and consequently the biofilms, are  
308 disaggregated by using paddles to homogenize the food sample immersed into a liquid  
309 medium. This method is widely used in the literature to count microorganisms in solid  
310 foods in which 1-2 min of application is currently used (Grounta and Panagou 2014;  
311 Medina et al. 2007).

312 After application of stomacher for different periods of time, the population levels of  
313 LAB obtained (from 6.5 to 7.0  $\log_{10}$  cfu·g<sup>-1</sup>) were much higher than those of yeasts (in many  
314 cases lower than 1  $\log_{10}$  cfu·g<sup>-1</sup>) with no statistical significant differences between times of  
315 application within the same microbial group (Table 1, Figure 4). In table olives, Grounta  
316 and Panagou (2014) used a stomacher time of 2 min to recover microorganisms present in  
317 fruits, obtaining a maximum recovery of 7  $\log_{10}$  cfu·g<sup>-1</sup> for bacteria and 5  $\log_{10}$  cfu·g<sup>-1</sup> for  
318 yeasts.

319 *Ultrasonic bath method*

320 Ultrasonic baths are commonly used for the sterilization of laboratory and medical  
321 material (Raffin et al. 2008). By immersing the samples into a liquid medium, the ultrasonic  
322 wave is applied in different directions setting a specific frequency. The immersion of the  
323 naturally fermented olives for diverse periods of time in an ultrasound bath working at 50  
324 Hz, showed that LAB population levels obtained ( $\sim 5 \log_{10} \text{cfu}\cdot\text{g}^{-1}$ ) were higher than those of  
325 yeasts (frequently lower than  $1 \log_{10} \text{cfu}\cdot\text{g}^{-1}$ ), with no statistical significant differences  
326 between application times within the same microbial group (Table 1, Figure 4).

327 *Statistical comparison between methods*

328 Table 1 shows the average counts obtained for the different methods and levels  
329 assayed. As can be deduced, there were not statistical significant differences within the  
330 same detachment methodology among the different levels, except for sonication in the  
331 release of LAB.

332 As a summary, Table 2 shows the statistical comparison (Scheffé test) among the  
333 greater LAB and yeast counts obtained within methodologies. The statistical analysis  
334 shows that the best method (highest counts) for recovery of LAB was stomacher applied  
335 for 1 min ( $6.6 \log_{10} \text{cfu}\cdot\text{g}^{-1}$ ) whereas sonication for 5 min ( $3.53 \log_{10} \text{cfu}\cdot\text{g}^{-1}$ ) was the  
336 treatment which led to the best results for yeasts. However, we must bear in mind that  
337 with the stomacher method is not possible to distinguish between microorganisms which  
338 are only present in the superficial biofilms, or inside the fruits. In fact, Nychas et al. (2002)  
339 showed by SEM that a rich biofilm was developed on the epicuticular wax of the olive skin  
340 during fermentation, with yeasts dominated in the stomatal openings, but bacteria were  
341 more numerous in intercellular spaces in the sub-stomatal flesh.



342 Characterization and identification of microorganisms obtained from biofilms

343 Twenty LAB (10 of them isolated from olive epidermis and other 10 isolated from  
344 brines) and 11 yeast isolates (2 isolated from fruits and 9 from brines) were randomly  
345 obtained at the end of the fermentation process. A reduced number of yeast isolates was  
346 obtained because of the lower counts obtained from olive surface for this type of  
347 microorganisms at the end of fermentation.

348 The dendrogram generated by rep-PCR with primer GTG<sub>5</sub> using the patterns profile  
349 of the 20 LAB isolates randomly obtained at the end of fermentation (Figure 5) showed that  
350 the isolates formed two groups clearly differentiated, sharing 78.8% similarity in their  
351 banding profile. The first group included isolates obtained from brines (7) and fruits (8),  
352 with a coefficient of similarity of 90.7%. The second group presented a coefficient of  
353 similarity of 93.6%, being formed by 2 isolates of fruits and 3 isolates of brine. Because of  
354 the reproducibility of the rep-PCR analysis for LAB was determined in 85.1%, it was  
355 inferred that only two genotypes were present among the LAB population in the  
356 fermentation of natural green Gordal olives. Two representative isolates from each  
357 genotype (S5, S7, F10 and S9) were selected for identification purposes. Using the  
358 multiplex PCR method based on *recA* gene (Torriani et al. 2001) and RFLP analysis based  
359 on *dnaK* gene (Huang et al. 2010), all selected isolates were identified as *Lactobacillus*  
360 *pentosus* (multiplex PCR amplification of *recA* gene of 218 bp; profile of RFLP *dnaK* gene  
361 with *TSP509I* enzyme of 470+290+200+140 bp), thus indicating the presence of two  
362 different strains of the same species in the fermentation process. The presence of *L.*  
363 *pentosus* in vegetable fermentations, and particularly in biofilms of olives, has already been  
364 previously described (Arroyo-López et al. 2012a; Domínguez-Manzano et al. 2012; Grounta  
365 and Panagou 2014).

366 The dendrogram obtained by RAPD-PCR with primer M13 using the patten profiles  
367 of eleven yeast isolates, randomly selected from brines (9) or fruits (2) (Figure 6) showed  
368 the presence of two major groups sharing a low homology among them according to their  
369 banding profiles (9.6%). Taking into account the technique reproducibility for yeasts  
370 (80.5%), four different genotypes were distinguished. One representative isolate from each  
371 group (F2, S8, S3 and S4) was selected for identification purposes.

372 The restriction profiles generated by a battery of endonucleases on the 5.8-ITS  
373 region (Table 3) and further comparison in yeast data base, showed that isolates S4 and S8  
374 obtained from brines belong to the same species (*P. galeiformis*), while the isolate F2  
375 obtained from fruits was identified as *P. membranifaciens*. Both yeast species have  
376 previously been isolated from diverse table olive elaborations (Arroyo-López et al. 2012b)  
377 and biofilms (Grounta and Panagou 2014). The profile restriction obtained for S3 isolate  
378 has not been found in the yeast database or in the literature, and further studies must be  
379 performed for its identification.

## 380 **Conclusions**

381 In the present study, it has been shown for the first time the formation of poly-  
382 microbial biofilms on natural green Gordal olives. The highest recovery of LAB from these  
383 biofilms was achieved by using the stomacher for 1 min, while the highest yeast  
384 detachment was observed after sonication for 5 min. Thus, a combined treatment  
385 consisting of sonication and subsequent physical disaggregation of olives with stomacher  
386 could be very useful for a complete release of the different group of microorganisms, which  
387 should be confirmed in further studies. *L. pentosus* and *P. membranifaciens* were recovered  
388 from these biofilms at the end of the fermentation, with higher counts obtained for the

389 bacteria. Hence, the study of the microorganisms forming biofilms on the epidermis of  
390 natural green table olives and the searching of those with beneficial properties is an  
391 interesting challenge because these fruits can also carry a high number of microorganisms  
392 ( $>6.5 \log_{10} \text{ cfu}\cdot\text{g}^{-1}$ ). The use of natural olives for the development of potential probiotic  
393 olives is interesting due to is friendly (absence of lye treatment) and low energy cost  
394 processing.

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### 404 **Conflict of interest**

405 The authors declare that they have not conflict of interest.

### 406 **References**

407 Aponte M, Ventrino V, Blaiotta G, Volpe G, Farina V, Avellone G, Lanza CM, Moschetti G  
408 (2010) Study of green Sicilian table olive fermentations through microbiological,  
409 chemical and sensory analyses. Food Microbiol 27:162-170.

410 Arroyo-López FN, Bautista-Gallego J, Domínguez-Manzano J, Romero-Gil V, Rodríguez-  
411 Gómez F, García-García P, Garrido-Fernández A, Jiménez-Díaz R (2012a) Formation of  
412 lactic acid bacteria-yeasts communities on the olive surface during Spanish-style  
413 Manzanilla fermentations. *Food Microbiol* 32:295-301.

414 Arroyo-López FN, Romero-Gil V, Bautista-Gallego J, Rodríguez-Gómez F, Jiménez-Díaz R,  
415 García-García P, Querol A, Garrido-Fernández A (2012b) Yeasts in table olive  
416 processing: desirable or spoilage microorganisms?. *Int J Food Microbiol* 160:42-49.

417 Balatsouras G (1990) Edible olive cultivars, chemical composition of fruit, harvesting,  
418 transportation, processing, sorting and packaging, styles of black olives, deterioration,  
419 quality standards, chemical analysis, nutritional and biological value of the end  
420 product. *Olio d'oliva e olive da tavola: tecnologia e qualità* 25:291-330.

421 Böckelmann U, Szewzyk U, Grohmann E (2003) A new enzymatic method for the  
422 detachment of particle associated soil bacteria. *J Microbiol Methods* 55:201-211.

423 Brenes M (2004) Olive Fermentation and Processing: Scientific and Technological  
424 Challenges. *J Food Sci* 69:FMS33-FMS34.

425 Brizuela MA, Serrano P, Pérez Y (2001) Studies on probiotics properties of two  
426 lactobacillus strains. *Brazilian Arch Biol Technol* 44:95-99.

427 Domínguez-Manzano J, Olmo-Ruiz C, Bautista-Gallego J, Arroyo-López FN, Garrido-  
428 Fernández A, Jiménez-Díaz R (2012) Biofilm formation on abiotic and biotic surfaces  
429 during Spanish style green table olive fermentation. *Int J Food Microbiol* 157:230-238.

430 Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP  
431 analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int  
432 J Syst Bacteriol 49:329-337.

433 Garrido Fernández A, Fernandez-Diez M, Adams MR (1997) Table olives: production and  
434 processing. Chapman and Hall, London (U.K).

435 Gevers D, Huys G, Swings J (2001) Applicability of rep-PCR fingerprinting for identification  
436 of *Lactobacillus* species. FEMS Microbiol Lett 205:31-36.

437 Grounta A, Panagou EZ (2014) Mono and dual species biofilm formation between  
438 *Lactobacillus pentosus* and *Pichia membranifaciens* on the surface of black olives under  
439 different sterile brine conditions. Annals Microbiol 64:1757-1767.

440 Huang C, Lee F, Liou J (2010) Rapid discrimination and classification of the *Lactobacillus*  
441 *plantarum* group based on a partial dnaK sequence and DNA fingerprinting  
442 techniques. Antonie van Leeuwenhoek 97:289-296.

443 Hurtado A, Reguant C, Bordons A, Rozès N (2012) Lactic acid bacteria from fermented table  
444 olives. Food Microbiol 31:1-8.

445 IOC (International Olive Oil Council) (2004) Trade standard applying to table olives.  
446 Resolution RES-2/91-IV/04. Madrid, Spain.

447 Kroupitski Y, Golberg D, Belausov E, Pinto R, Swartzberg D, Granot D, Sela S (2009)  
448 Internalization of *Salmonella enterica* in leaves is induced by light and involves  
449 chemotaxis and penetration through open stomata. Appl Environ Microbiol 75:6076-  
450 6086.

451 Mason TJ, Paniwnyk L, Lorimer JP (1996) The uses of ultrasound in food technology.  
452 Ultrason Sonochem 3:S253-S260.

453 Medina E, Romero C, Brenes M, De Castro A (2007) Antimicrobial activity of olive oil,  
454 vinegar, and various beverages against foodborne pathogens. J Food Prot 70:1194-  
455 1199.

456 Nagpal R, Kaur A (2011) Synbiotic effect of various prebiotics on in vitro activities of  
457 probiotic lactobacilli. Ecol Food Nutr 50:63-68.

458 Nychas GE, Panagou EZ, Parker ML, Waldron KW, Tassou CC (2002) Microbial colonization  
459 of naturally black olives during fermentation and associated biochemical activities in  
460 the cover brine. Lett Appl Microbiol 34:173-177.

461 Pérez-Díaz IM, Breidt F, Buescher RW, Arroyo-López FN, Jiménez-Díaz R, Garrido-  
462 Fernández A, Bautista-Gallego J, Yoon SS, Johanningsmeire SD (2013) Fermented and  
463 Acidified Vegetables. In: Compendium of methods for the microbiological examination  
464 of foods. 4<sup>th</sup> Edition (Ed. Pouch-Downes, F., and Ito, K.). American Public Health  
465 Association, Washington, USA. pp- 521-532.

466 Perricone M, Bevilacqua A, Corbo MR, Sinigaglia M (2010) Use of *Lactobacillus plantarum*  
467 and glucose to control the fermentation of “Bella di Cerignola” table olives, a  
468 traditional variety of Apulian region (southern Italy). J Food Sci 75:M430-M436.

469 Piyasena P, Mohareb E, McKellar R (2003) Inactivation of microbes using ultrasound: a  
470 review. Int J Food Microbiol 87:207-216.

471 Raffin J, Peyron I, Sarfati A (2008) Ultrasounds in sterilization: Study on recommendations  
472 follow-up. Pharmacie Hospitalier 43:81-86.

473 Sala Trepas FJ (1995) Food preservation through combined processes. Microbiol SEM  
474 11:23-32.

475 Sánchez AH, De Castro A, Rejano L, Montaña A (2000) Comparative study on chemical  
476 changes in olive juice and brine during green olive fermentation. J Agric Food Chem  
477 48:5975-5980.

478 Tassou CC, Panagou EZ, Katsaboxakis KZ (2002) Microbiological and physicochemical  
479 changes of naturally black olives fermented at different temperatures and NaCl levels  
480 in the brines. Food Microbiol 19:605-615.

481 Tofalo R, Chaves-López C, Di Fabio F, Schirone M, Felis GE, Torriani S, Paparella A, Suzzi G  
482 (2009) Molecular identification and osmotolerant profile of wine yeasts that ferment a  
483 high sugar grape must. Int J Food Microbiol 130:179-187.

484 Torriani S, Felis GE, Dellaglio F (2001) Differentiation of *Lactobacillus plantarum*, *L.*  
485 *pentosus*, and *L. paraplantarum* by *recA* Gene Sequence Analysis and Multiplex PCR  
486 Assay with *recA* Gene-Derived Primers. Appl Environ Microbiol 67:3450-3454.

487 Tsukamoto I, Yim B, Stavarache CE, Furuta M, Hashiba K, Maeda Y (2004) Inactivation of  
488 *Saccharomyces cerevisiae* by ultrasonic irradiation. Ultrason Sonochem 11:61-65.

489 Willey JM, Sherwood L, Woolverton CJ (2011) Prescott's microbiology, McGraw-Hill.

490 Zwietering MH, Jongenburger I, Rombouts FM, Van't Riet K (1990) Modeling of the  
491 bacterial growth curve. Appl Environ Microbiol 56:1875-1881.

492 **Figure Legends**

493  
494 *Figure 1.* Evolution of pH (a) and titratable acidity (b) throughout fermentation of Gordal  
495 directly brine natural table olives.

496  
497 *Figure 2.* Reparameterized Gompertz equation fit to the plate counts ( $\log_{10}$  cfu·ml<sup>-1</sup>) of LAB  
498 (a) and yeast (b) populations in brines throughout the fermentation process of directly  
499 brined Gordal variety olives.

500  
501 *Figure 3.* SEM pictures obtained from the epidermis of natural green Gordal olives after 90  
502 days of fermentation. Arrows indicate LAB and yeasts surrounded by a matrix in the  
503 biofilms.

504  
505 *Figure 4.* Counts ( $\log_{10}$  cfu·g<sup>-1</sup>) of the LAB and yeasts populations obtained after application  
506 of different sonication times, enzymatic concentrations, stomacher and ultrasonic bath  
507 times to the biofilms formed on the skin of directly brined Gordal olives. The means and the  
508 associated deviations were obtained from n=6 measurements for each level. Temperature  
509 in the ultrasonic bath was kept constant at 30 °C by ice addition.

510  
511 *Figure 5.* Dendrogram generated after bioinformatic analysis with Bionumerics 6.6  
512 software package of the rep-PCR profiles obtained with GTG<sub>5</sub> primer for the different LAB  
513 randomly isolated from brines (S) or biofilms (F) at the end of fermentation (90 d).

514  
515 *Figure 6.* Dendrogram generated after bioinformatic analysis with Bionumerics 6.6  
516 software package of the RAPD-PCR profiles obtained with M13 primer for the different  
517 yeast isolates randomly obtained from brines (S) or biofilms (F) at the end of fermentation  
518 (90 d).



**Table 1.** Average plate counts ( $\log_{10}$  cfu·g<sup>-1</sup>) (n=6) of the LAB and yeasts populations adhered to the olive surface after application of the different detachment methods and levels.

Stomacher	Levels	1 min	5 min	10 min	15 min	20 min							
	LAB		6.57 (0.40) <sup>a</sup>	6.79 (0.36) <sup>a</sup>	6.74 (0.44) <sup>a</sup>	7.02 (0.14) <sup>a</sup>	6.88 (0.37) <sup>a</sup>						
Yeast		1.13 (0.88) <sup>a</sup>	1.46 (1.28) <sup>a</sup>	0.00 (0.00) <sup>a</sup>	0.00 (0.00) <sup>a</sup>	0.81 (1.26) <sup>a</sup>							
Enzymatic	Levels	1/2	Standard	×2	×4								
	LAB	5.26 (0.37) <sup>a</sup>	5.30 (0.49) <sup>a</sup>	5.21 (0.79) <sup>a</sup>	5.37 (0.41) <sup>a</sup>								
Yeast	1.30 (0.82) <sup>a</sup>	1.29 (0.25) <sup>a</sup>	1.24 (0.72) <sup>a</sup>	1.81 (0.60) <sup>a</sup>									
Sonication	Levels	0.08 min	0.16 min	0.25 min	0.33 min	0.50 min	1 min	2 min	5 min	10 min	15 min	20 min	30 min
	LAB	4.23 (0.58) <sup>a</sup>	4.31 (0.45) <sup>a</sup>	4.39 (0.23) <sup>a,b</sup>	4.63 (0.31) <sup>a,b</sup>	4.37 (0.53) <sup>a,b</sup>	4.57 (0.38) <sup>a,b</sup>	4.71 (0.43) <sup>a,b</sup>	5.43 (0.35) <sup>a,b</sup>	5.59 (0.42) <sup>a,b</sup>	5.93 (0.31) <sup>b</sup>	5.63 (0.88) <sup>a,b</sup>	6.07 (0.15) <sup>b</sup>
Yeast	2.22 (0.40) <sup>a</sup>	1.75 (1.38) <sup>a</sup>	2.02 (0.15) <sup>a</sup>	1.92 (0.19) <sup>a</sup>	2.81 (0.36) <sup>a</sup>	1.88 (1.48) <sup>a</sup>	3.05 (0.04) <sup>a</sup>	3.53 (0.14) <sup>a</sup>	3.82 (0.03) <sup>a</sup>	2.47 (0.09) <sup>a</sup>	2.53 (0.49) <sup>a</sup>	1.73 (1.53) <sup>a</sup>	
Ultrasonic bath	Levels	1 min	5 min	10 min	15 min	20 min	40 min						
	LAB	4.79 (0.38) <sup>a</sup>	5.11 (0.56) <sup>a</sup>	5.07 (0.37) <sup>a</sup>	5.11 (0.44) <sup>a</sup>	5.21 (0.39) <sup>a</sup>	4.69 (0.47) <sup>a</sup>						
Yeast	0.77 (0.85) <sup>a</sup>	0.63 (1.09) <sup>a</sup>	0.79 (0.87) <sup>a</sup>	1.17 (1.09) <sup>a</sup>	1.16 (0.92) <sup>a</sup>	0.62 (1.07) <sup>a</sup>							

Note: Standard deviation in parentheses. Values followed by different superscript letters, within the same row, are significantly different according to Scheffé post-hoc comparison test.

**Table 2.** One-way ANOVA analysis for the comparison among the best levels of the diverse detachment methods for lactic LAB and yeasts populations.

<b>Procedure/Level</b>	<b>LAB (<math>\log_{10}</math> cfu·g<sup>-1</sup>)</b>	<b>Yeast (<math>\log_{10}</math> cfu·g<sup>-1</sup>)</b>
Stomacher (1 min)	6.57 (0.40) <sup>a</sup>	1.13 (0.88) <sup>a</sup>
Enzymatic (1/2)	5.25 (0.37) <sup>b</sup>	1.30 (0.82) <sup>a</sup>
Sonication (5 min)	5.43 (0.35) <sup>b</sup>	3.53 (0.14) <sup>b</sup>
Ultrasonic bath (1 min)	4.79 (0.38) <sup>b</sup>	0.77 (0.85) <sup>a</sup>

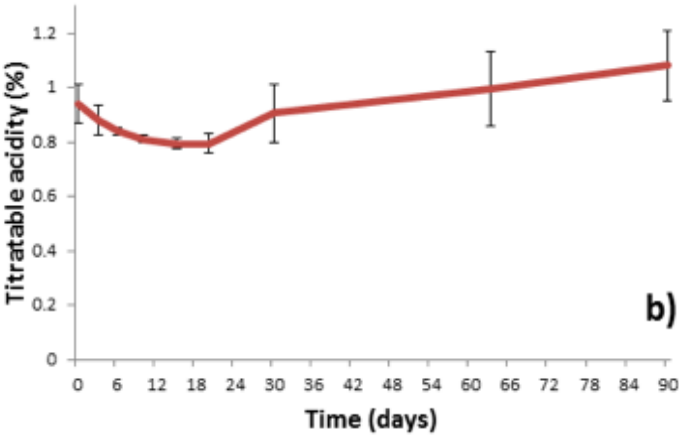
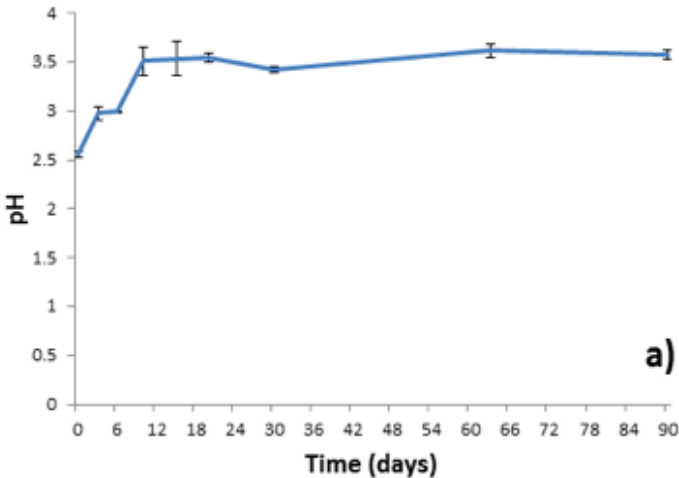
Note: Standard deviations are in parentheses. Values followed by different superscript letters, within the same column, are significantly different according to Scheffé post-hoc comparison test.

**Table 3.** RFLP profiles (in bp) for the 5.8-ITS region of the four selected yeast isolates from rep-PCR analysis with M13 primer.

Isolates	<i>Restriction enzyme</i>				Species
	PCR	<i>CfoI</i>	<i>HaeIII</i>	<i>HinI</i>	
S-3	480	270+250+100+70	320+90+50	300+250+200+190	Unknown profile
S-4	460	250+100+60	320+90+50	250+200	<i>Pichia galeiformis</i>
S-8	460	250+100+60	320+90+50	250+200	<i>Pichia galeiformis</i>
F-2	490	190+110+90	320+90+50	275+200	<i>Pichia membranifaciens</i>

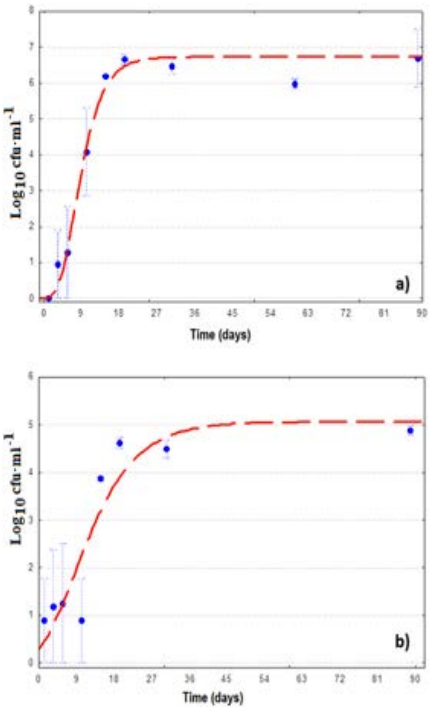
519

Figure 1



520

Figure 2



521

Figure 3

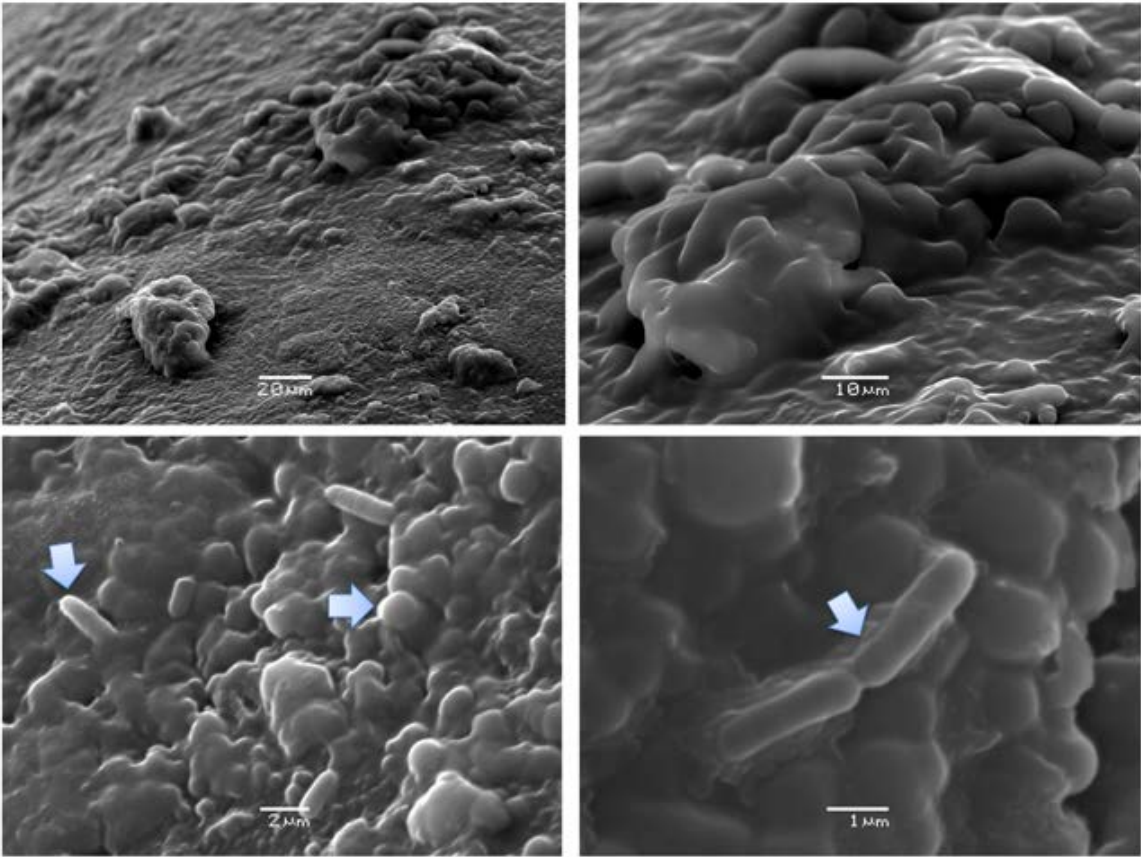
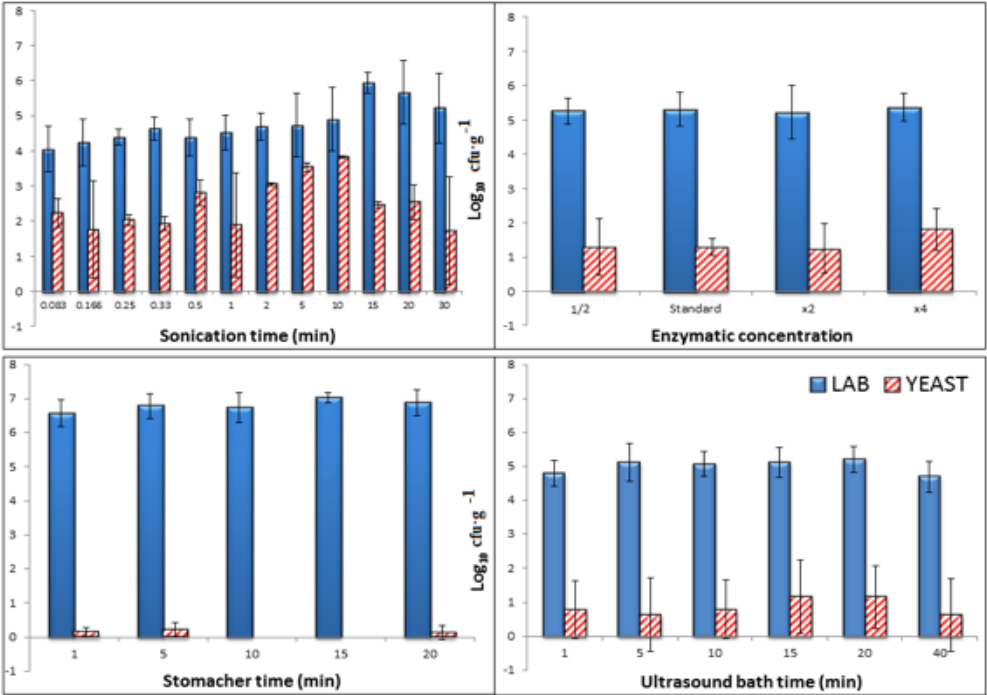
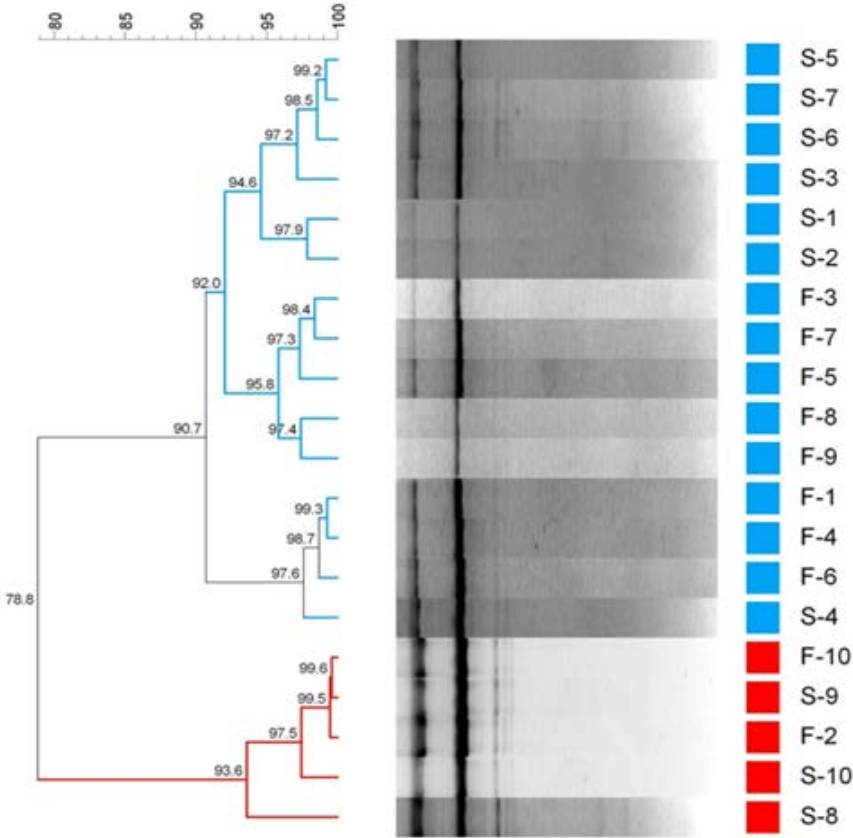


Figure 4



523

Figure 5



524



Figure 6

