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Abstract: Knowledge of the microbial biodiversity through the different stages of black ripe olive processing is minimal. This study applied a metagenomic approach to evaluate the bacterial and fungal communities present in samples of two olive cultivars collected from an industrial producer. High-throughput sequencing and analysis of the V2-V3 region of the bacterial 16S rRNA genes showed that genus *Acetobacter* and the lactic acid bacteria (LAB) *Lactobacillus* and *Oenococcus* were the most abundant during most of the process. Other LAB genera (*Enterococcus*, *Lactococcus*, *Weissella*, *Leuconostoc* and *Streptococcus*), as well as *Enterobacteriaceae* and *Vibrio* were also detected, increasing their abundance in the last step before packaging. Some genera, such as *Pseudoalteromonas*, *Alteromonas*, *Marinomonas* and *Oenococcus*, had never been previously detected in table olives. The fungal community was characterized by the presence of *Pichia membranifaciens*, *Kregervanrija fluxuum* and members of the family *Dispodascaceae*. Although black ripe olives are sterilized before trading, the consequences that these findings may have on the quality of this product are discussed.

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2 **Microbial ecology along the processing of Spanish olives darkened by oxidation**

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16 **Abstract**

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18 processing is minimal. This study applied a metagenomic approach to evaluate the
19 bacterial and fungal communities present in samples of two olive cultivars collected
20 from an industrial producer. High-throughput sequencing and analysis of the V2-V3
21 region of the bacterial 16S rRNA genes showed that genus *Acetobacter* and the lactic
22 acid bacteria (LAB) *Lactobacillus* and *Oenococcus* were the most abundant during most
23 of the process. Other LAB genera (*Enterococcus*, *Lactococcus*, *Weisella*, *Leuconostoc*
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25 increasing their abundance in the last step before packaging. Some genera, such as
26 *Pseudoalteromonas*, *Alteromonas*, *Marinomonas* and *Oenococcus*, had never been
27 previously detected in table olives. The fungal community was characterized by the
28 presence of *Pichia membranifaciens*, *Kregervanrija fluxuum* and members of the family
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38 1. Introduction

39 Black ripe olives are those table olives called “darkened by oxidation” according
40 to the Trade Standard for Table Olives issued by the International Olive Council (IOC,
41 2004). Spain and the United States are the main producers and consumers of this kind of
42 commercial table olive. This preparation may account for around 30% of the world table
43 olive production, which rose to about 2,500,000 tons in the last five years (IOC, 2016).
44 Processing includes the preservation stage: previous storage of the fruits for several
45 months (Figure 1). Olive fruits are kept in underground fiberglass tanks with acidic
46 cover solutions with or without salt for at least 6 months to more than 18 months (de
47 Castro, García, Romero, Brenes, & Garrido, 2007). Then, olives are treated with a
48 diluted sodium hydroxide solution (lye). After lye treatment, olives are put in water and
49 air is bubbled throughout the mixture for 24 - 30 hours (Washing 1). The acid solutions
50 for preservation or a mix with water can be used for this washing step. Throughout this
51 operation, both the epicarp and the flesh of the olives darken progressively, which
52 become brown to black due to the polymerization of hydroxytyrosol and caffeic acid
53 under the effect of oxygen (Brenes-Balbuena, García-García, & Garrido-Fernández,
54 1992). After this first washing step, a ferrous gluconate or lactate solution is added
55 (Ferrous Gluconate step) to retain the dark color formed (Garrido, García, Brenes &
56 Romero, 1995). Subsequently, the product can undergo pitting, slicing, or other
57 modifications, and is washed again with water (Washing 2 stage) before it being canned
58 and sterilized at least for 15 minutes at 121 °C, as appropriate for a low-acid food
59 (Sánchez-Gómez, García-García, & Rejano-Navarro, 2006). A single lye treatment
60 followed by a single water wash has been proposed in order to reduce the high volume
61 of wastewater generated during the darkening process (García, Brenes, & Garrido,

1991). This new process gives rise to a product which is richer in bioactive substances than the traditionally processed product (Romero, García-García, & Brenes, 2016).

Black ripe olives are referred as safe as they are a sterilized product, providing they have been correctly manufactured and manipulated by the end user. However, in most companies the period between the initiation of the washing 2 stage and the packaging and sterilization may last for more than 30 h, which is long enough for the growth of some microorganisms that affect on the organoleptic characteristics of the final product. Black ripe olives have a long history of microbial safety. However, botulism outbreaks due to *Clostridium botulinum* have been associated with this product. The most severe outbreaks took place in the United States in 1919–1920 and the products implicated were olives darkened by oxidation that were improperly sterilized (Horowitz, 2011). Also, other botulism outbreaks have been reported due to an incorrect storage or manipulation in Italy (Fenicia, Ferrini, & Aureli, 1992) and Finland (Jalava et al., 2011).

The microbiota in the preservation phase consists of mainly yeasts, acetic acid bacteria under acidic conditions without NaCl, and the presence of lactic acid bacteria (LAB) when the temperature rises in spring and summer (de Castro et al., 2007). Also, a high microbial load has been observed in solutions after lye treatment, color retention with ferrous gluconate, and water washing steps. While the microbiota in the preservation step is very well characterized, there is not any study regarding the microbiota present in the rest of the phases of the black ripe olive processing. The pH values in these phases are close to neutrality and olives remain a variable time period before sterilization.

High-throughput sequencing has revolutionized the field of food microbial ecology. It is a new culture-independent tool to study the microbial communities in foods. Recently, metagenetics studies have been carried out to study the microbial ecology in several table olive elaborations (Cocolin et al., 2013; De Angelis et al., 2015; Medina et al., 2016a; Randazzo et al., 2017). Therefore, the aims of this work were to carry out a physicochemical characterization and to evaluate the bacterial communities using high-throughput sequencing techniques in order to obtain the necessary information regarding the process so as to design new strategies to improve the quality and safety in the black ripe olives.

2. Material and Methods

2.1. Sampling

Industrial samples were obtained from a renowned table olive producer in the province of Seville (Spain). Three samples from each stage depicted in Figure 1 were withdrawn (only two samples of Washing 2 stage were available), transported to the laboratory in sterile containers and kept at 4 °C (chemical and microbiological analyses), and -20° C (HPLC and genetic analyses), until they were examined. Two samples belonged to cv. Hojiblanca (H1, H2) and the third to cv. Manzanilla (M), the most important cultivars used for black ripe olive processing in Spain. Since after the alkaline treatment is presumable that the microbial growth should initiate and reach higher populations in the surrounding liquids than in the fruits, the research was carried out with the liquids in which the olives were submerged. Taking into account the reality of the industrial practice, the objective of the sampling plan was to cover the greatest diversity of cases. With this aim, the preservation stage samples corresponded to olives

108 preserved with salt (2.7 g NaCl 100 mL⁻¹) in the case of sample M, whereas
109 preservation samples H1 and H2 corresponded to acidified water (2 mL acetic acid 100
110 mL⁻¹) without salt. Moreover, all M and H1 samples came from olives preserved for 6-7
111 months, whereas samples H2 came from olives kept for 18 months (Table S1).

112 2.2. Chemical analyses

113 The pH values of the solutions were measured with a Crison Basic 20 pH-meter.

114 2.2.1. Organic acids

115 Organic acids were analyzed by HPLC as described by Sánchez, de Castro,
116 Rejano, & Montaña (2000). Duplicate samples (1 mL) were acidified with 10 µL of
117 concentrated phosphoric acid and finally the samples were filtered through 0.22 µm
118 before injection into the HPLC system. The chromatographic system consisted of a
119 2695 Alliance that includes a quaternary pump, an automatic injector, a column heater
120 module (30 °C), with the detection being performed with a 410 refractive index (40°C of
121 internal temperature). The entire system was operated with Millenium 32 software
122 (Waters, Milford, USA). A Spherisorb ODS-2 (5 µm, 25 cm, 4.6 mm i.d., Waters Inc.)
123 column was used and the separation was achieved by isocratic elution using water
124 acidified with phosphoric acid (pH 2.5) as mobile phase. The flow rate was 1.2 mL min⁻¹
125 and the volume injected was 20µL. Quantification of organic acids was made by using
126 the reference compound obtained from a commercial supplier. All analyses were carried
127 out in duplicate.

128 2.2.2. Sugars

Glucose, fructose, sucrose, ethanol and mannitol were analyzed by HPLC as described by Sánchez et al. (2000). The HPLC system is the same as described above. An Aminex HPX-87C carbohydrate analysis column (BioRad Labs) held at 65 °C was used. Deionized water was used as eluent at 0.7 mL min⁻¹. Sample analysis were performed in duplicate.

2.2.3. Phenolic compounds

The analysis of phenolic compounds was carried out as described elsewhere (Medina, Brenes, Romero, García, & de Castro, 2007) with variations. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module, and a Waters 996 photodiode array detector operated with Empower 2.0 software (Waters Inc.). Chromatograms were recorded at 280 and 260 nm. The evaluation of each compound was performed using a regression curve with the corresponding standard. Standards were purchased from Sigma and Extrasynthese (Fenay, France) companies or isolated by semi-preparative HPLC (Medina et al., 2007; Medina, Romero, de Castro, Brenes, & García, 2008). The analyses were performed in duplicate. Total polyphenols are the sum of hydroxytyrosol (Hy), Hy-glycol, Hy 1-Glucoside, Hy 4-glucoside, tyrosol (Ty), Ty -glucoside, vanillic acid, caffeic acid, ester of caffeic acid, *p*-coumaric acid, verbascoside, the dialdehydic form of decarboxymethyl elenolic acid linked to Hy (HyEDA), luteolin, luteolin -7-glucoside, oleuropein, rutin, comselogoside, apigenin, secoxyloganin, secologanoside, and ligustroside. Samples were run in duplicate.

2.3. Microbiological analysis

Both culture dependent and independent techniques were applied to investigate the microbiota present in the different process stages. The viable and culturable populations of *Enterobacteriaceae*, lactic acid bacteria (LAB), yeast and molds, and mesophilic aerobic bacteria were determined by plating the brines and their decimal dilutions (in 0.9 % NaCl) with a Spiral Plater (Don Whitley Sci. Ltd., Shipley, England). The culture media used were VRBG agar (Biokar diagnostics, Beauvais, France); De Man, Rogosa, Sharpe MRS agar (Biokar) with and without 0.02 % sodium azide (Sigma-Aldrich); oxytetracycline-glucose-yeast extract (Oxoid Ltd., Basingstoke, England) agar; and Standard Methods Agar (PCA) (Laboratorios Conda S.A., Madrid, Spain) for the aforementioned groups, respectively. MRS plates were incubated under anaerobic conditions (AnaeroGen, Oxoid). VRBG plates were incubated at 37 °C and all others at 32 °C for up to five days, and the numbers of colony forming units were counted with a Scan 500 (Interscience, St Nom la Bretèche, France) colony counter.

2.4. Microbial DNA extraction, preparation of libraries and MiSeq sequencing

Culture-independent methods were applied to assess the whole microbiota in the samples. Microbial genomic DNA was extracted as described by Medina et al. (2016b) and sent to the Sequencing and Bioinformatic Service of FISABIO (Valencia, Spain). 16S rRNA gene amplicons were amplified following the 16S rRNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Part # 15044223 Rev. A). The gene-specific sequences used in this protocol target the 16S rRNA gene V3 and V4 region (Klindworth et al., 2013). Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. The primer pair were: forward primer PCR_1 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer PCR_1 (5'-

175 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAA
 176 TCC- 3'). In addition, Intergenic transcribed spacers (ITS) of fungal organisms were
 177 amplified with primers ITS1_KYO2_F (5'-
 178 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGAGGAAGTAAAAGTCGT
 179 AA-3') and ITS2_KYO2_R (5'-
 180 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTYRCTRCTCGTTCTTCATC-
 181 3') according to the work of Toju, Tanabe, Yamamoto, & Sato (2012). The multiplexing
 182 step was performed using Nextera XT Index Kit (FC-131-1096). 1 µL of the PCR
 183 product was run on a Bioanalyzer DNA 1000 chip to verify the size; the expected size
 184 on a Bioanalyzer trace is ~550 bp. Libraries were sequenced using a 2x300 pb paired-
 185 end run (MiSeq Reagent kit v3 (MS-102-3001) on a MiSeq Sequencer according to
 186 manufacturer's instructions (Illumina). Quality assessment was performed by the use of
 187 prinseq-lite program (Schmieder & Edwards, 2011) applying the following parameters:
 188 minimum sequence length of 50 bp, trim_qual_right of 20, trim_qual_type of mean and
 189 trim_qual_window of 20 bp. Bioinformatic analysis was carried out using an ad-hoc
 190 pipeline written in RStatistics environment (R Core Team, 2012), making use of several
 191 Open Source libraries such as gdata, vegan, etc. Taxonomic affiliations of 16S rRNA
 192 dataset and ITS sequences have been assigned using the RDP_classifier from the
 193 Ribosomal Database Project (Cole et al., 2009; Wang, Garrity, Tiedje & Cole, 2007); for
 194 16S rRNA assignation, we used the proper RDP training set, while for ITS sequences
 195 we used the UNITE fungal classification database (<https://unite.ut.ee/>) also included in
 196 the *rdp_classifier*.

197 2.8. Statistical analysis

Data were expressed as mean values and standard deviations (SD) were calculated. Statistica software version 7.0 was used for data processing (Statistica for Windows, Tulsa, OK, USA).

3. Results and Discussion

3.1. Chemical and microbiological characteristics of the ripe olive processing stages

California-style black ripe olives, as a low-acid canned food, require sterilization temperatures above 110 °C to guarantee their microbiological safety (Tang et al., 2016). Spanish industry usually applies a sterilization process autoclaving for 15 minutes at 121 °C, at least. This final heat treatment could explain the lack of microbial studies between the initial preservation step and the final product (to the best of our knowledge). However, sensorial characteristics of this product can be influenced by the growth of certain microorganisms during the several steps of black olive processing. The chemical parameters and the population of monitored microorganisms of the three samples at each processing stage are presented in Table 1 and 2, respectively. Despite the different cultivars and origins, the samples presented similar values for each stage, which justifies that the mean values are shown. Meaningful differences among the successive stages were found, which can be explained according to the industrial procedure.

The pH values at the preservation stage are clearly acidic in order to keep the olives in good condition. Acetic acid, rather than lactic acid, is responsible for this acid environment. Acetic acid is customarily added to the initial olive cover liquids, either brine or water, in order to prevent potential spoiling microorganisms, for instance

220 *Enterobacteriaceae*. Significant amounts of ethanol and mannitol were found, whereas
221 the concentrations of sucrose, glucose and fructose were low (Table 1). The main
222 phenolic compound detected in these preservation solutions was hydroxytyrosol,
223 originating from the hydrolysis of the bitter glucoside oleuropein. It has to be said that
224 two of these preservation samples (H1 and H2) were acidified water, but the third (M)
225 was a brine (2.7 g NaCl 100 mL⁻¹). However, few differences among the main chemical
226 characteristics were found and the standard deviations were low (Table 1). Scant
227 microbial populations were detected in the preservation samples (Table 2). No
228 *Enterobacteriaceae*, lactic acid bacteria, or molds were detected at the time of sampling.
229 The predominant microbiota were mesophilic aerobic bacteria, and yeasts were also
230 present. Despite of the high ethanol concentration, which is a product of the yeast
231 metabolism, the yeast population was very low at the time of sampling, but these results
232 are in agreement with previous studies (De Castro et al., 2007).

233 The most noteworthy chemical characteristic of the lye treatment samples was
234 their high pH values, which is obvious taking into account that 3 % NaOH
235 concentrations are used in this second stage. The lye facilitates diffusion through the
236 olive epidermis between the flesh and the surrounding liquid and it explains why acids,
237 sugars, ethanol and polyphenols were detected in the samples, albeit their
238 concentrations were lower than in the preservation samples. No viable microorganisms
239 were detected in the second stage samples (lye).

240 On the contrary, all the microbial groups examined by culture technics were
241 detected in the Washing 1 stage (Table 2). This step lasts for 24-30 hours, and a mixture
242 of water with preservation liquids is currently applied by the industrial producer with
243 the aim of reducing the olives' high pH values due to the anterior lye treatment. As a

consequence, the pH values of the Washing 1 samples (Table 1) were suitable for microbial **survival** or even growth. Aerobic mesophiles organisms were the most abundant, reaching more than 10^6 cfu mL⁻¹ in two of the three samples. Yeast and LAB, which constitute the normal microbiota in aged preservation liquids, presented populations ranging from 4.2×10^3 to 1.7×10^5 cfu mL⁻¹. Molds were also detected and a low number of *Enterobacteriaceae*. In relation to the chemical characteristics in this stage (Table 1), in general the monitored compounds presented values between the concentrations detected in stages 1 and 2. As it has been said above, the Washing 1 step is carried out with mixtures of water and preservation liquids, which justifies these results, and in some way the important standard deviations, as a consequence of the liquid variabilities applied in this stage.

Once the olives have been oxidized (blackened) during the Washing 1 step, the next stage aims to retain color by using the agent ferrous gluconate (E-579). The olives stayed in this solution for 7-12 hours and, as it was observed in the previous stage 3, the pH values and the low but remaining substrate concentrations allowed the existence of diverse microbiota dominated by mesophilic aerobic bacteria (around 1×10^7 cfu mL⁻¹), followed by lactic acid bacteria, yeasts and, to a lesser extent, molds and *Enterobacteriaceae*.

However, this “initial” colonization increased dramatically throughout the fifth and last stage (Washing 2) before packing. Counts above 1×10^8 cfu mL⁻¹ were recorded for aerobic mesophiles and LAB, whereas yeasts and molds also displayed higher numbers than in the previous stage, and it was noteworthy that *Enterobacteriaceae* reached values close to 1×10^7 cfu mL⁻¹ during this Washing 2 stage (Table 2).

3.2. Molecular ecology of the bacterial and fungal communities

The results obtained by using culture dependent techniques highlighted the importance that the black ripe olive processing could have on both the final product's quality-sensory properties and on the safety consequences should the product be marketed without any thermal treatment. In addition, these results justify the suitability of applying DNA sequencing methods to unveil what microorganisms are present through the different stages and their importance.

The high throughput sequencing of the 16S-PCR products generated a mean of 53,017 high quality sequences per sample with an average length of 460 bp (Table S2). Across all taxa, a total of 24 predominant bacterial OTUs were classified at genus level (Table S4). Also, ITS sequencing showed an average of 60,534 sequences per sample with an average length of 477 bp (Table S3). Table S5 in Supplementary material shows the main 15 fungal OTUs assigned at family or species shared among all samples in the different steps. The microbial community was also analyzed using diversity estimator (Shannon, Simpson and Chao1) (Tables S2 and S3). The least biodiversity was found in the preservation stage and the highest in samples from the second washing step.

A change of bacterial (Figure 2) and fungal (Figure 3) genera and species was observed through the different steps of black ripe olive processing in the samples studied. In relation to bacteria, a very significant difference was found in the preservation stage between sample M (which contained 2.7 g NaCl 100 mL⁻¹), and the samples H1 and H2 that contained no salt. Almost all the DNA in sample M belonged to the genus *Lactobacillus*, whereas *Acetobacter* prevailed in samples H1 and H2 (Figure 2 and Table S4). It is in some way surprising that LAB were not detected in this stage by

cultural methods (Table 2). Taking into account that the same methodology was used for the successive stages and viable LAB were recovered from them, the most reasonable explanation is that this group of bacteria lost viability in the preservation liquids but their DNA was present and available for sequencing. Nevertheless, the fact that both LAB and acetic acid bacteria may be present during the preservation stage confirms previous findings (de Castro et al., 2007). With regard to the yeast species, *Kregervanrija fluxuum* and *Pichia membranifaciens* predominated in the preservation liquids, the first species in the salt containing M sample and the second in the H1 and H2 samples (Figure 3 and Table S5). *Pichia membranifaciens* has long been cited in olive environments, both in Spanish-style green olives (González-Cancho, 1960) and in directly brined olives (Duran, García, Brenes, & Garrido, 1994). On the contrary, *Kregervanrija fluxuum* has been found in wine, beer and cider (Kurtzman, 2006), but to the best of our knowledge, it has never been referenced in table olives.

Proceeding to the second stage, no DNA was obtained from lye treatment samples and microbiological results of this step have not been attained either by sequencing or by cultivation (Table 2).

Acetobacter (64.6 – 76.4 %) and *Lactobacillus* (22.0 – 31.7 %) were also the most abundant bacterial genera found in the Washing 1 stage (Figure 2 and Table S4). However, it is important to highlight that *Vibrio* (up to 8.3 % relative abundance in sample H1) and *Enterobacteriaceae* (up to 1.6 %) were firstly detected in this stage. Low numbers of viable *Enterobacteriaceae* were already detected by cultural methods in this stage (Table 2), and although this family is frequently found in olive brines and other environments without causing side effects, it is well known that certain species or strains can cause illnesses. Fortunately, the usually hard characteristics achieved during

314 the time that olives are kept in brine (low pH, low a_w , antimicrobial phenolic
315 compounds) prevent the presence of these microorganisms in the preservation step.
316 However, this was not the case in the Washing 1 samples, since neither pH values nor
317 salt concentration were limiting factors. The genus *Vibrio* also includes pathogenic
318 species and this possibility cannot be ruled out. A new species of *Vibrio* has been
319 described in Spanish-style green olive fermentations (Lucena-Adrós, González,
320 Caballero-Guerrero, Ruiz-Barba, & Maldonado-Barragán, 2015) and *Vibrio* spp. have
321 also been found in fermented cucumbers (Breidt et al., 2013). The most probable origin
322 of this species is the salt, which is almost always from marine sources in Spanish olive
323 industries, and other microorganisms from saltern habitats have been found in olive
324 brines (Lucena-Adrós & Ruiz-Barba, 2016). As regards yeasts found in this step,
325 *Pichia membranifaciens* and *Kregervanrija fluxuum* were still present, but the highest
326 relative abundance corresponded to the family *Dipodascaceae* (Figure 3 and Table S5).
327 Within this family, *Geotrichum candidum*, *Galactomyces geotrichum* and *Dispodascus*
328 *australiensis* have been identified as sequences with 99 % of similarity (data not
329 shown). Likewise, other species such as *Magnusiomyces capitatus* (*Dipodascaceae*) and
330 *Dekkera anomala* (*Pichiaceae*) were present in low quantities.

331 Yeasts are the predominant microorganisms in black ripe olive preservation and
332 natural olive elaboration (Brenes, García, Durán, & Garrido, 1986). In this study, a total
333 of 15 yeast taxa have been detected and many of them have been described as common
334 yeast in green natural olives, Spanish-style Green olives (Arroyo-López et al., 2012),
335 and in cracked green olives (Mateus, Santo, Saúde, Pires-Cabral, & Quintas, 2016).
336 Yeasts play a dual role in the elaboration of table olives. They are in part responsible for
337 the aroma production in the final products, but can also provoke negative effects such as

excessive CO₂ release, the formation of gas-pockets in the fruits, or different unwanted enzymatic activities (Arroyo-López et al., 2012).

Once the olives have finished the washing after the lye treatment, they are kept in the ferrous gluconate solution. Ferrous gluconate acts as a color retention agent after polyphenol polymerization has darkened the olives. The microbiota in this fourth stage was similar to the previous third in both bacteria and yeasts. The most marked difference was that *Oenococcus* took the place of *Lactobacillus* as the most abundant LAB in the gluconate solutions. In fact, *Oenococcus* was more abundant than *Acetobacter* in the sample M (63.7 % vs 35.5 %, respectively), whereas *Acetobacter* surpassed LAB in the H1 and H2 samples (80-96.4 % vs 3.1-19.2 %, respectively) (Figure 2 and Table S4). *Oenococcus* is an important genus in wine-making, where it is applied as a starter culture to perform the malolactic fermentation (Mills, Rawsthorne, Parker, Tamir, & Makarova, 2005). Although it has been cited in olive mill wastes (Ntougias, Bourtzis, & Tsiamis, 2013), this is the first time it has been reported in olive samples. Apart from that, *Dipodascaceae*, *Pichia membranifaciens*, and *Kregervanrija fluxuum* remained the yeasts detected more abundantly.

As it was previously said, the highest microbial diversity was found in samples from the Washing 2 step. The high number of bacteria detected by cultural methods was mirrored in more than 20 genera identified by sequencing (Figure 2 and Table S4). Within LAB, *Enterococcus*, *Streptococcus* and *Lactococcus* unveiled compared to the previous stages, *Lactobacillus* and *Leuconostoc* stayed present, but *Oenococcus* faded dramatically. Altogether, LAB accounted for approximately 13.4 – 31.6 % in this Washing 2 stage. An important decrease was also detected in the relative abundance of *Acetobacter*, although the figures are still notable (7.9 -10.5 %). However, the most

striking result was that between 54.1 and 74.3 of the readings belonged to Gammaproteobacteria, with *Enterobacteriaceae* (21.3-27.8 %) and *Vibrio* (14.2-31.2 %) being the most abundant, but other six genera were found. Among them, *Pseudoalteromonas*, *Alteromonas* and *Marinomonas* have never been detected before in table olives, and *Shewanella* has been found recently in Nocellara Etnea table olives (Randazzo et al., 2017). Suspiciously, all these genera, along with *Vibrio*, share sea water, coast and salt marshes as main habitats (Gauthier, Gauthier, & Christen, 1995). The genus *Acinetobacter* (2.9-3.9 % relative abundance) has been described in table olives with alkaline treatment by Cocolin et al. (2013). *Acinetobacter* is not considered pathogenic for healthy human beings, but some strains are opportunistic pathogens responsible for respiratory and urinary diseases in immunosuppressed patients (Bergogne-Bérézin & Towner, 1996). Going back to *Enterobacteriaceae*, it has to be mentioned that, apart from the pathogenic species included in this family, these bacteria cause table olive spoilages such as gas-pocket formation in the olive fruits and produce metabolites that generate off-odours and off-flavours in the final products (Garrido-Fernandez, Fernández-Díaz, & Adams, 1997).

Less significant were the differences found among the yeasts detected in the Washing 2 step when comparing with the previous steps. *Dipodascaceae*, *Pichia membranifaciens*, and *Kregervanrija fluxuum* were the predominant taxa.

4. Conclusions

The microbial ecology of the different black ripe olive processing stages had not been studied before this work. Our findings have confirmed that a high diversity of bacteria and, to a lesser extent, yeasts can be present in the different solutions used

385 throughout the process. Although ripe olives are finally sold after a sterilization
386 treatment, the microbial growth through the previous steps may have sensorial effects
387 and compromise the product quality.

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524 **Figure captions**

525 **Fig. 1.** Flowchart with the detailed stages of the black ripe olive processing from which
526 samples were collected.

527 **Fig. 2.** Relative abundance (%) of the bacterial genera or family obtained by
528 pyrosequencing analysis through the different stages of black ripe olive processing. M:
529 cv. Manzanilla; H1 and H2: cv. Hojiblanca.

530 **Fig. 3.** Relative abundance (%) of the fungal genera or family obtained by
531 pyrosequencing analysis through the different stages of black ripe olive processing. M:
532 cv. Manzanilla; H1 and H2: cv. Hojiblanca.

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535 Table 1. Chemical characteristics of samples at the different stages of black ripe olive processing (mmoles L⁻¹).

Stage	pH	Lactic Acid	Acetic Acid	Ethanol	Sucrose	Glucose	Fructose	Mannitol	Total Polyphenols
Preservation	3.9 ± 0.0*	1.1 ± 1.1	118.2 ± 5.0	125.9 ± 4.3	0.6 ± 0.0	8.3 ± 2.1	2.6 ± 0.5	31.8 ± 3.2	15.1 ± 1.0
Lye treatment	14.2 ± 0.1	2.2 ± 1.1	38.3 ± 0.0	47.8 ± 0.0	0.1 ± 0.1	1.7 ± 0.4	1.7 ± 0.4	5.0 ± 1.8	1.9 ± 0.2
Washing 1	6.4 ± 0.9	4.4 ± 2.2	68.3 ± 6.7	28.2 ± 4.3	0.0 ± 0.0	2.9 ± 1.6	1.5 ± 0.1	5.4 ± 2.8	3.9 ± 1.1
Ferrous gluconate	6.2 ± 1.2	2.2 ± 1.1	24.9 ± 3.3	6.5 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	1.1 ± 0.1	0.9 ± 0.4	1.2 ± 0.4
Washing 2	6.4 ± 0.3)	124.3 ± 2.2	44.9 ± 6.7	6.5 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.2	0.0 ± 0.0	2.3 ± 0.7

536 * Data are the mean of samples belonging to the same stage ± Standard Deviation.

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544 Table 2. Viable microbial population in samples during black ripe olive processing (Log cfu mL⁻¹).

Stage	<i>Enterobacteriaceae</i>	LAB	Yeast	Molds	Aerobic mesophiles
Preservation	<1.30 ± 0.00*	<1.30 ± 0.00	1.65 ± 0.14	<1.30 ± 0.00	3.36 ± 1.23
Lye treatment	<1.30 ± 0.00	<1.30 ± 0.00	<1.30 ± 0.00	<1.30 ± 0.00	<1.30 ± 0.00
Washing 1	1.70 ± 0.43	4.49 ± 0.81	4.48 ± 0.67	3.61 ± 1.04	5.95 ± 1.16
Ferrous gluconate	1.55 ± 0.21	5.90 ± 0.96	4.01 ± 1.18	3.81 ± 0.50	6.96 ± 0.19
Washing 2	6.90 ± 0.35	8.23 ± 0.21	5.80 ± 0.54	4.08 ± 0.16	8.32 ± 0.08

545 * Data are the mean of samples belonging to the same stage ± Standard Deviation. The detection limit of the
 546 plating method is 1.30 cfu mL⁻¹.

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19 Fig. 2.

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1 Table S1. Characteristics of samples collected from black ripe olive processing.

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Sample	Cultivar	Preservation time (months)	Type of cover solution
M	Manzanilla	6 - 7	NaCl
H1	Hojiblanca	6 - 7	Acid water
H2	Hojiblanca	18	Acid water

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9 Table S2. Number of reads and genus diversity estimators for 16s rRNA amplicons from black ripe olive processing samples.

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Stage	Sample	Reads (bp)	Mean Length (bp)	Shannon	Simpson	Chao1
Preservation	M	20495	464.82	0.07	0.02	18
	H1	41707	464.74	0.22	0.07	49
	H2	64410	464.77	0.33	0.12	53
Washing 1	M	56168	464.20	1.23	0.67	55
	H1	51002	463.66	1.54	0.73	52
	H2	86299	465.19	1.46	0.72	6
Ferrous Gluconate	M	47799	453.27	0.78	0.47	32
	H1	54937	459.93	0.98	0.57	47
	H2	81621	456.45	0.77	0.49	24
Washing 2	M	26814	465.54	2.68	0.87	10
	H1	51939	440.23	2.85	0.91	13

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15 Table S3. Number of reads and genus diversity estimators for ITS rRNA amplicons from black ripe olive processing samples.

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Stage	Sample	Reads (bp)	Mean Length (bp)	Shannon	Simpson	Chao1
Preservation	M	24696	507.64	0.59	0.38	14
	H1	21988	505.20	0.30	0.14	18
	H2	30697	489.42	0.72	0.43	8
Washing 1	M	146773	441.23	0.68	0.30	1
	H1	116392	457.57	1.14	0.60	1
	H2	57589	419.52	1.13	0.54	2
Ferrous Gluconate	M	65964	432.97	0.63	0.28	17
	H1	89709	504.31	0.76	0.36	17
	H2	52842	476.93	1.23	0.65	2
Washing 2	M	22989	507.59	0.73	0.32	1
	H1	36237	505.52	1.01	0.53	17

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22 Table S4. Relative abundance (%) of the more representative bacterial OTUs at genus and family level in samples analyzed.

Taxonomy	Processing stage										
	Preservation			Washing 1			Ferrous Gluconate			Washing 2	
	M	H1	H2	M	H1	H2	M	H1	H2	M	H1
g_ <i>Bacteroides</i>	-	-	-	-	-	-	-	-	-	0.1	0.3
g_ <i>Dysgonomonas</i>	-	-	-	-	-	-	-	-	-	0.1	0.2
g_ <i>Prevotella</i>	-	-	-	-	-	0.1	-	-	-	0.5	0.5
g_ <i>Aerococcus</i>	-	-	-	-	-	0.4	-	-	-	0.1	0.1
g_ <i>Alkalibacterium</i>	-	-	0.1	-	-	0.1	-	-	-	-	-
g_ <i>Enterococcus</i>	-	-	-	-	0.3	-	-	-	-	12.6	5.2
g_ <i>Lactobacillus</i>	99.3	0.9	4.1	22.2	22.0	31.7	0.2	-	-	3.3	2.6
g_ <i>Leuconostoc</i>	-	-	-	-	2.0	0.1	0.1	5.6	1.0	0.8	0.6
g_ <i>Oenococcus</i>	0.3	1.7	-	0.8	0.9	1.3	63.7	13.1	1.8	-	-
g_ <i>Weissella</i>	-	-	-	-	-	-	-	0.5	0.3	0.1	0.3
g_ <i>Lactococcus</i>	-	-	-	-	-	-	-	-	-	8.1	1.5
g_ <i>Streptococcus</i>	-	-	-	-	-	-	-	-	-	6.7	3.2
g_ <i>Veillonella</i>	-	-	-	-	-	-	-	-	-	0.1	0.1
g_ <i>Acetobacter</i>	0.1	96.6	95.0	76.4	64.6	64.7	35.5	80.0	96.4	10.5	7.9
g_ <i>Comamonas</i>	-	-	-	-	-	-	-	-	-	0.2	0.2
g_ <i>Arcobacter</i>	-	-	-	-	-	-	-	-	-	0.3	0.5
g_ <i>Alteromonas</i>	-	-	-	-	-	-	-	-	-	2.8	3.0
g_ <i>Shewanella</i>	-	-	-	-	-	-	-	-	-	3.2	9.6
f_ <i>Enterobacteriaceae</i>	-	0.2	-	-	1.6	0.8	0.2	0.1	-	27.8	21.3
g_ <i>Marinomonas</i>	-	-	-	-	-	-	-	-	-	0.9	2.6
g_ <i>Acinetobacter</i>	-	-	-	-	-	-	-	-	-	2.9	3.9
g_ <i>Pseudomonas</i>	-	-	-	-	-	-	-	-	-	0.1	0.2
g_ <i>Pseudoalteromonas</i>	-	-	-	-	-	-	-	-	-	2.2	2.5
g_ <i>Vibrio</i>	-	0.1	0.3	0.1	8.3	0.3	-	-	-	14.2	31.2
Unclassified and minor groups	0.3	0.5	0.4	0.6	0.3	0.6	0.2	0.7	0.5	2.3	2.5

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24 Table S5. Relative abundance (%) of the more representative fungi OTUs at family and species level in samples analyzed.

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Taxonomy	Processing stage										
	Preservation			Washing 1			Ferrous Gluconate			Washing 2	
	M	H1	H2	M	H1	H2	M	H1	H2	M	H1
<i>sp_Candida apicola</i>	-	-	0.1	-	-	-	-	-	-	-	-
<i>sp_Candida bituminiphila</i>	-	-	0.2	-	-	0.3	-	-	0.1	0.8	1.1
<i>sp_Candida etchellsii</i>	-	-	0.1	-	-	-	-	-	-	-	-
<i>sp_Candida magnoliae</i>	-	-	0.1	-	-	-	-	-	-	-	-
<i>sp_Candida pararugosa</i>	-	-	0.2	-	-	-	-	-	-	-	-
<i>sp_Cyberlindnera jadinii</i>	-	-	-	-	-	-	-	-	0.1	-	-
<i>sp_Dekkera anomala</i>	0.1	-	1.1	0.1	0.3	0.7	-	-	0.1	-	-
<i>f_Dipodascaceae</i>	0.2	0.9	1.7	89.5	59.6	70.5	90.9	85.0	50.4	88.9	67.2
<i>sp_Kregervanrija fluxuum</i>	75.1	6.3	25.9	2.0	7.6	9.3	2.1	1.7	15.2	1.8	4.1
<i>sp_Magnusiomyces capitatus</i>	-	-	0.2	-	1.2	2.6	-	0.1	0.2	0.5	0.4
<i>sp_Pichia membranifaciens</i>	24.5	92.6	70.1	8.0	30.7	13.9	6.7	12.7	32.6	7.2	25.5
<i>sp_Pichia terricola</i>	-	-	-	-	-	-	-	-	0.1	-	0.1
<i>sp_Thichosporon cutaneum</i>	-	-	-	-	-	-	-	-	-	0.1	-
<i>sp_Thichosporon guehoae</i>	-	-	-	-	-	-	-	-	-	0.1	0.1
<i>sp_Thichosporon veenhuisii</i>	-	-	-	-	-	-	-	-	-	0.1	0.8
Unclassified and minor groups	0.1	0.3	0.4	0.4	0.6	2.6	0.3	0.5	1.2	0.7	0.7

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Highlights

- Microbiota present in black ripe olive processing determined for the first time
- Microbial diversity increased throughout the successive stages
- High-throughput sequencing found out taxa never before cited in table olives