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Effect of Spanish-style processing steps and inoculation with *Lactobacillus pentosus* starter culture on the volatile composition of cv. Manzanilla green olives

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ABSTRACT

The effects of the main steps of Spanish-style processing (alkaline treatment and fermentation) on the volatile composition of cv. Manzanilla green olives were studied. Both spontaneous and controlled fermentations were considered. In the latter case, a *Lactobacillus pentosus* strain from green olive fermentation brine was used as starter culture. The volatile profile was determined by headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC–MS). Most of the volatile compounds detected in fresh olives decreased or were undetected after alkaline treatment, while several compounds (mostly acetic acid, 2-methylbutanoic acid, and ethanol) were formed as a result of this treatment. Over 50 new volatile components, mostly esters and phenols, appeared as a result of fermentation. The most outstanding finding was a considerable increase in 4-ethyl phenol (almost 100-fold increase) in inoculated olives compared to the uninoculated product. However, a sensory panel did not find significant differences in odor perception.

1. Introduction

Flavor is one of the key drivers in consumer appreciation for the Spanish-style green table olive, which is considered the main fermented vegetable product in western countries (Breidt, McFeeters, Perez-Diaz, & Lee, 2013). Flavor formation in food fermentation processes is caused by the accumulation of volatile aroma compounds, as well as the formation of compounds that are related to taste (bitterness, umami, sweetness, sourness, and saltiness) (Smid & Kleerebezem, 2014). Spanish-style green table olives contain a complex blend of volatile compounds, with phenols, alcohols, and acids being the predominant chemical classes (Cortés-Delgado et al., 2016; Sánchez et al., 2018). The volatile profile of this product can be influenced by a number of factors, including olive cultivar and production area (Cortés-Delgado et al., 2016; Garrido-Fernández, Montaño, Sánchez, Cortés-Delgado, & López-López, 2017), and post-fermentation and packing stages (Sánchez et al., 2018). The processing of Spanish-style green table olives consists of two main steps: (1) alkaline treatment including the operation of "cocido" (addition of 1.8–2.5% (*w/v*) NaOH) and washing to remove the excess alkali, and (2) addition of brine (10–13% (*w/v*) NaCl), where a fermentation process takes place (Rejano, Montaño, Casado, Sánchez, & de Castro, 2010). However, up to now, the effects of these steps separately on the volatile composition of olives have not been determined.

It has been generally established that lactic acid bacteria (LAB) are mainly responsible for the fermentation, with *Lactobacillus pentosus* and *Lactobacillus plantarum* being the predominant species (Hurtado, Reguant, Bordons, & Rozès, 2012). Many studies recommend the use of these species, in single or mixed combinations, as starter cultures, in order to reduce the risk of unexpected growth of spoilage strains and to lead to a controlled and more predictable fermentation process (Ruiz-Barba, Cathcart, Warner, & Jiménez-Díaz, 1994; Sánchez, Rejano, Montaño, & de Castro, 2001; De Castro, Montaño, Casado, Sánchez, & Rejano, 2002; Leal-Sánchez et al., 2003; Hurtado et al., 2012; Ruiz-Barba & Jiménez-Díaz, 2012; Martorana et al., 2017). However, very few studies have been conducted to determine the effect of a starter culture inoculation on the volatile profile of the product. The selected strain in table olive processing should have the ability to produce desirable aroma and taste through the production of volatile compounds, among other characteristics (Bonatsou, Tassou, Panagou, & Nychas, 2017). Panagou and Tassou (2006) studied the effect of inoculation with single strains of *L. pentosus* and *L. plantarum* on the volatile profile in Spanish-style green olives of the cv. Conservolea, but the number of

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compounds analyzed was quite limited and their identification was only based on retention times. More recently, a more complete analysis of the volatile compounds in green olives (cv. Nocellara del Belice) inoculated with a *L. pentosus* strain, based on headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC–MS), was carried out by Martorana et al. (2017), showing significant qualitative and quantitative differences in comparison with uninoculated olives.

The objectives of this work were (1) to determine the effects of alkaline treatment and fermentation steps on the volatile composition of olives, and (2) to determine the effect of inoculation with a strain of *L. pentosus* on the volatile profile of Spanish-style green olives, in comparison with an uninoculated product. The study was carried out with olives of the Manzanilla cultivar, which is the most prominent cultivar used for green table olives in Spain (ASEMESA, 2018).

2. Materials and methods

2.1. Materials and chemicals

The olives were harvested in Arahal (Seville province, Spain) at their mature-green stage and transported to our laboratories to be processed. Processing was carried out in cylindrical fermenters made of polyethylene (5.2 kg fruits plus 3.4 L liquid capacity).

All volatile compounds used as reference standards were purchased from Sigma-Aldrich (St Louis, MO). The internal standard 2,3,5,6-d_4-4-ethylphenol was supplied by SPEX CertiPrep (Metuchen, NJ). Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA). All other chemicals and solvents were of analytical or chromatographic grade from various suppliers (Panreac, Barcelona, Spain; VWR, Barcelona, Spain; Merck, Darmstadt, Germany).

2.2. Olive processing

The olives were divided into four fermenters (coded M1-M4) and alkali treated with 2.3% (*w/v*) NaOH for 5.5 h. Then, the fruits were washed with tap water for 11 h and, finally, a brine with 12% NaCl (*w/v*) as initial concentration was used to sustain the fermentation. Two fermenters (M1 and M2) were left as spontaneous fermentation control and the other two (M3 and M4) were inoculated with the strain *Lactobacillus pentosus* LP99, originally isolated from green olive fermentation brines. Taking into account the initial high alkalinity of the brine, a massive inoculation was carried out in order to warrant the starter culture implantation from the beginning (Sánchez et al., 2001).

Preparation of the inoculum was as follows. For propagation from stocks maintained at −80 °C, the strain was cultivated twice in MRS broth, the second time with 4.5% (*w/v*) NaCl to allow adaptation to the saline environment. After incubation at 32 °C for 30 h, the cultures were centrifuged, washed with saline (0.9% NaCl), and the washed pellets were re-suspended in the brine from the corresponding fermenters. A double inoculation was performed: *ca.* 2.1×10^8 cfu mL^{-1} was the initial population first added at Day 5, and then *ca.* 5.3×10^7 cfu mL^{-1} seven days after, at Day 12. No pH control by acid addition was carried out. However, the brines were supplemented with glucose at Day 159 in order to attain the acidity and pH values to warrant a convenient final preservation.

2.3. Sampling

Brine samples from each fermenter were taken during fermentation to control the main physicochemical and microbiological characteristics. Analyses of volatile compounds in pulp were performed at three different times throughout the elaboration: before processing, just before brining (i.e. once olives were NaOH treated and washed), and after 7 months of brining.

2.4. Physicochemical and microbiological analyses

The pH and titratable acidity of the brines were measured using a Metrohm 670 Titro processor (Herisau, Switzerland). Titratable acidity was determined by titrating to pH 8.3 with 0.2 mol L^{-1} NaOH and expressed as lactic acid.

The viable and culturable populations of lactic acid bacteria (LAB), and yeast and molds were determined by plating the brines and their decimal dilutions (in 0.9% NaCl) with a Spiral Plater (Don Whitley Sci. Ltd., Shipley, UK). The culture media used were de Man, Rogosa, Sharpe MRS agar (Biokar Diagnostics, Beauvais, France) with and without 0.02% sodium azide (Sigma-Aldrich), and oxytetracycline-glucose-yeast extract (Oxoid Ltd., Basingstoke, UK) agar for the aforementioned groups, respectively. MRS plates were incubated under anaerobic conditions (AnaeroGen, Oxoid). Plates were incubated 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.

2.5. Analysis of volatile compounds

The olives (approximately 200 g) were pitted and homogenized, and 2.5 g of homogenized pulp were placed in a 15-mL glass vial. After the addition of 7.5 mL NaCl solution (300 g L^{-1}), and 100 μL of 3-octanol (2 mg L^{-1}) as internal standard, the vial was closed and extraction by HS-SPME was performed with subsequent analysis by GC–MS, following the procedure reported by Sánchez et al. (2018). Concentrations were expressed as μg/kg of 3-octanol. Compound identification was based on mass spectra matching with the standard NIST 08 MS library and on the comparison of retention indices (RI) sourced from the NIST Standard Reference Database and from authentic reference standards when available. All analyses of the volatile compounds were made in triplicate.

In order to detect the volatile acids formed as a result of the alkaline treatment, and before extraction by HS-SPME, 150 μL of 2 N HCl solution were added to the vial containing 2.5 g pulp and 7.5 mL of NaCl solution to adjust the pH to 4 units.

A quantitative analysis of 4-ethylphenol was carried out in the olive juice according to the method described by Pollnitz, Pardon, and Sefton (2000) for red wine. For olive juice preparation, the olives were pitted and homogenized using a mixer. Then the juice was obtained by filtration through cheesecloth and centrifugation of the filtrate at 20,000*g* for 15 min. A solution of 2,3,5,6-d_4-4-ethylphenol (100 μL, 10 μg/mL) in methanol was added to the olive juice (2.5 mL) in a screw cap vial. Diethyl ether-pentane (1:2, 1 mL) was added and the mixture was shaken briefly. A portion of the organic layer was then transferred to a vial and an aliquot (1 μL) was injected into the GC–MS system, using the splitless injection mode. The GC–MS system used was a 7890A GC coupled to mass detector model 5975C (Agilent Technologies, Santa Clara, CA). Separation was achieved on a VF-WAX MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness) from Agilent. The injector temperature was 200 °C, and the oven temperature program was as follows: isothermal for 1 min at 150 °C, then 150–210 °C at 10 °C min^{-1}, and then 210–240 °C at 40 °C min^{-1} and held there for 15 min. The carrier gas was helium at a constant flow of 1 mL min^{-1}. For the mass selective detector conditions, the quadrupole, ion source and transfer line temperatures were maintained at 150, 230, and 250 °C, respectively. For quantification, mass spectra were recorded in selected ion monitoring (SIM) mode. The ions monitored were: *m/z* 107 and 122 for 4-ethylphenol and *m/z* 111 and 126 for 2,3,5,6-d_4-4-ethylphenol. The ions used for quantitation were: *m/z* 122 for 4-ethylphenol and *m/z* 126 for the internal standard. The other ions were used as qualifiers. Replicate ($n = 6$) analysis of a sample (M1) gave a relative standard deviation (RSD) of 1.5%. To verify the accuracy, we carried out recovery tests by adding known amounts of 4-ethylphenol (0.2 and 1 μg/mL) to a sample of olive juice that had been shown to contain 0.1 μg/mL of 4-