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## Research Highlights

* Pyrosequencing analysis revealed 131 bacterial genera in fermented olive and brines.

* The biodiversity was higher at the onset of fermentation compared to the end.

* *Celerinatantimonas* was the most important genera found at the end of fermentation.

* *Pseudomonas* and *Propionibacterium* were also common during course of fermentation.

* Food may be considered safe by the practical absence of foodborne pathogens.

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# Assessment of the bacterial community in directly brined *Aloreña de Málaga* table olive fermentations by metagenetic analysis

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**Running title:** Bacterial pyrosequencing in olive fermentations

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**Abstract**

This study uses an "omics" approach to evaluate the bacterial biodiversity changes during fermentation process of natural green cracked *Aloreña de Málaga* table olives, from raw material to fermented fruit. For this purpose, two industries separated by almost 20 km in Guadalhorce Valley (Málaga, Spain) were analysed for obtaining both brines and fruit samples at different moments of fermentation (0, 7, 30 and 120 days). Physicochemical and microbial counts during fermentation showed the typical evolution of this type of processes, apparently dominated by yeasts. However, high-throughput barcoded pyrosequencing analysis of V2-V3 hypervariable region of the bacterial 16S rRNA gene showed at 97% identity the presence of 131 bacterial genera included in 357 operational taxonomic units, not detected by the conventional approach. The bacterial biodiversity was clearly higher in the olives at the moment of reception in the industry and during the first days of fermentation, while decreased considerably as elapse the fermentation process. The presence of *Enterobacteriaceae* and *Lactobabacillaceae* species was scarce during the four months of study. On the contrary, the most important genus at the end of fermentation was *Celerinatantimonas* in both brine (95.3% of frequency) and fruit (89.4%) samples, while the presence of well-known spoilage microorganisms (*Pseudomonas* and *Propionibacterium*) and halophilic bacteria (*Modestobacter, Rhodovibrio, Salinibacter*) was also common during the course of fermentation. Among the most important bacterial pathogens related to food, only *Staphylococcus* genus was found at low frequencies (<0.02% of total sequences). Results show the need of this type of studies to enhance our knowledge of the microbiology of table olive fermentations. It is also necessary to determine the role played by these species not previously detected in table olives on the quality and safety of this fermented vegetable.

**Keywords:** *Aloreña de Málaga*; Bacterial ecology; *Celerinatantimonas*; Olive fermentations; Pyrosequencing.

28 **1. Introduction**

29 Table olives worldwide production nowadays exceeds 2.5 million tons/year,
30 with Spain, Turkey, Egypt, Greek and Italy as the main producer countries (IOC, 2015).
31 This fermented vegetable is prepared with fruits obtained from cultivated *Olea*
32 *eoropaea* subsp. *europaea* var. *europea* trees and it has an important role in the culture
33 and diet of many Mediterranean countries. Olive fruit cannot be consumed directly from
34 the tree due to its peculiar characteristics (presence of the bitter glucoside compound
35 oleuropein). For this reason, diverse methods have been developed to make them
36 palatable. Although many of them share the general process of brining/salting,
37 fermentation and acidification, they can differ slightly between areas of production.
38 Green Spanish-style, Greek naturally black and ripe Californian styles are the most
39 popular commercial preparations (Garrido-Fernández et al., 1997). However, in the last
40 years, consumers are demanding more traditional and natural homemade seasoned
41 olives. *Aloreña de Málaga* is a traditional green olive preparation from Guadalhorce
42 Valley (Málaga, Spain) with a Protected Designation of Origin (PDO) recognized by the
43 European Union (DOUE, 2012). This olive variety has unique features, related to the
44 production area, which make them quite different from others: its fruits are
45 characterized by an excellent flesh-to-stone ratio, a green–yellow colour, a crispy
46 firmness, and a peculiar mild bitter taste. The manufacturing process is carried out by
47 small and medium enterprises placed in, or very close to, the region of production. Due
48 to its low-to-moderate concentrations of oleuropein, the processing does not include
49 alkaline debittering. Thus, they are produced as directly brined olives and seasoned at
50 the moment of packaging (López-López and Garrido-Fernández, 2006).

51 In many cases, table olives are produced thorough spontaneous fermentations
52 performed by the indigenous microbiota activity initially present in olive fruit,
53 ingredients, and the environment (fermentation vessels, pipelines, etc.). It is widely
54 accepted that the main microbiota with a positive role during table olive fermentations
55 are lactic acid bacteria (mainly *Lactobacillus plantarum* and *Lactobacillus pentosus*
56 species) and yeasts (*Saccharomyces cerevisiae, Wickerhamomyces anomalus, Candida*
57 *boidinii,* among others), opposite to the role played by *Enterobacteriaceae*, *Clostridium,*
58 and *Propionibacteriaceae* which are considered undesirable microorganisms (Arroyo-
59 López et al., 2012; Garrido-Fernández et al., 1997; Hurtado et al., 2012).

60 Diverse molecular methods have been used to study the bacterial community
61 associated to *Aloreña de Málaga* table olives fermentation and packaging. Because the
62 presence of high concentrations of antimicrobial compounds, as occurs in other directly
63 brined table olive specialities, it is assumed that the fermentation process is mainly
64 dominated by yeasts (López-López and Garrido-Fernández, 2006). However, recently
65 Abriouel et al. (2012) uses REP-PCR clustering and further identification of strains by
66 sequencing of *phes* and *rpo* genes for the study of the LAB population associated to this
67 table olive speciality, while Romero-Gil et al. (2016) used sequencing of ribosomal 16S
68 gene and multiplex PCR of *recA* gene for the study of the *Enterobacteriaceae* and
69 *Lactobacillaceae* populations, respectively. Unfortunately, the use of methods that rely
70 on the cultivation of microorganism in selective media do not offer a complete profile of
71 the microbial diversity that is present in olive fruit fermentation ecosystem and only a
72 small portion of the true microbial population is detected. For this reason, Abriouel et
73 al. (2011) used a culture-independent approach (PCR-DGGE) for the study of the
74 bacterial biodiversity in *Aloreña de Málaga* fermentations. All these studies were
75 performed exclusively with brines and they did not take into consideration the study of

the microbial population adhered to olive surface, which is finally the food intake by consumers.

Metagenetics has become ubiquitous in the field of microbial ecosystem exploration and diverse natural environments (water, air, soil, plants, digestive tract, etc.) have been thoroughly explored by this approach. High-throughput sequencing has also emerged as a new culture-independent tool to quantitatively investigate the biodiversity of microbial communities in foods in order to look at dominant as well as minor microbial populations, gaining at the same time information of the fermentative process and the microbiota of raw materials (Ercolini, 2013; Kergourlay et al., 2015). It has revolutionized the field of food microbial ecology via more accurate identification of microbial taxa without the need for cultivation-dependent methods. In the specific case of table olive fermentations, recently Cocolin et al. (2013) and De Angelis et al. (2015) have used this powerful methodology for the study of the bacterial biodiversity adhered to the surface of diverse Italian olive varieties (*Nocellare etnea* and *Bella di Cerignola*) using the 16S rRNA encoding gene as masker.

The aim of this study was to use a phylogeny metagenetic approach to evaluate the changes in bacterial community through raw material until end of fermentation of PDO *Aloreña de Málaga* table olives, to rationally assess the influence of industry, substrate and time on their population dynamics. Insight into the bacterial life of table olive fermentation will allow us to obtain valuable information of the fermentation process for the design of new strategies to improve the quality and safety of this fermented vegetable.

## 2. Material and Methods

99 *2.1. Sampling of industrial fermentations*

100 Samples were obtained from industrial fermentations of PDO *Aloreña de*
101 *Málaga* table olives during October 2014 to January 2015. Fruits were harvested at
102 green maturation stage, washed to remove impurities, cracked and directly brined in a
103 110 g/L NaCl solution in fermentations vessels with 220 L capacity (130 kg fruits).
104 When necessary, fermentation vessels were required with new brine of 120 g/L NaCl
105 and 13 g/L citric acid. Two different industries (labelled as COP and TOL) located at
106 Guadalhorce Valley (Málaga, Spain) were sampled by duplicate. Both industries are
107 separated by a distance of 19.2 km but they produce the same denomination of product
108 (traditional PDO *Aloreña de Málaga* olives). Samples were obtained from fermentation
109 brines (B) and fruits (F) at the time of reception in the factory (fresh fruit, FF) and after
110 7 (initial stage of fermentation), 30 (minimum time of brining contemplated by PDO
111 *Aloreña de Málaga* normative) and 120 (moment of packaging established by demand)
112 days of fermentation (0, 1 and 4 months, respectively). Table 1 shows the references of
113 the samples analysed in the present study and their characteristics.

114 *2.2. Monitoring of the fermentation process*

115 The analyses of olive brine for pH, NaCl, titratable and combined acidity were
116 carried out using the routine methods described by Garrido-Fernández et al. (1997).
117 Firmness and surface colour of fruits followed methods described elsewhere (Bautista-
118 Gallego et al., 2011), determining the CIE parameters: $L^*$ (lightness), $a^*$ (freshness,
119 negative values indicate green while positive values are related to red tones), and $h_{ab}$
120 (hue angle). Individual reducing sugars (glucose, fructose, sucrose and mannitol) were
121 determined by HPLC according to the methods developed by Sánchez et al. (2000).

122 For the counts of the *Enterobacteriaceae,* yeasts and *Lactobacillaceae*
123 populations in both brine and fruit samples were spread in selective media according to
124 methods described by Rodríguez-Gómez et al. (2015). Counts were expressed as $\log_{10}$
125 CFU/mL for brines or $\log_{10}$ CFU/g for olives.

126 *2.3. DNA extraction from olive matrix, preparation of libraries and pyrosequencing*

127 All samples were treated in the same day for DNA extraction from solid (fruit)
128 or liquid (brine) matrixes. In the case of fermentation brine samples, a volume of 50 mL
129 was taken from fermentation vessels and spun at 14,000 rpm for 20 min at 5ºC. Then,
130 the pellet was washed twice in saline solution (0.9% NaCl). In the case of fruit samples,
131 20 g of pulp (approximately 4-5 pitted olives) was homogenized with 50 mL of saline
132 solution in a stomacher for 2 min and the aqueous phase was spun to get a pellet with
133 same conditions describe above. DNA isolation was done using the PowerFood®
134 Microbial DNA Isolation Kit (MoBio, Carlsbad, Calif.) according to the manufacturer
135 instructions. Purified DNA samples (~10 ng/µL) were stored at –20ºC until use.

136 A total of 14 different samples by duplicate (Table 1) were used for bacterial
137 community analysis. The 28 DNA samples were submitted to PCR-amplification of the
138 V2-V3 hypervariable region of the bacterial 16S rRNA gene. Three independent 20-µL
139 PCRs were performed for each sample using a two-step PCR protocol with the 16S
140 rRNA gene primers 27F (5'-*AGTTTGATCCTGGCTCAG*-3') and 357R (5'-
141 *CTGCTGCCTYCCGTA*-3') linked to universal M13/pUC forward (5'-
142 *GTTGTAAAACGACGGCCAGT*-3') and M13/pUC reverse (5'-
143 *CACAGGAAACAGCTATGACC*-3') primers (M13F-*27F* and M13R-*357R*) in an
144 approach similar to that described before (Gholami et al., 2012). Then, second PCR
145 reactions were performed using a 10x dilution of the first PCR product with the fusion

146 forward primer of the Lib-L consisting of the A-adaptor sequence 5'-
147 *CCATCTCATCCCTGCGTGTCTCCGAC*-3' followed by the 4-base calibration
148 sequence 5'-*TCAG*-3', a 10-base MID oligonucleotide to differentiate each of the 28
149 samples and the 20-base M13F/pUC forward oligonucleotide. The reverse fusion primer
150 consists of the Lib-L B-adaptor sequence 5'-*CCTATCCCCTGTGTGCCTTGGCAGTC*-
151 3' followed by the 4-base calibration sequence, and the 20-base M13/pUC reverse
152 oligonucleotide. HPLC-purified oligonucleotides were synthesized by TIB MOLBIOL
153 (Berlin, Germany). All PCR reactions were run in a T100TM Thermal Cycler (Bio-rad,
154 Madrid Spain) using the FastStart High Fidelity Polymerase (Roche Diagnostics
155 GmBH, Mannheim, Germany) and conditions recommended by the manufacturer for
156 pyrosequencing analysis for each type of amplicons. The PCR products were purified
157 twice with AgencourtH AMPureH XP PCR purification system (Agencourt Bioscience
158 Co., Beverly, MA, USA) and quantified using the QuantiT dsDNA BR assay kit
159 (Invitrogen, Carlsbad, CA, USA) and a fluorometer (BioTek Instruments, Winooski,
160 VT, USA). Subsequently, all samples from each run were pooled in equimolar
161 concentrations and purified again twice with AgencourtH AMPureH XP PCR. Pools of
162 the 28 samples were diluted to obtain a total of $1x10^5$ copies/µL and two independent
163 emulsion PCRs were performed with the Lib-L kit (454 Life Sciences) according to
164 manufacturer's instructions for short (16S) reads. DNA positive beads were enriched,
165 counted on the GS Junior Bead Counter, and loaded onto a picotiter plate for
166 pyrosequencing on the 454 Life Sciences (Roche) Junior platform according to the
167 standard platform protocols for short (16S) sequencing runs. Two independent runs
168 were obtained for each 16S sequences. Additionally, other 18 samples were run in a
169 third run to increase the number of sequences from some samples and test the
170 reproducibility of results.

171 *2.4. Statistical analysis of pyrosequencing reads*

172 Samples were processed and analysed following the procedure described by
173 Caporaso et al. (2010) using the Quantitative Insights into Microbial Ecology (QIIME)
174 pipeline (version v1.9.1. http://qiime.sourceforge.net/) using default parameters unless
175 otherwise noted. Sequences were first screened for quality using the following
176 parameters: minimum quality score of 25, minimum sequence length of 200 bp,
177 maximum length of 600 bp (16S), and no ambiguous bases in the entire sequence or
178 mismatches in the primer sequence. Any sequences not meeting these parameters were
179 excluded from downstream analyses. Sequences were then sorted by barcode into their
180 respective samples and the barcode and primer sequences were removed. Chimeras
181 were removed and operational taxonomic units (OTUs) were clustered de novo
182 (pick_de_novo_otus.py script) using USEARCH at 97% identity (Edgar, 2010).
183 Sequences are available at the Sequence Read Archive of Genbank under BioProject ID
184 PRJNA315418. Taxonomy was assigned to the OTUs against the SILVA 108 database
185 preclustered at 97% identity (McDonald et al., 2012)
186 (http://qiime.org/home_static/dataFiles.html). A phylogenetic tree was constructed
187 using the FastTree 2.1.3 with default parameters (Price et al., 2010) for use in
188 phylogenetic diversity calculations. Singleton OTUs were filtered out of the entire
189 dataset to reduce the noise caused by PCR or sequencing error.

190 Data from the replicated fermentations vessels were combined before statistical
191 analysis. Data analyses were performed at the genus taxonomy level. Differences
192 between bacterial communities were calculated in QIIME using rarefaction curves of
193 alpha-diversity indexes including estimates of community richness (such as the Chao1
194 estimator, Richness or the observed number of OTUs present in each sample, Good's

195 coverage, and Phylogenetic diversity (PD) or the amount of phylogenetic branch length
196 observed in each sample). These alpha-diversity indexes were chosen to estimate the
197 total diversity in the different microbial communities for each substrate and industry
198 and each sampling time (Lozupone and Knight, 2008). Rarefaction analysis was
199 performed using rarefied OTU tables (rarefied to 350 sequences); the lowest number of
200 reads obtained for any of the 28 DNA samples analysed to control for differing depths
201 of sequencing across the samples), 100 replications, and cut-offs of 97% sequence
202 similarity. Beta-diversity UniFrac distance matrices were built for 16S sequences only
203 after subsampling all samples to an even depth of 350 sequences per sample. UniFrac
204 distances were based on the PD beta-diversity measures to evaluate the extent to which
205 microbial communities changed over time, or between substrates or industries
206 (Lozupone and Knight, 2008). Taxonomic abundances within each identified Phylum to
207 genus level were visualized using Krona hierarchical data browser (Ondov et al., 2011).
208 Principal coordinates analysis (PCoA) was also performed on the UniFrac distance
209 matrices to show the differences between the sample types, and visualized using the
210 KiNG graphics program (http://kinemage.biochem.duke.edu/software/king.php). Statistical
211 significance of differences in alpha- and beta-diversity were performed with QIIME
212 using a nonparametric two sample t-test with 999 Monte Carlo permutations on number
213 of observations, Chao1 and PD and nonparametric ANOSIM tests on unweighted
214 UniFrac (16S).

## 215 3. Results

### 216 *3.1. Monitoring of the fermentation process*

217 The fermentation process of traditional *Aloreña de Málaga* table olives was
218 followed during four months by routine physicochemical and microbiological analyses.

219 Table 2 shows the evolution of the main physicochemical characteristics assayed in
220 both brines (pH, salt, titratable and combined acidity, sugar concentration) and fruits
221 (colour and texture). In general, the evolution of physicochemical parameters was
222 similar in both industries, except salt concentration which was slight higher in COP
223 industry at the onset of fermentation (80.1 COP *vs* 66.8 g/L TOL). Then, the salt
224 concentration increased up to practically 95 g/L at the end of fermentation process in
225 both factories by the addition of new brine. The profile of pH in brines was kept
226 practically constant during all fermentation process, ranging from 4.31 to 4.53. The
227 combined acidity was also kept constant during all time approximately at 0.10 Eq/L,
228 while titratable acidity slight increased from approximately 0.40% to 0.60%. The initial
229 total sugar concentration in brine was around 21 g/L, principally composed by glucose.
230 Glucose, fructose and sucrose were consumed by microorganisms during fermentation
231 process. However, the content in mannitol remained unchanged during four months,
232 with a higher content in COP industry (Table 2). Regarding physicochemical
233 characteristics of the fruits, texture was not affected during four months of fermentation,
234 ranging from 6.27 to 6.82 KN/100g, while colour of fruits was characterized by a loss
235 of green appearance ($a^*$ parameter increased from negative to positive values) and
236 luminosity ($L^*$ parameter decreased from initial 60 to approximately 54 through
237 fermentation process).

238 As regards microbial counts, *Enterobacteriaceae* and *Lactobacillaceae* were
239 below limit of detection (<1.3 $\log_{10}$ CFU/mL) during all fermentation process, in both
240 brines and fruits. On the contrary, yeasts increased during time studied, with population
241 levels higher in TOL than in COP industry for much of the time of fermentation. After
242 four months of fermentation, this fungal group reached practically the same population
243 in both industries, with ~5.0 $\log_{10}$ CFU/mL in brines, and ~4.5 $\log_{10}$ CFU/g in fruits.

244 Thus, the main physicochemical and microbiological changes which occurred
245 during fermentation were related with sugar consumption (mainly glucose), a slight salt
246 and titratable acidity increase, loss of green colour and luminosity of fruits (darkening),
247 and yeast dominance. All these changes were very similar in both factories and can be
248 considered as usual during fermentation of this speciality of natural, cracked, green
249 olives. The flavour and aroma of fermented olives were also tested by a training panel,
250 not detecting the presence of abnormal taste or smells and resulting in the typical
251 product (data not shown). Hence, the samples obtained for pyrosequencing analysis can
252 been considered as representative of this type of process for both industries.

253 *3.2. Phylogenetic analysis of the bacterial community*

254 The pyrosequencing of the 16S-PCR products generated a total of 337,114 raw
255 sequences for the 28 olive samples. After screening our data for poor quality sequences,
256 we recovered 274,141 high quality sequences with an average of 9,791 sequences per
257 sample. From those, we obtained a total of 254,147 sequences that could be assigned
258 into OTUs with a mean of 5,906 classifiable sequences per sample. After removing
259 chloroplasts and taxonomically unassigned 16S sequences, a total of 32,801 sequences
260 were finally used for metagenetic analysis making a mean of 1,171 sequences per
261 sample.

262 Across all taxa, 131 bacterial genera and 357 OTUs, with an average of 81
263 observed OTUs per sample, were identified (see Table 1). The bacterial phylogenetic
264 characterization of all samples showed big differences between fresh fruits, fermented
265 fruit and brine samples, and covered four main bacterial phyla including *Actinobacteria*,
266 *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Fig 1). However, whereas for fresh
267 fruits samples, *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacilli* and

268 *Actinomycetales* classes were present in similar proportions (13.6 to 27.0%), for
269 fermented fruit and brine samples this proportions where completely shifted to a
270 significantly higher proportion of *Gammaproteobacteria* of the family
271 *Alteromonadaceae* (66.9% for fermented fruit and 81.8% for brines; being significantly
272 higher for brine samples). Also, the proportion of *Alphaproteobacteria*, *Actinobacteria*,
273 *Bacilli* and *Betaproteobacteria* were significantly higher for fermented fruits than for
274 brines. From the total of 131 genera identified, only 21 genera represented >90% of the
275 total sequences, which ranged between 0.5% (*Pedobacter*) to 50.6%
276 (*Celerinatantimonas*). Globally, for fermented fruits the genera that accounted for
277 >80% of the sequences were: *Celerinatantimonas* (53.5%), *Pseudomonas* (9.7%),
278 unknown *Acetobacteraceae* (6.8%), *Modestobacter* (5.1%), *Propionibacterium* (5.0%),
279 and an unknown *Lactobacillaceae* (3.0%), whereas for brines were *Celerinatantimonas*
280 (63.9%), *Pseudomonas* (13.4%), and *Propionibacterium* (5.4%) (Fig. 1).

281 A change of bacterial genera was observed during fermentation process. Thus,
282 the fermented fruit samples at $7^{th}$ day of fermentation showed high levels of
283 *Pseudomonas* sp. (27.1%), *Modestobacter* sp. (15.1%), *Propionibacterium* (13.5%,
284 mainly *P. acnes*), and an unidentified *Lactobacillaceae* (8.8%), and very low numbers
285 of *Celerinatantimonas* (1.8%) or members of *Acetobacteraceae* (<1%), whereas at $30^{th}$
286 day of fermentation most bacteria belonged to *Celerinatantimonas diazotrophica*
287 (69.4%) and unidentified *Acetobacteraceae* (19.7%), or *Acetobacter* sp. (4%) and a
288 *Gammaproteobacteria* (2.8%) with very low proportion of *Pseudomonas* sp. and
289 *Propionibacterium* (<0.2%) or undetectable (*Modestobacter* sp., and *Lactobacillaceae*).
290 Finally, at $120^{th}$ day of fermentation, most bacteria belonged to *C. diazotrophica*
291 (89.4%) and *Gammaproteobacteria* (3.2%) with the remaining genera being present at
292 very low proportions or not being detectable (Fig. 2). On the other hand, samples from

293 brines at 7th day of fermentation were dominated by *C. diazotrophica* (46.6%),
294 *Pseudomonas* sp. (34.7%), *Rhodovibrio* sp. (2.3%) and *Enterobacteriaceae* (2.1%),
295 whereas at 30th day of fermentation most bacteria from brines samples belonged to *C.*
296 *diazotrophica* (49.7%), *P. acnes* (14.2%) and *Streptococcus* sp. (7.0%), and at 120th day
297 of fermentation most bacteria were represented by *C. diazotrophica* (95.3%) (Fig. 2).
298 Tables S1 and S2 in supplementary material shows the relative abundance of the most
299 representative OTUs, at genera and family level, in all samples analysed.

300 *3.3. Biodiversity of the bacterial community*

301 The Venn diagrams show that a total of 63 OTUs (48%) were shared among the
302 three substrates (fresh fruits, brines and fermented fruits), with fermented fruit and brine
303 samples sharing the highest number of OTUs (113 OTUs; 86%) and fresh fruit and
304 brine the lowest (69 OTUs; 53%), and a few or none OTUs being unique (non-shared
305 by any other sample) for each sample type (Fig. 3a). Table S3 in supplementary material
306 shows the OTUs assigned at genera level shared among the three types of substrates.
307 The number of unique and shared bacterial OTUs changed with the type of substrate
308 and during the fermentation process. Thus, 15 bacterial OTUs (12%) were shared by all
309 fruits (including fresh fruits) through fermentation process, but the highest number of
310 OTUs was shared between fresh fruits and fruits at 7th day after starting the
311 fermentation (F-0). The number of unique bacterial OTUs diminished during
312 fermentation time from F-0 to F-4 (Fig. 3b). Table S4 in supplementary material shows
313 the OTUs assigned at genera level shared among the fruits in all sampling time. In brine
314 samples, a total of 28 bacterial OTUs (23%) were shared among all sample types with
315 the brines samples at 30th day of fermentation (B-1) showing the highest number of
316 unique OTUs (Fig. 3c). Table S5 in supplementary material shows the OTUs assigned

317 at genera level shared among the brine samples in the different sampling time. The
318 bacterial genera *Celerinatantimonas, Pseudomonas, Propionibacterium, Salinibacter,*
319 *Staphylococcus, Rhodovibrio, Streptococcus,* and *Alicyclobacillus* were shared among
320 substrates, fruits and brines in the different sampling times (see Tables S3-S5 in
321 supplementary material).

322 The bacterial community was also analysed using rarefaction curves and
323 richness estimator (Chao1 index). The Chao1 index varied from 17.1 (brine sample
324 obtained from TOL industry after 4 months of fermentation) to 230.2 (fruit sample
325 obtained from COP industry at the onset of fermentation) (Table 1). Overall, despite the
326 diversity of sequencing depth between samples, the rarefaction analysis indicated that
327 the number of bacterial reads above 350 per sample was satisfactory to obtain a good
328 coverage. Thus, there was a satisfactory coverage of the bacterial diversity for all the
329 samples analysed with Good's coverage values above 90% with the exception of two
330 treatments (see Table 1). This result was also confirmed by the analysis of rarefaction
331 curves (Fig. 4). When analysing alpha-diversity rarefaction curves for bacteria, we
332 found differences among fermented fruit and brines samples, industries and during the
333 fermentation process, with similar pattern for all alpha-diversity indexes (Fig. 4**;** only
334 Richness data are shown). For both factories, alpha-diversity was higher for fresh fruits
335 and after 7$^{th}$ days of fermentation; then significantly decreased at 30$^{th}$ and 120$^{th}$ days of
336 fermentation. For brine samples, there were significant differences among industries
337 with samples from TOL industry showing low alpha-diversity values, whereas for COP
338 industry there was a trend to increase alpha-diversity values at 30$^{th}$ day of fermentation
339 and then a dramatically decreased occurred at 120$^{th}$ day of fermentation reaching value
340 similar to those obtained for COP industry at same period of fermentation.

341 Unweighted UniFrac analysis based in principal coordinates analysis of 16S
342 sequences segregated olive fruits samples unprocessed (FF) and those at the beginning
343 of the fermentation process (F-0) irrespective of the industry along PC1 that explained
344 more than 79% of total variance. These samples were also closer to brines samples from
345 COP industry at $7^{th}$ and $30^{th}$ days of fermentation due to their higher alpha and beta-
346 diversity values. On the contrary, all fermented fruit and brines samples for both
347 industries tended to group together at $30^{th}$ and after $120^{th}$ days of fermentation with low
348 distance values among them indicating a closer similarity in their bacterial communities
349 (similar PC1 and PC2 values) pointing out that the changes occurring during the
350 fermentation process (time) were the main drivers of microbial community composition
351 irrespective of the substrate or industry (Fig. 5). However, ANOSIM test indicated that
352 there were not statistical significant differences ($p>0.05$) among the Unweighted
353 UniFrac distances when comparing samples among the different categories (i.e.,
354 industry, substrate, or fermentation time).

355 **4. Discussion**

356 Metagenetic analysis has been used to investigate the changes in bacterial
357 communities in diverse vegetables in brines such as asparagus, cucumbers, kimchi, and
358 table olives. This way, the bacterial population of green asparagus was composed
359 mainly by *Proteobacteria* (mainly *Pantoea* and *Pseudomonas* genera), followed by
360 *Firmicutes* (mainly *Lactococcus* and *Enterococcus*) (del Árbol et al., 2016). Bacterial
361 community of kimchi were represented mainly by the genera *Leuconostoc* and
362 *Lactobacillus,* but also of *Weissella, Pantoea* and *Pseudomonas* (Jeogn et al., 2013).
363 Medina et al. (2016) reported recently the presence of *Acetobacter, Gluconobacter* and
364 *Lactobacillus* as the majority genera during fermentation of cucumbers. In the specific

365 case of table olives, Cocolin et al. (2013) used pyrosequencing analysis for the study of
366 the bacterial ecology during fermentation of directly brined *Nocellare etnea* olives.
367 They found also a change of the bacterial population through fermentation process. This
368 way, the surface and brines of the olives at the onset of fermentation was characterized
369 by a high level of halophilic bacteria, mainly *Chromohalobacter, Halomonas,* and
370 *Marinilactibacillus* genera, while after 3 months of fermentation the structure of the
371 population changed dramatically, especially in olive surface with *Lactobacillus* as the
372 main bacterial population present. These authors also reported the presence of
373 *Pseudomonas* and *Propionibacterium* among the DNA samples, but at low frequencies
374 (<1.5% in the highest case). De Angelis et al. (2015), using also pyrosequencing
375 analysis for the study of bacterial changes trough fermentation of not lye-treated *Bella*
376 *di Cerignola* olives, found that the main genus present in the fresh olives and the onset
377 of fermentation were *Hafnia* and *Methylobacterium,* whilst at the end of fermentation
378 were *Lactobacillus* and *Propionibacterium.*

379 In this study, *Pseudomonas, Modestobacter, Acetobacter* and *Propionibacterium*
380 (*P. acnes*) were the genera that accounted for the majority of sequences in both fruit and
381 brine samples at the onset of fermentation, whilst at the end of fermentation most
382 bacteria belonged to *Celerinantimonas* genera (*C. diazotrophica*). The presence of
383 *Enterobacteriaceae* and *Lactobabacillaceae* during all fermentation process (4 months)
384 was scarce, in contrast with studies described before in table olives and other fermented
385 vegetables. This way, *Lactobacillaceae* only represented globally the 2% of total
386 sequences in the fresh fruit and 3% in fermented fruits, while the frequency of
387 *Enterobacteriaceae* was approximately 2% in the different type of substrates. The
388 presence of both bacterial taxa in the fermentation of table olives and other vegetables is
389 habitual, with a well-known negative role during fermentation for *Enterobacteriaceae*,

and positive for *Lactobacillaceae* (Garrido-Fernández et al., 1997). Among the most important bacterial pathogens related to foods (*Listeria, Clostridium, Escherichia, Salmonella,* etc.), only *Staphylococcus* genus was found at low frequencies (<0.02% of total sequences) in the present study. This result has a special relevance regarding safety issues. In general, the presence of food-borne pathogen in table olives is scarce, as it was also confirmed by pyrosequencing analysis by Cocolin et al. (2013) and De Angelis et al. (2015), who only found a low abundance of the genera *Escherichia, Staphylococcus, Clostridium* and *Listeria* during fermentation process of diverse *Italian* olive varieties.

The bacterial biodiversity in the different samples was affected by the type of industry, with in general lower biodiversity indexes in TOL factory than in COP, and by the time of fermentation, with this later factor being the major driver of both alpha- and beta-diversity changes. Thus, the chao1 and richness indexes had lower values at the end of fermentation compared to initial points, indicating that both the total number of OTUs (richness) and the number of rare or less frequent OTUs (Chao1) tended to decrease during the fermentation process, and the beta-diversity UniFrac distances tended to decrease in the same direction. This is indicative that during course of fermentation, only determined species (mainly *C. diazotrophica*) were adapted to the fermentation process. This contrast with data obtained by Cocolin et al. (2013) and De Angelis et al. (2015), who noticed similar biodiversity indexes between the initial and end points of olive fermentations. *C. diazotrophica* has been recently described by Cramer et al. (2011) as a facultative anaerobic, nitrogen-fixing, short, motile, polar monotrichous rods bacterium, belonging to the class *Gammaproteobacteria* and order *Alteromonadales*. The type strain of this species was originally isolated from the roots of estuarine grasses *Spartina alterniflora* and *Juscuns roemerianus* (Cramer et al.,

415 2011). This species growth between pH 3.5 to 8.0 and it has halotolerance above 80 g/L
416 NaCl, so presumably can support the usual pH and salt conditions found in olive
417 fermentations (Garrido-Fernández et al., 1997). *C. diazotrophica* is metabolically
418 versatile, with ability to ferment glucose to acid products and utilization of a wide
419 variety of carbohydrates (many of them present in olive fruits) and carboxylic acids, as
420 performed by many *Lactobacillaceae* species (Cramer et al., 2011). Recently, this
421 species was detected among the bacterial community present at the final stage of
422 Sichuan fermentation, a typical representative of Chinese traditional food where
423 different vegetables (cabbage, radish, leaf mustard, bamboo, shoot, tender ginger, and
424 chili) are immersed in salt brine (60-80 g/L) at pH 4.5 (Li et al., 2014), similar to
425 conditions found in this work. Curiously, other genus never detected before in olive
426 samples, *Modestobacter,* has been also isolated from the roots of halophyte plants
427 (*Salicornia europaea*). This genus is classified into the family *Geodermatophilaceae*
428 and has a considerable tolerance to salt (Quin et al., 2012). We hypothesize that the
429 presence in *Aloreña de Málaga* fermentations of both halophyte genera, related with
430 marine environment, could be due to the use of sea salt in olive fermentations, which is
431 usually added to prepare the cover brines. This hypothesis is reinforced by the presence
432 also in *Aloreña* samples, although at lower abundance (<0.03%), of the genera
433 *Rhodovibrio* and *Salinibacter,* two halophilic bacteria related with salterns (Johannes,
434 2005). Their influence (positive or negative) on the fermentative process must be
435 elucidated in further studies.

436 On the contrary, other microorganisms detected in *Aloreña de Málaga* samples
437 by pyrosequencing analysis are clearly considered undesirable in the fermentation
438 process. The presence of *Pseudomonas* genera during fermentation of *Aloreña de*
439 *Málaga* table olives was previously described by Abriouel et al. (2011) using a culture-

440 independent approach based in PCR-DGGE analysis. This ubiquitous microorganism
441 has been also detected previously on the surface of unfermented black olives,
442 fermentation of naturally black olives and directly brined *Nocellare etnea* olives
443 (Cocolin et al., 2013; Ercolini et al., 2006; Nychas et al., 2002). *Pseudomonas* are
444 usually associated to fresh foods. Due to its high metabolic potential, diverse species of
445 this group can produce alteration of foods. The development of proteolytic pathways in
446 table olives, followed by decarboxylation and deamination of the resulting amino acids
447 by heterofermentative lactobacilli could cause an unusual type of spoilage characterized
448 by a decrease in the acidity of brines and swelling (Harmon et al., 1987), and could also
449 lead to biogenic amine formation. *Propionibacterium* and *Acetobacter* genera were also
450 detected during the course of this research. *Acetobacter* sp. have been related with
451 spoilage of cucumber fermentations, producing the consumption of lactic acid and
452 consequent formation of acetic acid in aerobic conditions (Medina et al. 2016). An
453 undesired secondary fermentation or spoilage may be initiated by propionic acid
454 bacteria, in particular *Propionibacterium* spp, which is a well-known species in table
455 olive fermentations (Garrido-Fernández et al., 1997). This genera metabolizes sugars or
456 the lactic acid form during the primary fermentation, to produce propionic acid, acetic
457 acid and $CO_2$, inducing an increase in pH and volatile acidity (Gonzalez-Cancho et al.,
458 1980). These conditions also encourage the development of *Clostridium* species, which
459 together with *Propionibacterium* can promote *zapatería* spoilage, giving abnormal
460 odours and tastes in table olives (Garrido-Fernández et al., 1997). Control of pH and salt
461 concentration in brine would prevent the growth of these spoilage microorganisms and
462 their off-odours fermentations, especially when temperatures are warmer during the
463 summer months (Gonzalez-Cancho et al., 1970).

464 **5. Conclusions**

465 New bacterial species have been detected for the first time in natural green olive
466 fermentations by the use of high-throughput pyrosequencing analysis. Spread of
467 samples on specific selective media only provides partial and very limited information
468 of the microbiology of table olive fermentations. Thus, results show the need of this
469 type of work to improve our knowledge of the microbiology of table olive
470 fermentations. Further studies are also necessary to determine the influence of these
471 new microbial species on the quality and safety of table olives. Apparently, the presence
472 of spoilt microorganisms did not alter the physic-chemical characteristics of fermented
473 olives, whilst the absence of pathogens genera reinforces the safety issues of this
474 fermented vegetable.

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589 **Figure legends**

590 *Fig 1.* Taxonomic abundances (%) from Phylum to genus level in the fresh fruits at the
591 moment of reception in the industry, fermented fruit and brine samples. The different
592 industries and sampling times were considered together for elaboration of the graphs.

593 *Fig 2.* Relative abundance (%) of genera or family obtained by pyrosequencing analysis
594 through fermentation process. FF, F, and B stands for fresh fruits, fermented fruits and
595 fermentation brines, respectively, while 0, 1 and 4 stands for the different sampling
596 times (0, 1 and 4 months of fermentation, respectively).

597 *Fig 3.* Venn diagrams showing the number of unique and shared OTUs among
598 substrates (A), sampling times in fruits (B) and sampling times in cover brines (C). FF,
599 F, and B stands for fresh fruits, fermented fruits and cover brines, respectively, while 0,
600 1 and 4 stands for the different sampling times (0, 1 and 4 months of fermentation,
601 respectively).

602 *Fig 4.* Rarefaction curves for the different industries and substrates. FF, F, and B stands
603 for fresh fruits, fermented fruits and cover brines, respectively, while 0, 1 and 4 stands
604 for the different sampling times (0, 1 and 4 months of fermentation, respectively).

605 *Fig 5.* Unweighted UniFrac analysis based in principal coordinates analysis of 16S
606 sequences obtained from different samples. FF, F, and B stands for fresh fruits,
607 fermented fruits and brines, respectively, TOL and COP stands for different industries,
608 while 0, 1 and 4 stands for the different sampling times (0, 1 and 4 months of
609 fermentation, respectively).

**Table 1.** Number of sequences and OTUs assigned (after removing chloroplast), observed diversity and estimated sample coverage for 16S (bacteria) amplicons for the different type of samples after grouping duplicated experiments.

| Sample | Matrix | Industry | Time | Number of reads | Number of OTUs | Good's coverage | PD whole tree* | Chao1* | Richness* |
|---|---|---|---|---|---|---|---|---|---|
| FF-COP | Fresh Fruit | COP | 0 month (0 days) | 817 | 85 | 96.94 | 5.40 | 79.20 | 64.40 |
| F-COP-0 | Fruit | COP | 0 month (7 days) | 723 | 152 | 88.38 | 8.36 | 230.23 | 101.10 |
| F-COP-1 | Fruit | COP | 1 month (30 days) | 2398 | 69 | 98.37 | 2.63 | 49.76 | 25.10 |
| F-COP-4 | Fruit | COP | 4 months (120 days) | 1051 | 75 | 96.29 | 4.20 | 102.76 | 35.90 |
| B-COP-0 | Brine | COP | 0 month (7 days) | 355 | 65 | 90.70 | 4.57 | 111.39 | 64.40 |
| B-COP-1 | Brine | COP | 1 month (30 days) | 510 | 145 | 84.90 | 9.19 | 221.03 | 117.20 |
| B-COP-4 | Brine | COP | 4 months (120 days) | 4387 | 69 | 99.07 | 1.57 | 23.78 | 11.70 |
| FF-TOL | Fresh Fruit | TOL | 0 month (0 days) | 1404 | 137 | 96.94 | 7.14 | 135.73 | 83.80 |
| F-TOL-0 | Fruit | TOL | 0 month (7 days) | 410 | 78 | 90.49 | 5.74 | 126.30 | 72.50 |
| F-TOL-1 | Fruit | TOL | 1 month (30 days) | 1956 | 27 | 99.23 | 1.27 | 25.48 | 10.60 |
| F-TOL-4 | Fruit | TOL | 4 months (120 days) | 2312 | 36 | 98.96 | 1.28 | 31.95 | 11.10 |
| B-TOL-0 | Brine | TOL | 0 month (7 days) | 4345 | 89 | 98.76 | 1.93 | 56.23 | 16.00 |
| B-TOL-1 | Brine | TOL | 1 month (30 days) | 7700 | 61 | 99.51 | 1.35 | 17.96 | 8.90 |
| B-TOL-4 | Brine | TOL | 4 months (120 days) | 4433 | 47 | 99.35 | 1.59 | 17.13 | 9.10 |

*Values were estimated after rarefaction to 350 sequences.

**Table 2.** Physicochemical evolution of the brines and fruits during fermentation process in the different industries. Standard deviation from duplicate experiments in parentheses.

| | **pH** | | **Salt (g/L)** | | **Titratable acidity (%)** | | **Combined acidity (Eq/L)** | |
|---|---|---|---|---|---|---|---|---|
| **Time** (days) | **COP** | **TOL** | **COP** | **TOL** | **COP** | **TOL** | **COP** | **TOL** |
| 7 | 4.47 (0.02) | 4.53 (0.03) | 80.1 (0.80) | 66.8 (2.10) | 0.39 (0.01) | 0.42 (0.01) | 0.09 (0.00) | 0.11 (0.00) |
| 30 | 4.48 (0.03) | 4.31 (0.12) | 81.6 (1.11) | 76.0 (0.50) | 0.59 (0.04) | 0.57 (0.04) | 0.11 (0.01) | 0.10 (0.00) |
| 120 | 4.48 (0.03) | 4.45 (0.01) | 92.4 (0.50) | 95.5 (0.40) | 0.54 (0.07) | 0.62 (0.05) | 0.09 (0.00) | 0.08 (0.00) |
| | **Glucose (g/L)** | | **Fructose (g/L)** | | **Sucrose (g/L)** | | **Mannitol (g/L)** | |
| **Time** (days) | **COP** | **TOL** | **COP** | **TOL** | **COP** | **TOL** | **COP** | **TOL** |
| 7 | 12.12 (0.69) | 14.49 (1.39) | 2.55 (1.05) | 3.05 (0.58) | 1.29 (0.01) | 1.22 (0.17) | 4.61 (0.55) | 2.78 (0.00) |
| 30 | 6.54 (0.40) | 4.15 (0.03) | 1.75 (0.00) | 0.89 (0.01) | 0.79 (0.18) | 0.37 (0.04) | 4.75 (0.31) | 2.86 (0.08) |
| 120 | 2.85 (0.32) | 3.30 (0.22) | 0.89 (0.00) | 0.99 (0.02) | 0.10 (0.02) | 0.12 (0.00) | 4.16 (0.52) | 2.68 (0.11) |
| | **Texture (KN/100 g)** | | **Colour $a^*$** | | **Colour $h_{ab}$** | | **Colour $L^*$** | |
| **Time** (days) | **COP** | **TOL** | **COP** | **TOL** | **COP** | **TOL** | **COP** | **TOL** |
| 7 | 6.27 (0.28) | 6.36 (0.02) | -4.97 (1.03) | 0.10 (1.46) | 74.07 (1.89) | 66.45 (2.31) | 61.70 (2.16) | 58.96 (2.38) |
| 30 | 6.34 (0.34) | 6.82 (0.35) | 5.24 (0.63) | 4.36 (0.08) | 82.43 (1.43) | 83.24 (0.32) | 55.20 (1.99) | 53.96 (1.35) |
| 120 | 6.76 (0.67) | 6.76 (0.19) | 6.24 (0.14) | 6.44 (0.71) | 80.62 (0.50) | 79.52 (0.86) | 54.50 (0.50) | 53.16 (0.85) |

Figure

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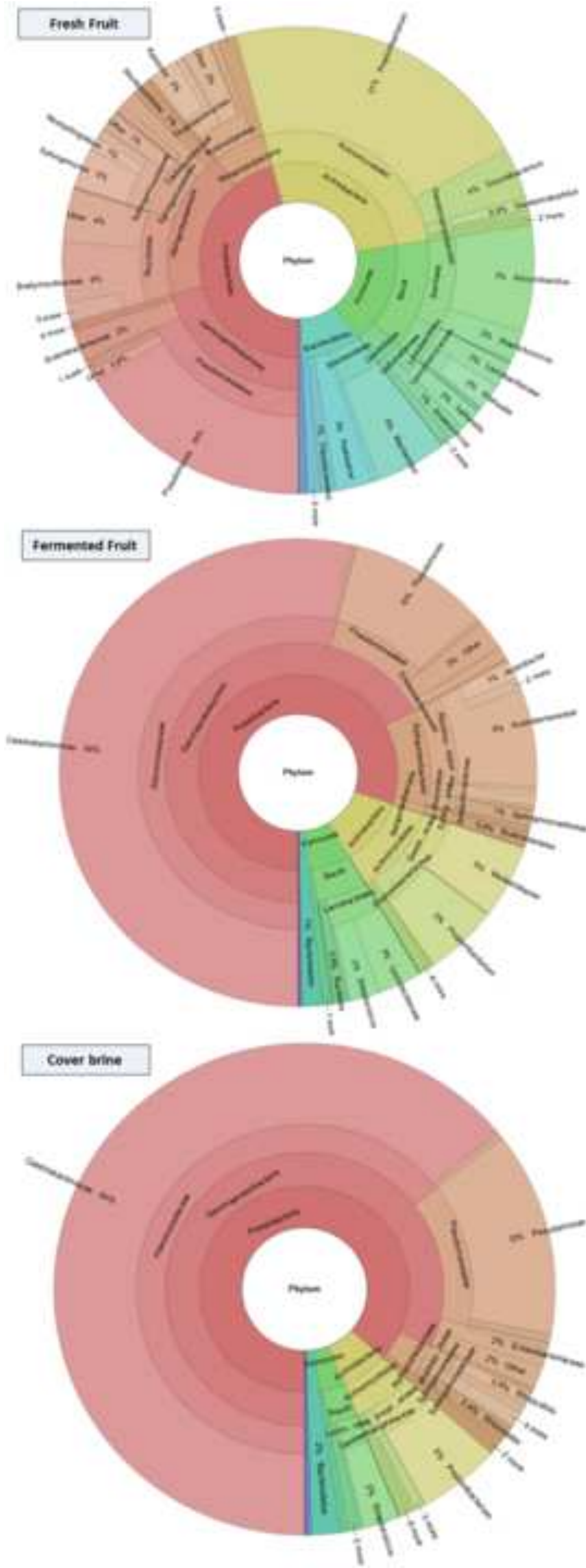

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FF
F - 0
F - 1
F - 4
B - 0
B - 1
B - 4
TOTAL

g_Pedobacter
g_Staphylococcus
o_Pseudomonadales
g_Veillonella
g_Rhodovibrio
g_Acetobacter
o_Rhizobiales
g_Bacteroides
f_Bradyrhizobiaceae
g_Corynebacterium
g_Sphingomonas
f_Enterobacteriaceae
g_Alicyclobacillus
f_Lactobacillaceae
c_Gammaproteobacteria
g_Streptococcus
g_Modestobacter
f_Acetobacteraceae
g_Propionibacterium
g_Pseudomonas
g_Celerinatantimonas

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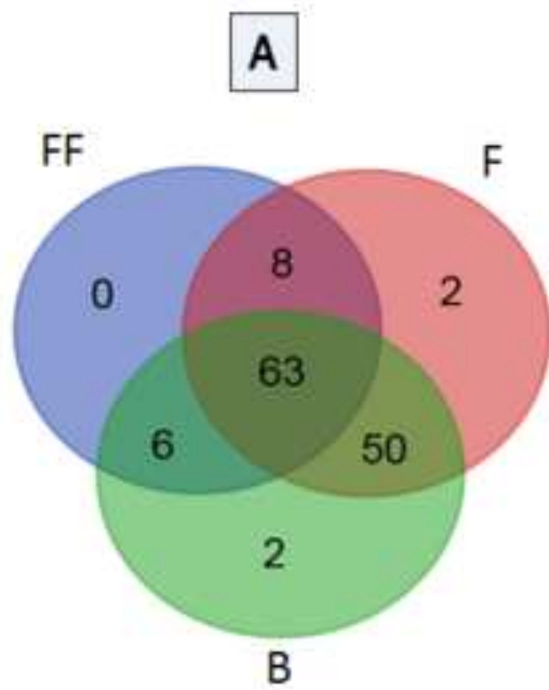


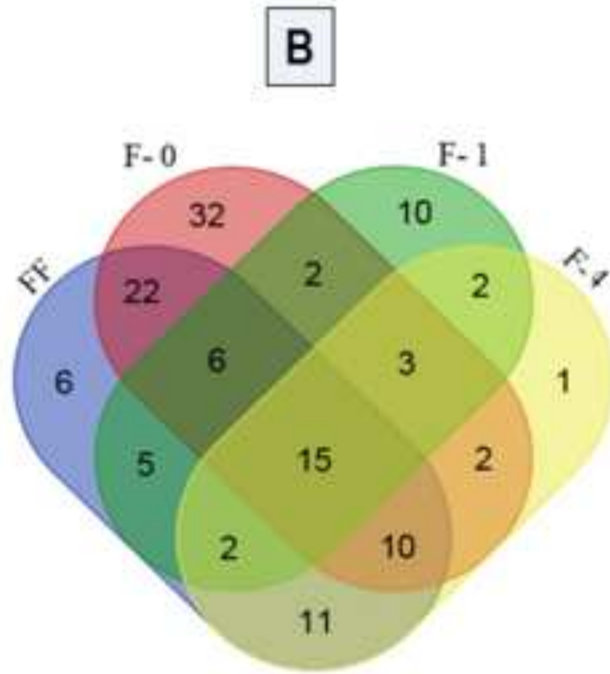


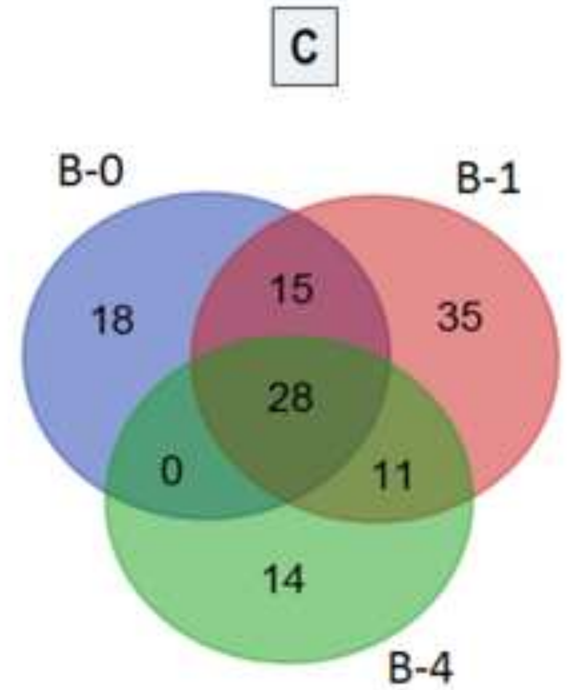

Figure
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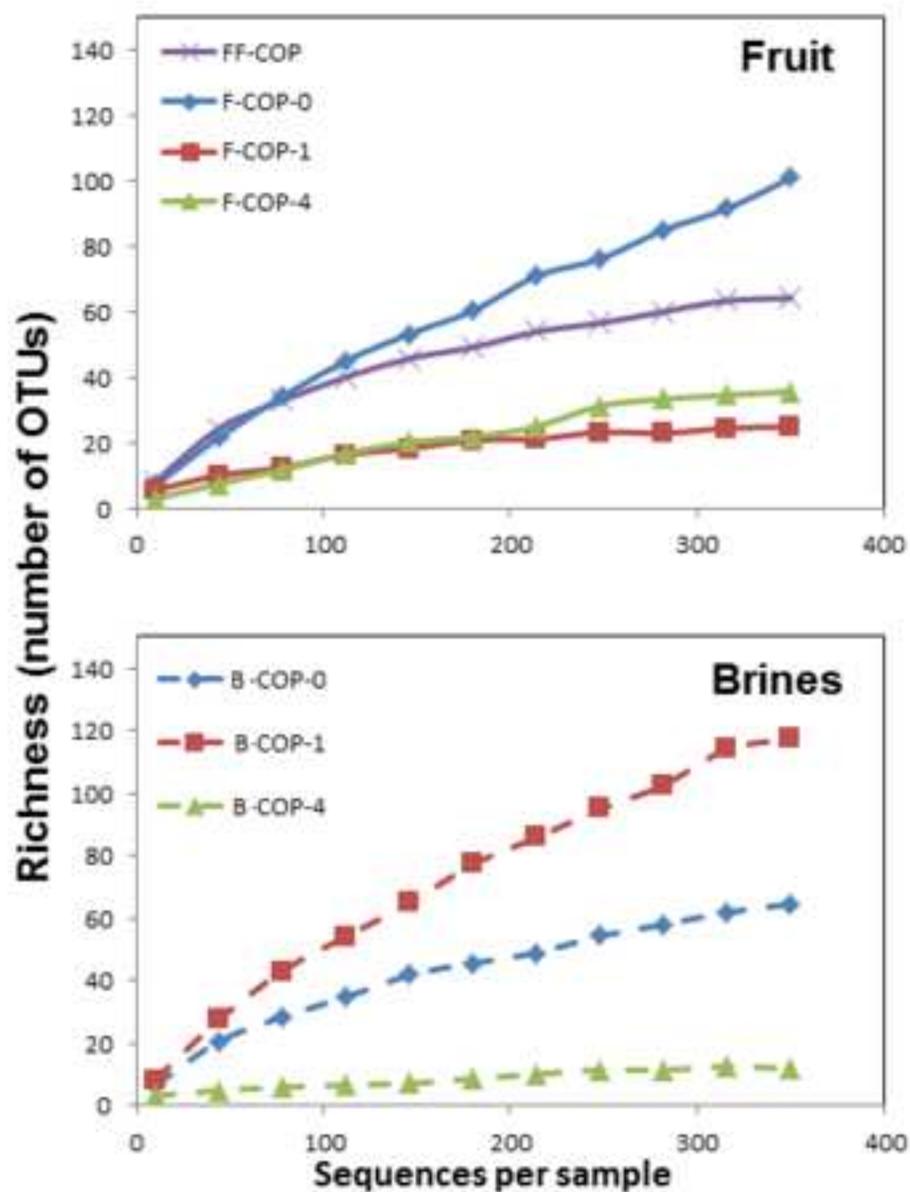


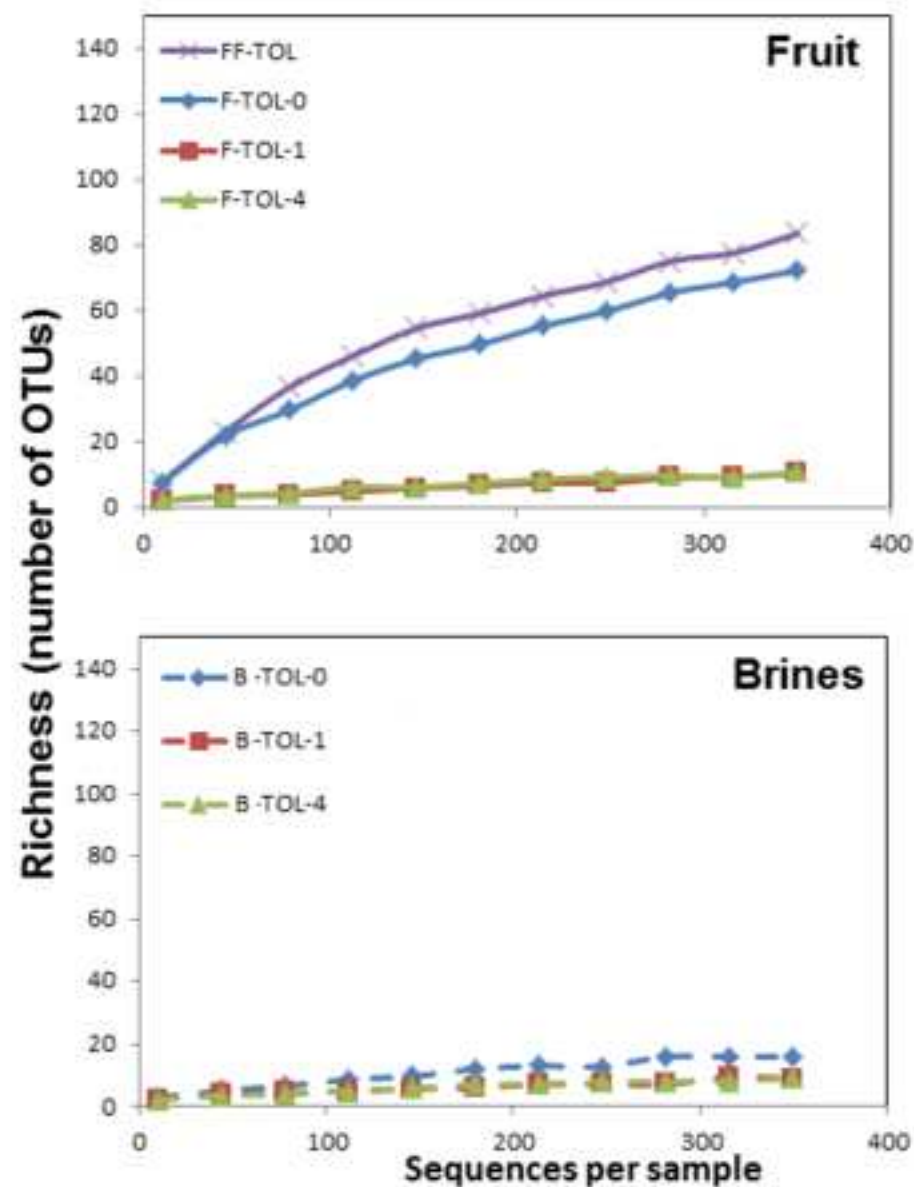

Figure
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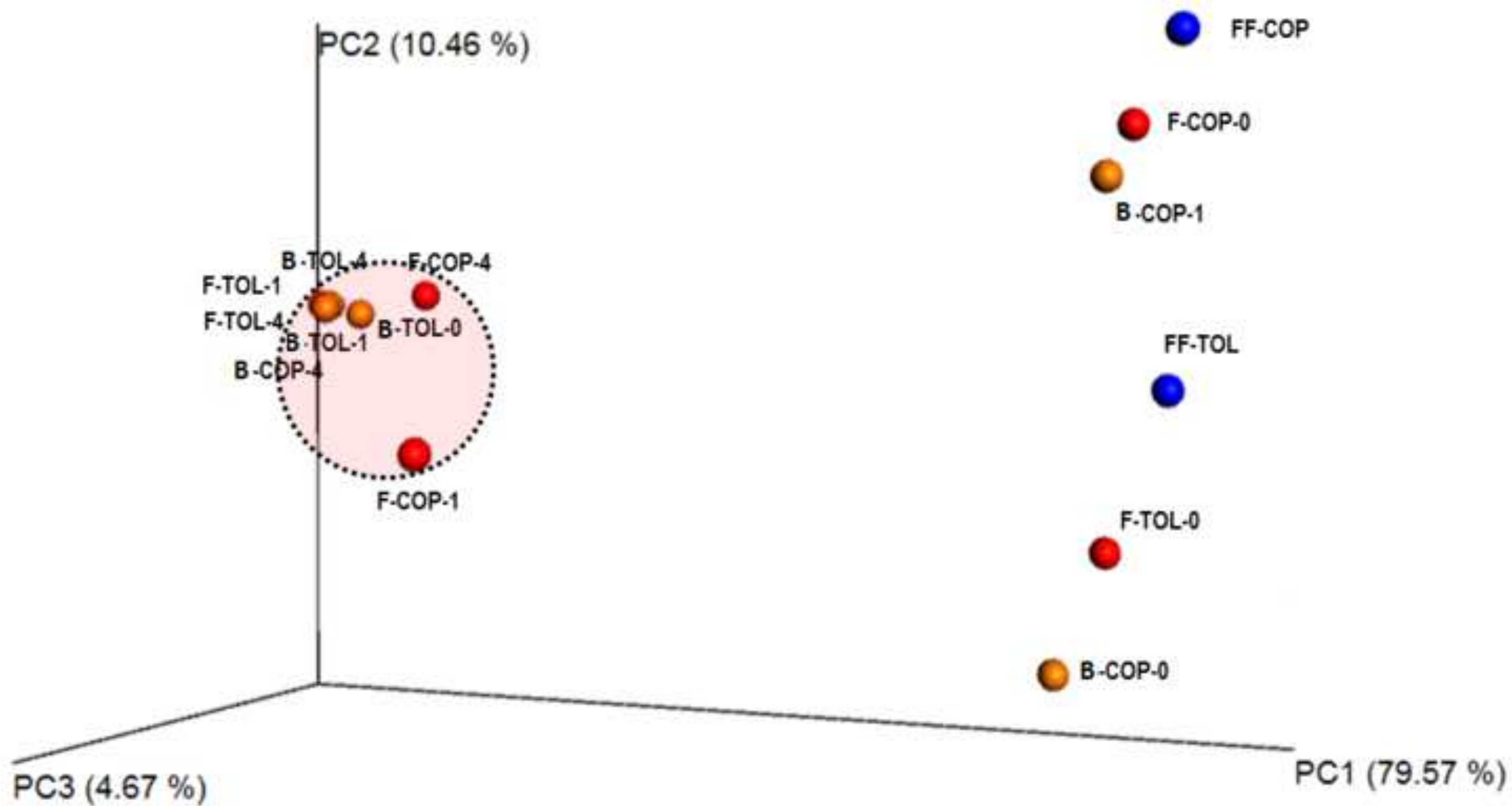

**Supplementary material to**: *Assessment of the bacterial community in directly brined Aloreña de Málaga table olive fermentations by metagenetic analysis*

**Table S1.** Relative abundance (%) of the more representative OTUs at genus level in the different type of samples analysed.

| Taxonomy | FF.TOL | F.TOL.0 | F.TOL.1 | F.TOL.4 | B.TOL.0 | B.TOL.1 | B.TOL.4 | FF.COP | F.COP.0 | F.COP.1 | F.COP.4 | B.COP.0 | B.COP.1 | B.COP.4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *g__Celerinatantimonas* | 0.4% | 1.5% | 94.8% | 95.2% | 92.9% | 96.3% | 96.3% | 0.5% | 2.1% | 44.1% | 83.5% | 0.3% | 3.1% | 94.3% |
| *g__Alicyclobacillus* | 2.8% | 1.0% | 0.1% | - | - | - | - | 12.0% | 0.7% | 0.1% | 0.8% | - | 1.8% | - |
| *g__Pseudomonas* | 32.4% | 47.8% | 0.1% | - | 2.4% | - | - | 0.2% | 6.4% | 0.1% | 3.5% | 67.0% | 11.2% | - |
| *g__Propionibacterium* | 18.2% | 6.3% | 0.1% | - | 0.1% | - | 0.2% | 24.4% | 20.9% | 0.4% | 2.1% | 3.4% | 28.4% | 0.1% |
| *g__Rhodovibrio* | 1.4% | - | - | - | 0.1% | - | - | - | 0.6% | - | 0.1% | 4.5% | - | 0.1% |
| *g__Streptococcus* | 0.4% | 2.0% | 0.1% | - | - | - | - | 0.2% | 9.7% | 0.1% | 0.2% | 0.3% | 13.9% | - |
| *g__Staphylococcus* | - | 0.5% | 0.1% | - | - | - | - | 3.8% | 0.7% | - | 0.3% | - | 0.6% | 0.1% |
| *g__Corynebacterium* | 3.1% | 2.2% | - | - | - | - | - | 4.0% | 1.5% | - | 0.5% | - | 2.0% | - |
| *g__Bacteroides* | 3.8% | - | - | - | - | - | - | 6.1% | 0.7% | - | - | - | 1.0% | - |
| *g__Pedobacter* | 0.2% | - | - | - | - | - | - | 4.9% | 0.7% | - | - | - | 0.6% | - |
| *g__Modestobacter* | - | - | - | - | - | - | - | - | 30.2% | - | 0.1% | 0.6% | - | - |
| *g__Acetobacter* | - | - | - | - | - | - | - | - | - | 7.9% | - | - | - | - |
| *g__Gluconobacter* | - | - | - | - | - | - | - | - | - | 3.3% | - | - | - | - |
| Others | 37.3% | 38.8% | 4.9% | 4.6% | 4.4% | 3.5% | 3.5% | 43.8% | 26.0% | 44.0% | 8.9% | 23.9% | 37.5% | 5.3% |

**Supplementary material to**: *Assessment of the bacterial community in directly brined Aloreña de Málaga table olive fermentations by metagenetic analysis*

**Table S2.** Relative abundance (%) of the more representative OTUs at family level in the different type of samples analysed.

| Taxonomy | FF.TOL | F.TOL.0 | F.TOL.1 | F.TOL.4 | B.TOL.0 | B.TOL.1 | B.TOL.4 | FF.COP | F.COP.0 | F.COP.1 | F.COP.4 | B.COP.0 | B.COP.1 | B.COP.4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *f__Alteromonadaceae* | 0.4% | 1.5% | 95.1% | 95.6% | 93.4% | 96.8% | 96.6% | 0.5% | 2.9% | 44.2% | 83.5% | 0.3% | 4.1% | 94.5% |
| *f__Alicyclobacillaceae* | 2.8% | 1.0% | 0.1% | - | - | - | - | 12.0% | 0.7% | 0.1% | 0.8% | - | 1.8% | - |
| *f__Pseudomonadaceae* | 32.4% | 47.8% | 0.1% | - | 2.4% | - | - | 0.2% | 6.4% | 0.1% | 3.5% | 67.0% | 11.2% | - |
| *f__Propionibacteriaceae* | 18.2% | 6.1% | 0.1% | - | 0.1% | - | 0.2% | 24.4% | 21.2% | 0.4% | 2.1% | 3.4% | 28.8% | 0.1% |
| *f__Rhodospirillaceae* | 1.6% | 0.7% | - | - | 0.2% | - | - | - | 0.6% | - | 0.5% | 4.5% | 0.6% | 0.1% |
| *f__Streptococcaceae* | 0.4% | 2.0% | 0.1% | - | - | - | - | 0.2% | 9.7% | 0.1% | 0.2% | 0.3% | 13.9% | - |
| *f__Staphylococcaceae* | 0.4% | 0.5% | 0.1% | - | - | - | - | 3.8% | 0.7% | - | 0.3% | - | 0.6% | 0.1% |
| *f__Corynebacteriaceae* | 3.1% | 2.2% | - | - | - | - | - | 4.0% | 1.5% | - | 0.5% | - | 2.0% | - |
| *f__Bacteroidaceae* | 3.8% | - | - | - | - | - | - | 6.1% | 0.7% | - | 0.1% | - | 1.0% | - |
| *f__Sphingobacteriaceae* | 0.2% | - | - | - | - | - | - | 4.9% | 0.7% | - | - | - | 0.6% | - |
| *f__Enterobacteriaceae* | 3.7% | 2.0% | - | - | 0.3% | - | - | 0.9% | 2.5% | - | 0.8% | 5.6% | 3.5% | - |
| *f__Lactobacillaceae* | 3.6% | 17.6% | - | - | - | - | - | 0.1% | - | 0.1% | 0.1% | - | - | - |
| *f__Leuconostocaceae* | 0.3% | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *f__Sphingomonadaceae* | 3.6% | 4.4% | - | - | 0.4% | 0.1% | 0.1% | 5.4% | 1.7% | - | 0.2% | 3.1% | 3.5% | - |
| *f__Acetobacteraceae* | - | - | - | - | - | - | - | - | - | 52.0% | 0.2% | - | 0.8% | 2.5% |
| *f__Geodermatophilaceae* | - | - | - | - | - | - | - | - | 30.2% | - | - | - | - | - |
| Others | 27.7% | 14.4% | 4.6% | 4.2% | 3.2% | 2.9% | 3.0% | 37.5% | 20.7% | 3.0% | 7.3% | 15.8% | 27.6% | 2.6% |

**Supplementary material to**: *Assessment of the bacterial community in directly brined Aloreña de Málaga table olive fermentations by metagenetic analysis*

**Table S3.** OTUs shared among the three types of substrates (fresh fruits, fermented fruit and brines) considering sampling time and industry all together. Only OTUs assigned at genus level and *Enterobacteriaceae* and *Lactobacillaceae* families are shown.

| OTU |
|---|
| *p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae; g_Cyanothece* |
| *p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae; g_Sufflavibacter* |
| *p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae; g _Flavisolibacter* |
| *p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae; g__Bacillus* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas* |
| *p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae; g_Faecalibacterium* |
| *p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__Rhodothermaceae;g__Salinibacter* |
| *p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g_Chryseobacterium* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Rhodobacter* |
| *p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus* |
| *p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__Owenweeksia* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Balneimonas* |
| *p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Rhodovibrio* |
| *p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus* |
| *p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas* |
| *p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Herbaspirillum* |
| *p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Ectothiorhodospiraceae;g__Halorhodospira* |
| *p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium* |
| *p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia* |
| *p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter* |
| *p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium* |
| *p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Micrococcus* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Phenylobacterium* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Erwinia* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium* |
| *p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Leuconostocaceae;g__Weissella* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Celerinatantimonas* |
| *p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium* |
| *p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae* |

**Supplementary material to**: *Assessment of the bacterial community in directly brined Aloreña de Málaga table olive fermentations by metagenetic analysis*

**Table S4.** OTUs shared in fruit samples among all the different sampling time considering the two industries together. Only OTUs assigned at genus level and *Enterobacteriaceae* and *Lactobacillaceae* families are shown.

| |
|---|
| p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__Rhodothermaceae;g_Salinibacter |
| p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus |
| p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Rhodovibrio |
| p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus |
| p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas |
| p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus |
| p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium |
| p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium |
| p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Celerinatantimonas |
| p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae |
| p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae |

**Supplementary material to**: *Assessment of the bacterial community in directly brined Aloreña de Málaga table olive fermentations by metagenetic analysis*

**Table S5.** OTUs shared in brine samples among all the different sampling time considering the two industries together. Only OTUs assigned at genus level are shown.

| |
|---|
| *p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus* |
| *p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Cyanobacteriaceae;g__Cyanothece* |
| *p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas* |
| *p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium* |
| *p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__Rhodothermaceae;g_Salinibacter* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas* |
| *p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus* |
| *p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium* |
| *p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia* |
| *p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus* |
| *p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__Owenweeksia* |
| *p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Rhodovibrio* |
| *p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium* |
| *p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Celerinatantimonas* |