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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Abstract

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

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

Introduction

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

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

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

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

Material and methods

Olive fermentations

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

Analysis of the fermentation brines

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

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

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

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

where y is the microbial concentration ($\log_{10}$ cfu·ml^{-1}) at time t, x is the time (days), N_{max} is the maximum population reached ($\log_{10}$ cfu·ml^{-1}), μ_{max} is the maximum growth rate (d^{-1}) and λ is the lag phase (d). These parameters were obtained by a nonlinear regression procedure, minimizing the sum of squares of the difference between the experimental data and the fitted model, i.e. loss function (observed - predicted). This task was accomplished using the nonlinear module of the Statistica 7.1 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option. Fit adequacy was checked by the proportion of total 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 $g \cdot l^{-1}$ NaCl, 0.2 $g \cdot l^{-1}$ KCl,
115 1.44 $g \cdot l^{-1}$ Na_2HPO_4, 0.24 $g \cdot l^{-1}$ KH_2PO_4, 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^2 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 $\log_{10}$ $cfu \cdot g^{-1}$) 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, β-
137 galactosidase and α-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 $mg \cdot ml^{-1}$), β-galactosidase (0.64 $mg \cdot l^{-1}$), and α-glucosidase (1.05 $\mu L \cdot ml^{-1}$). α-
140 glucosidase and β-galactosidase were chosen for the cleavage of the α-D-glucoside residues
141 and β-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 (×2) and four
144 (x4) 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 ºC for 1 h in 50 ml of PBS buffer containing the different enzyme preparations. The
147 resultant suspension was centrifuged at 9,000 ×*g* for 10 min at 4 ºC, 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^{Tm} 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 ºC 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_5 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

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

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

Statistical analysis

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

Results and discussion

Evolution and control of fermentation

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

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

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

227 0.669±0.106 (d^{-1}) and a maximum population size (N_{max}) of 6.727±0.239 ($\log_{10}$ cfu·ml^{-1}). In
228 the case of yeasts (Figure 2b), the values obtained were: λ=0.227±3.483 d, μ_{max}=
229 0.228±0.079 d^{-1}, and N_{max}=5.066±0.687 $\log_{10}$ cfu·ml^{-1}. Therefore, the process was clearly
230 dominated by LAB, with higher growth rate than yeast (0.669 vs 0.228 d^{-1}) and also
231 maximum population levels (6.73 vs 5.1 $\log_{10}$ cfu·ml^{-1}) in brines, which were obtained
232 approximately at the 30th 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 exopolyssacharide 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 ~4 $\log_{10}$ cfu·g^{-1} and ~6
278 $\log_{10}$ cfu·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 ~ 2 $\log_{10}$ cfu·g^{-1}
281 (0.166 min) to ~ 4 $\log_{10}$ cfu·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 $\log_{10}$ cfu·g^{-1}) than yeasts (about 1.5 $\log_{10}$
295 cfu·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, α-D-glucoside residues
303 and β-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^{-1}) were much higher than those of yeasts (in many
314 cases lower than 1 $\log_{10}$ cfu·g^{-1}) 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^{-1} for bacteria and 5 $\log_{10}$ cfu·g^{-1} for
318 yeasts.

Ultrasonic bath method

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

Statistical comparison between methods

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

As a summary, Table 2 shows the statistical comparison (Scheffé test) among the greater LAB and yeast counts obtained within methodologies. The statistical analysis shows that the best method (highest counts) for recovery of LAB was stomacher applied for 1 min (6.6 $\log_{10}$ cfu·g^{-1}) whereas sonication for 5 min (3.53 $\log_{10}$ cfu·g^{-1}) was the treatment which led to the best results for yeasts. However, we must bear in mind that with the stomacher method is not possible to distinguish between microorganisms which are only present in the superficial biofilms, or inside the fruits. In fact, Nychas et al. (2002) showed by SEM that a rich biofilm was developed on the epicuticular wax of the olive skin during fermentation, with yeasts dominated in the stomatal openings, but bacteria were 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_5 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 *TSP*509I 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 patter 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 $\log_{10}$ cfu·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.

395 Acknowledgements

396 The research leading to these results has received funding from the Spanish
397 Government (project OliFilm AGL-2013-48300-R: www.olifilm.science.com.es), and the
398 Junta de Andalucía (through financial support to group AGR-125). A. Benítez and F.N.
399 Arroyo-López wish to express thanks to the Spanish Government for their pre-doctoral
400 fellowship and postdoctoral research contract (Ramón y Cajal), respectively. V. Romero-Gil
401 would like also to thank the AgriFood Campus of International Excellence (ceiA3), Bank of
402 Santander, Spanish Government and 'Aloreña de Málaga' Olive Manufacturing Association
403 for her pre-doctoral fellowship (training program of Ph.D. in companies).

404 Conflict of interest

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

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Figure Legends

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

Figure 2. Reparameterized Gompertz equation fit to the plate counts (log_{10} cfu·ml^{-1}) of LAB (a) and yeast (b) populations in brines throughout the fermentation process of directly brined Gordal variety olives.

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

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

Figure 5. Dendrogram generated after bioinformatic analysis with Bionumerics 6.6 software package of the rep-PCR profiles obtained with GTG_5 primer for the different LAB randomly isolated from brines (S) or biofilms (F) at the end of fermentation (90 d).

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

Table 1. Average plate counts (log_{10} cfu·g^{-1}) (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)^{a}	6.79 (0.36)^{a}	6.74 (0.44)^{a}	7.02 (0.14)^{a}	6.88 (0.37)^{a}							
	Yeast	1.13 (0.88)^{a}	1.46 (1.28)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.81 (1.26)^{a}							
Enzymatic	Levels	1/2	Standard	×2	×4								
	LAB	5.26 (0.37)^{a}	5.30 (0.49)^{a}	5.21 (0.79)^{a}	5.37 (0.41)^{a}								
	Yeast	1.30 (0.82)^{a}	1.29 (0.25)^{a}	1.24 (0.72)^{a}	1.81 (0.60)^{a}								
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)^{a}	4.31 (0.45)^{a}	4.39 (0.23)$^{a.b}$	4.63 (0.31)$^{a.b}$	4.37 (0.53)$^{a.b}$	4.57 (0.38)$^{a.b}$	4.71 (0.43)$^{a.b}$	5.43 (0.35)$^{a.b}$	5.59 (0.42)$^{a.b}$	5.93 (0.31)^{b}	5.63 (0.88)$^{a.b}$	6.07 (0.15)^{b}
	Yeast	2.22 (0.40)^{a}	1.75 (1.38)^{a}	2.02 (0.15)^{a}	1.92 (0.19)^{a}	2.81 (0.36)^{a}	1.88 (1.48)^{a}	3.05 (0.04)^{a}	3.53 (0.14)^{a}	3.82 (0.03)^{a}	2.47 (0.09)^{a}	2.53 (0.49)^{a}	1.73 (1.53)^{a}
Ultrasonic bath	Levels	1 min	5 min	10 min	15 min	20 min	40 min						
	LAB	4.79 (0.38)^{a}	5.11 (0.56)^{a}	5.07 (0.37)^{a}	5.11 (0.44)^{a}	5.21 (0.39)^{a}	4.69 (0.47)^{a}						
	Yeast	0.77 (0.85)^{a}	0.63 (1.09)^{a}	0.79 (0.87)^{a}	1.17 (1.09)^{a}	1.16 (0.92)^{a}	0.62 (1.07)^{a}						

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.

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

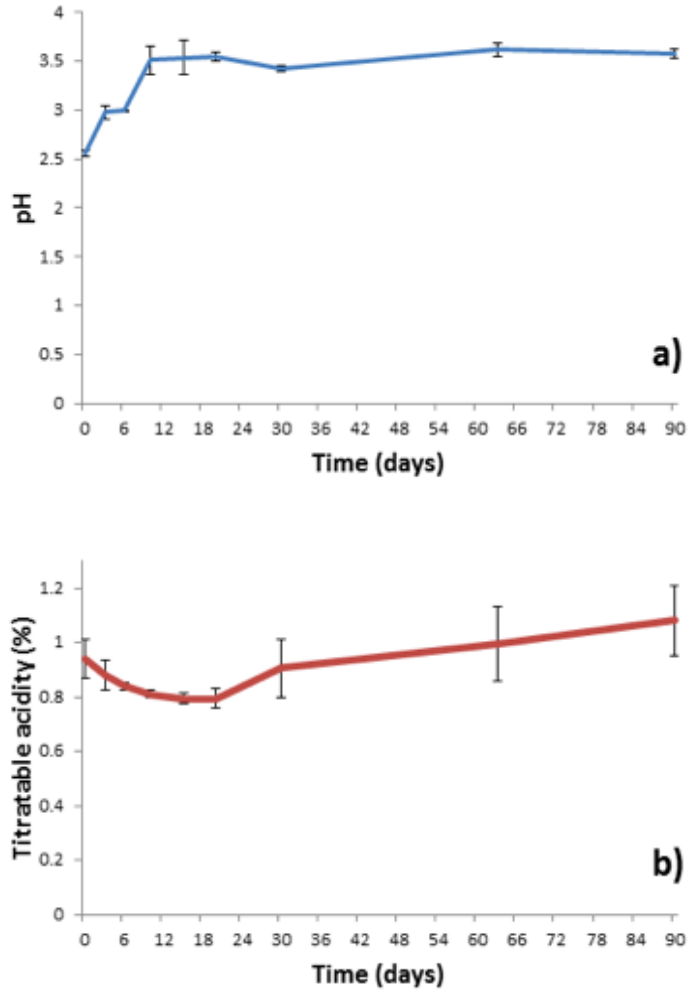
Note: Standard deviations are in parentheses. Values followed by different superscript letters, within the same column, are significantly different according to Scheffé post-hoc comparison test.

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.

		Restriction enzyme			
Isolates	**PCR**	***Cfo*I**	***Hae*III**	***Hinf*I**	**Species**
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	*Pichia galeiformis*
S-8	460	250+100+60	320+90+50	250+200	*Pichia galeiformis*
F-2	490	190+110+90	320+90+50	275+200	*Pichia membranifaciens*

519

Figure 1



520

Figure 2

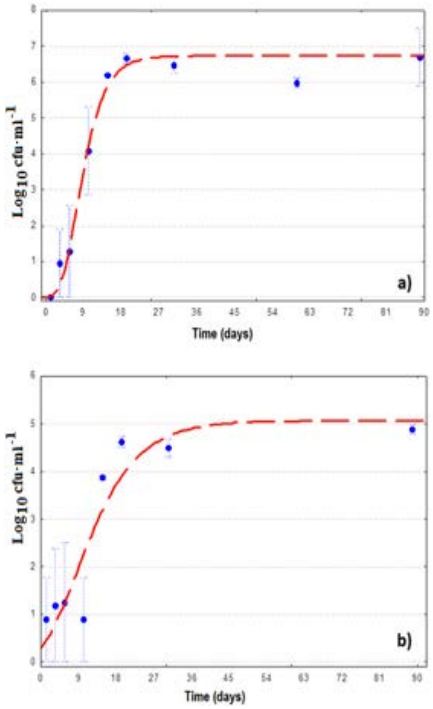


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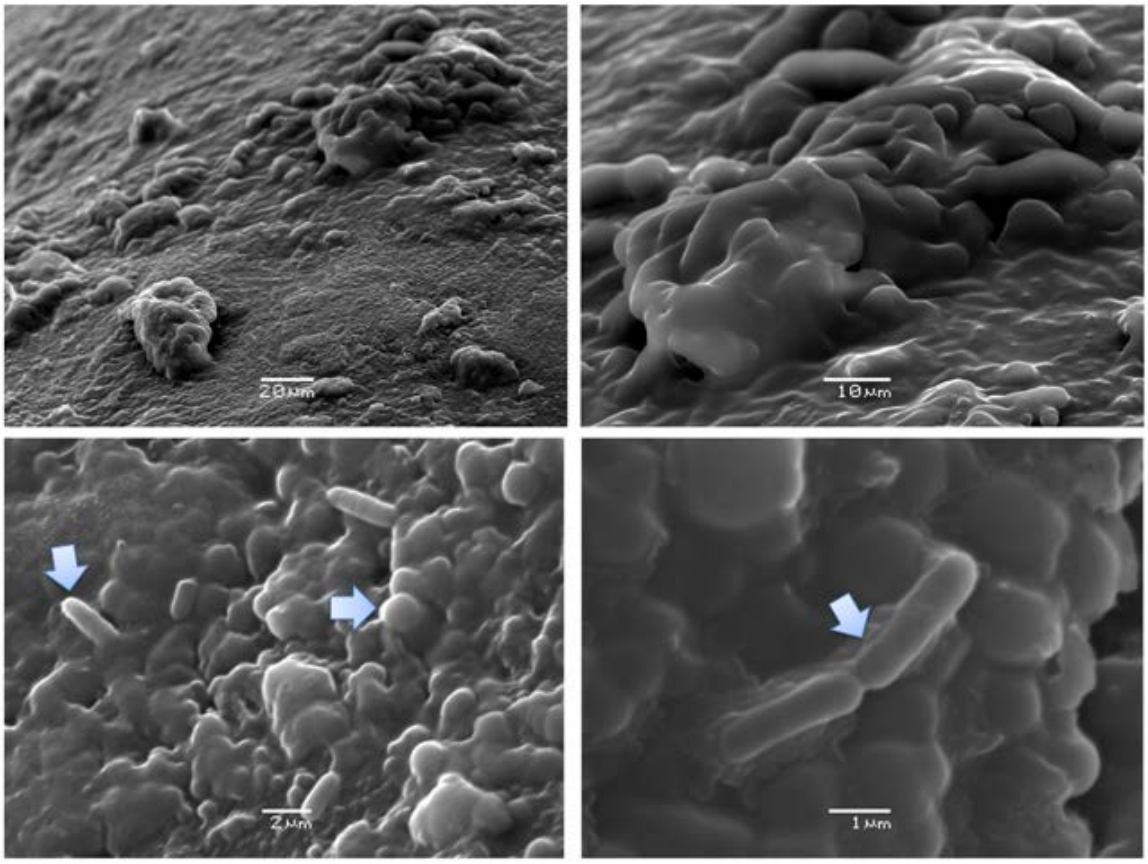
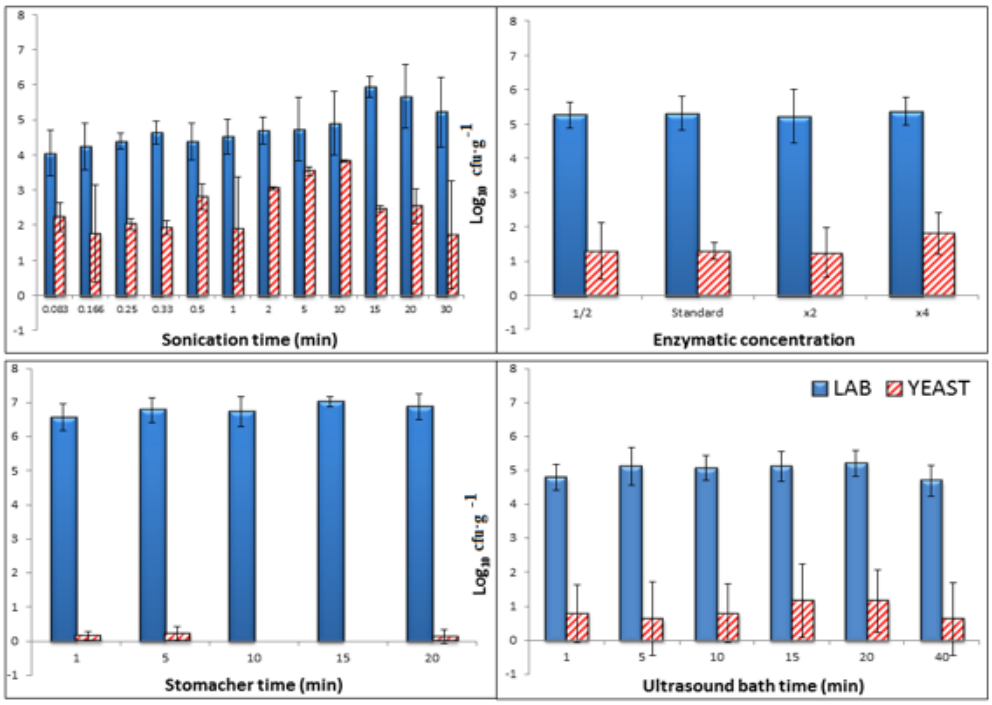
Figure 3



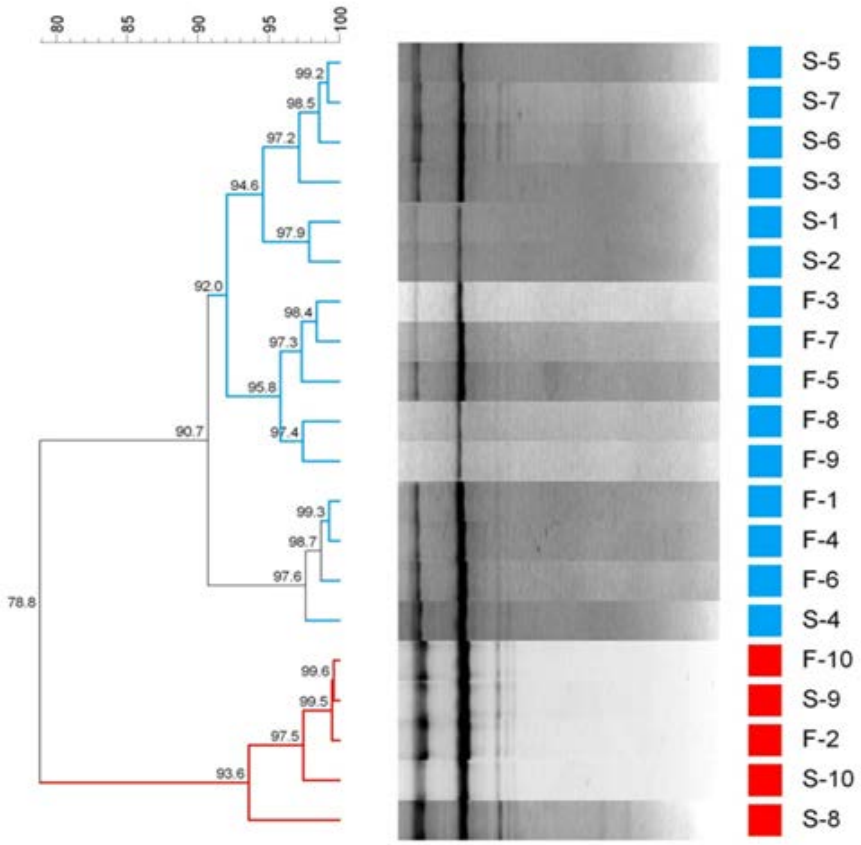
522

Figure 4


Sonication time (min)
Enzymatic concentration
Stomacher time (min)
Ultrasound bath time (min)
LAB
YEAST
Log10 cfu·g-1

523

Figure 5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S-5
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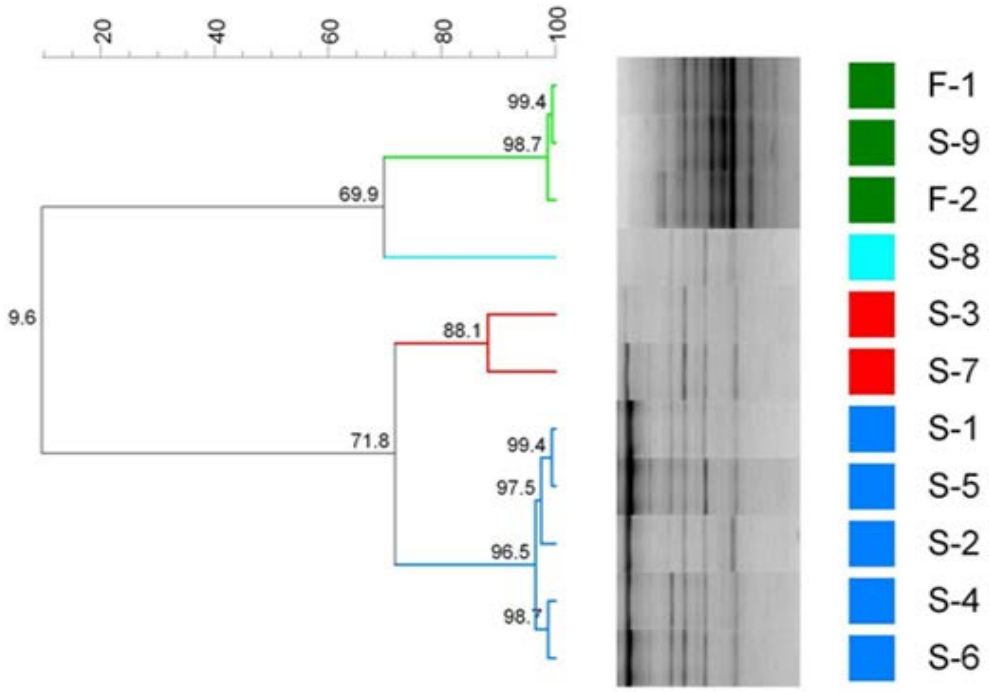
S-8

524

Figure 6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