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Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques

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ABSTRACT

We have studied the microbiota associated to Spanish-style green olive fermentations, attending to its dynamics along the time. Twenty 10-tonne fermenters were selected from two large table-olive manufacturing companies in southern Spain. While culture-dependent methodology was used to isolate the microorganisms, molecular methods were used to identify the isolates. A total of 1070 isolates were obtained, resulting in 929 bacterial and 141 yeast isolates. Thirty seven different bacterial species were isolated, belonging to 18 different genera, while 12 yeast species were isolated, belonging to 7 distinct genera. This fermentation was dominated by the species *Lactobacillus pentosus*, while its accessory microbiota was variable and depended on the fermentation stage and the actual fermentation yard ("*patio*"). It was noticeable the abundance of lactic acid bacteria isolates, belonging to 16 different species. Twenty bacterial species were isolated for the first time from Spanish-style green olive fermentations, while 17 had not been described before in any table olive preparation. The genera *Brachybacterium*, *Paenibacillus*, *Sporolactobacillus*, *Paracoccus* and *Yersinia* had not been cited before from any table olive preparation. *Saccharomyces cerevisiae* and *Candida thaimueangensis* appeared to dominate the yeast microbiota. *Candida butyri/asseri* and *Rhodotorula mucilaginosa* had not been described before from Spanish-style preparations, while *Saturnispora mendoncae* was isolated for the first time from any table olive preparation. Biodiversity was analysed through different alpha and beta indexes which showed the evolution of the microbiota over time.

1. Introduction

Table olives account for the largest volume of fermented vegetables in Western countries, especially in Mediterranean countries (Garrido Fernández et al., 1997). World production reached an average of 2.3 million tons per year in the period 2006–2012 (IOOC, 2012). Although table olives can be prepared for consumption in many different ways, Spanish-style preparation of green olives is one of the three most commercially important worldwide, along with natural black olives and oxidised black olives (Garrido Fernández et al., 1995; Sánchez et al., 2006; Rejano et al., 2010), representing 60% of the world production (Botta and Cocolin, 2012). Spanish-style preparation is characterised by the initial alkali treatment (1.8–3.5% [w/v] NaOH) of the green fruits, which removes bitterness and allows the subsequent growth of lactic acid bacteria (LAB) through the neutralisation and washing of inhibitory phenolic compounds (Rejano et al., 2010). Once removed the alkali, fruits are washed once or twice with water and finally covered with brine (10–12% [w/v] NaCl). In this brine a spontaneous fermentation takes place in which at least three different stages have been identified (Garrido Fernández et al., 1995). During the first stage, usually lasting 3–10 days, fermentation is conducted by the indigenous alkali-tolerant microbiota which contaminates the fruits as well as the environment (De Castro et al., 2002). This microbiota is responsible for lowering the initial high pH (10–11) to values close to 6–7, more appropriate for the growth of LAB, which are also present as contaminants (Sánchez et al., 2001). As soon as LAB take over and grow exponentially, during what it is considered the second stage in this fermentation, pH value drops as a result of their metabolism. Sugars are converted into lactic acid, as the major

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product, as a result of a mainly homolactic fermentation. This is carried out mostly by strains of the species *Lactobacillus pentosus* (De Castro et al., 2002; Rejano et al., 2010; Ruiz-Barba and Jiménez-Díaz, 2012), although in the past this role was attributed to strains of *Lactobacillus plantarum* (Ruiz-Barba et al., 1994; Garrido Fernández et al., 1995; Rejano et al., 2010) as a consequence of previous phenotypic criteria for the classification of species into what it is known as the "*L. plantarum* group", before molecular criteria were applied (Torriani et al., 2001). At the end of the second stage, typically 10–15-day long, pH value is about 4.5 and most sugars have been utilised (Montaño et al., 1993; Garrido Fernández et al., 1995). During the final, third stage of the fermentation all fermentative substrates are exhausted and LAB population declines steadily. Values of pH below 4.0 and free acidity of 0.7–1.2%, mainly as lactic acid, are considered indicative of a good fermentation. These conditions, combined with a NaCl concentration which is at this stage usually raised to 7–8%, should guarantee the long-term preservation of the final product.

Up to date, few comprehensive studies have been carried out on the microbiota of table olive fermentations, especially if we consider modern taxonomic criteria and molecular techniques (Ercolini et al., 2006; Botta and Cocolin, 2012; Cocolin et al., 2013). The aim of this study is to update the knowledge we have about the microbial diversity, in terms of both bacteria and yeast, which is inherent to the Spanish-style fermentation of green olives in large scale table-olive manufacturing companies. For this, we have used culture-dependent techniques for the isolation of the different microorganisms as well as molecular techniques to obtain as precise identifications as possible. We have selected two different large-scale table-olive fermentation yards (known in Spanish as "*patios*"), belonging to two large table-olive manufacturing companies in the province of Seville, southern Spain. In this province, up to 63% of the Spanish national production is concentrated (season 2012/2013; AAO, 2013), so that data obtained should be quite relevant. Actually, this table olive preparation is also known as "Sevillian-style" (Rejano et al., 2010). Finally, our goal is to obtain not only a picture of the microbial diversity along the time of this food fermentation but also get a well characterised collection of microorganisms to be used in the future as a comprehensive bank of wild-type strains for diverse biotechnological uses.

2. Materials and methods

2.1. Origin of the samples and sampling strategy

Samples of Spanish-style green-olive fermenting brines were taken during the 2010–2011 season from two large (4000–8000 tonnes of olives handled per season) table-olive manufacturing companies in the province of Sevilla, south-western Spain. These companies are located *ca.* 35 Km apart from each other. At each company, fermentation was followed in ten fermenters. These were of a total capacity of 10 tonnes of olives and 5500–6000 L of brine, made in polyester and glass fibre. They were all located outdoor, buried in the ground of what it is traditionally called in Spain a "*patio*". The traditional Spanish-style procedure to prepare green olives was followed (Rejano et al., 2010). Briefly, green olives were treated with a solution of NaOH (2–2.5% [w/v]) with the addition, only in the case of *patio* #1, of NaCl (15.3 g/L) and $CaCl_2$ (0.83 g/L), for 8–10 h; the olives were then washed with water to remove the excess of alkali and finally covered with brine (10–11% [w/v] NaCl). Again, only in the case of *patio* #1, brine contained 1.87 g/L $CaCl_2$. At this point, treated olives plus brine are used to fill up the 10-tonne fermenters located in the *patios*. Only in *patio* #1, brines were acidified by the addition of 25 L of food-grade HCl. After 1–2 months of fermentation, in both *patios ca.* 500 L of the fermenting brine taken from the bottom of the fermenters, and containing olive debris and more alkaline conditions, were discarded. The fermenters were then refilled with fresh brine containing lactic acid and HCl (usually 5 L each), being this a common practice in large table-olive manufacturing companies to avoid spoilage. Olives were all of the Manzanilla variety and no starter culture was used. Fermentations were set up during September 2010 and three consecutive 50-ml samples were taken from each fermenter at approximately monthly intervals, in coincidence with the initial, middle and final stages of green olives fermentation. As the harvesting of the fruits as well as the processing capacity of these industries had an obvious daily limitation, only a limited number of fermentations could be set up daily. Therefore, at each of the three sampling dates, brine samples collected from the fermenters at each *patio* felled into a range of time after brining. More specifically, fermentation had taken place for 1–14 (first two weeks), 35–48 (5th–7th week), and 69–72 (10th–12th week) days after brining, for sampling points #1, 2 and 3, respectively. Samples were added glycerol so that final concentration was 20% (v/v) and stored at −80 °C until use.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	References
OPL5	ACGCAGGCAC	Maldonado-Barragán et al., 2013
ISS1rev	GGATCCAAGACAACGTTTCAAA	Veyrat et al., 1999
plb16	AGAGTTTGATCCTGGCTCAG	Kullen et al., 2000
mlb16	GGCTGCTGGCACGTAGTTAG	Kullen et al., 2000
paraF	GTCACAGGCATTACGAAAAC	Torriani et al., 2001
pentF	CAGTGGCGCGGTTGATATC	Torriani et al., 2001
planF	CCGTTTATGCGGAACACCTA	Torriani et al., 2001
pREV	TCGGGATTACCAAACATCAC	Torriani et al., 2001
PAR	GACGGTTAAGATTGGTGAC	Ventura et al., 2003
CAS	ACTGAAGGCGACAAGGA	Ventura et al., 2003
RHA	GCGTCAGGTTGGTGTTG	Ventura et al., 2003
CPR	CAANTGGATNGAACCTGGCTTT	Ventura et al., 2003
NL1	GCATATCAATAAGCGGAGGAAAAG	Kurtzman and Robnett, 1998
NL4	GGTCCGTGTTTCAAGACGG	Kurtzman and Robnett, 1998

2.2. Isolation of microorganisms

Aliquots of samples stored at −80 °C were defrost at room temperature, serially diluted in 0.1% (w/v) peptone water and extended in duplicates onto agar plates of culture media. Five different culture media were used in this study: Brain Heart Infusion (BHI; Biokar Diagnostics, Beauvais, France) supplemented with 0.05% L-cysteine (AppliChem, Darmstadt, Germany); de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics) supplemented with 0.02 g/L bromophenol blue (AppliChem) and L-cysteine (MRS-BPB; Lee and Lee, 2008); Reinforced Clostridial Medium (RCM; Biokar Diagnostics); and MacConkey Broth Purple (Biokar Diagnostics). Seeded plates were incubated anaerobically at 30 °C for three days, except for RCM, when seven-day incubations were used. For anaerobic incubations we used a DG250 Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK), with a gas mixture consisting of 10% H_2-10% CO_2-80% N_2. Glucose–Yeast Extract Agar supplemented with oxytetracycline (0.1 g/L) (OGYE; Mossel et al., 1962) was incubated aerobically at 30 °C for 2 days. Agar was added to the broth media at 1.5% (w/v). Prior to spreading onto RCM agar plates, samples were pasteurised at 75 °C for 15 min in a water bath. For further studies, a single colony of each different morphotype identified in each culture medium at every sampling point was selected from plates with low counts, purified by repeated subculturing and observed under a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan) to distinguish its cell