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Microbial ecology along the processing of Spanish olives darkened by oxidation

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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*, *Weisella*, *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.

1. Introduction

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

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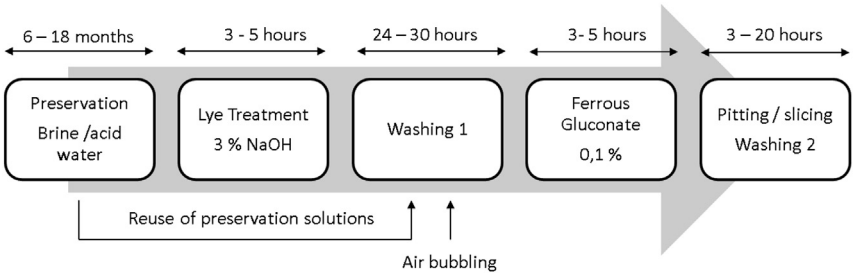

Fig. 1. Flowchart with the detailed stages of the black ripe olive processing from which samples were collected.

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

2.2. Chemical analyses

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

2.2.1. Organic acids

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

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, Romero, de Castro, Brenes, & García,