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Microbial population dynamics during the processing of *Arbequina* table olives

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ABSTRACT

Arbequina table olives are produced according to a traditional process involving an spontaneous fermentation in brine. The aim of this study was to evaluate for the first time the different microorganism populations in brine during the processing of *Arbequina* table olives.
Yeasts were the main organisms involved in fermentation but lactic acid bacteria were important when the olives were being matured before packaging. The main yeast species identified were *Candida boidinii*, *Candida diddensiae*, *Candida membranaefaciens*, *Kluyveromyces lactis*, *Pichia kluyveri*, *Pichia membranaefaciens* and *Rhodotolura glutinis*. *Lactobacillus pentosus* and *Lactobacillus paraplantarum* were the species of lactic acid bacteria involved in the process. Some of the microbial species identified in this work have not been previously reported in the fermentation processes of table olives. Moreover, no relevant differences in microbial species diversity were observed at different depths of the vat. Nevertheless, the development of lactic acid bacteria was delayed in deep brine.

1. Introduction

Table olives are one of the most important fermented vegetables in the world economy. Table olives are produced as Spanish-style green olives in brine, as naturally black olives in brine, and as ripe olives, according to well-established processes. There are other, innumerable ways of processing table olives according to traditional practices, which do not form part of the international markets but are very important at regional level (Fernández-Díez et al., 1985).

The *Arbequina* cultivar is greatly valued for its high yield. Its oil has low acidity and a fruity flavour and is originally from the village of Arbeca, near Lleida (Catalonia, Spain). *Arbequina* olive cultures are very important in the south of Catalonia but this cultivar has also recently been planted in other regions of Spain and other countries (e.g., Argentina, Tunisia, Morocco), mainly to produce oil.

Arbequina table olives are processed as 'untreated green olives' or 'naturally green olives'. This processing includes harvesting and transportation to the factory, sorting to remove damaged fruits, washing to eliminate superficial dirt and finally brining in 4–6% NaCl (Garrido-Fernández, Fernández-Díez, & Adams, 1997). *Arbequina* table olive processors use different methodologies varying salt concentration, vessel form and materials, and time of olive maturation in brine. Production methodologies are based on traditional methods and strongly influenced by local practices.

In naturally black olives, yeasts are the responsible for fermentation but in Spanish-style olives this role is played by lactic acid bacteria (LAB) (Garrido-Fernández et al., 1997). In other processing methodologies competition between yeasts and LAB has been reported (Tassou, Panagou, & Katsaboxakis, 2002). *Lactobacillus plantarum* and *Lactobacillus pentosus* are the main LAB involved in table olive fermentation although other LAB species have been isolated (Campaniello et al., 2005; Garrido-Fernández et al., 1997; Oliveira et al., 2004; Randazzo, Restuccia, Romano, & Caggia, 2002). The main yeast species identified in olive fermentations are *Candida boidinii*, *Candida diddensiae*, *Pichia anomala*, *Pichia kluyveri*, *Pichia membranaefaciens* and *Saccharomyces cerevisiae* (Arroyo-López, Durán-Quintana, Ruiz-Barba, Querol, & Garrido-Fernández, 2006; Coton, Coton, Levert, Casaregola, & Sohier, 2005; Oliveira et al., 2004).

The microbial ecosystem in brine is influenced by (a) the indigenous microbiota present in olives, (b) intrinsic factors of the fruit such as pH, water activity, diffusion of nutrients from the drupe (depending on the structure of the olive skin) and levels of antimicrobial compounds such as oleuropein, and (c) extrinsic factors such as temperature, oxygen availability and salt concentration in brine (Nychas, Panagou, Parker, Waldron, & Tassou, 2002). Traditionally, table olive industries let the process develop spontaneously without monitoring the microbiota (Spyropoulou, Chorianopoulos, Skandamis, & Nychas, 2001).

Usually the only parameter controlled by producers of *Arbequina* table olives is salt concentration, hence, the process is mainly

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spontaneous and uncontrolled. Nowadays there are neither physico-chemical nor microbiological controls to determine objectively the end of fermentation and so the producer decides, according to personal criteria, when olives are "ready to eat".

The aim of this work was to study the ecology and the population dynamics of yeast and LAB present in brine and to know if the depth of the fermentation tanks affects these dynamics. This knowledge will help to design future experiments in order to control and improve naturally fermented *Arbequina* olives processes.

2. Materials and methods

2.1. Olive brine samples

Samples were provided by the "Mas d'en Pou" olive manufacturer (Vinyols i els Arcs, Tarragona, Spain). They were collected at different depths (2 cm, 20 cm, 1 m and 2 m) from three subterranean vats, and were taken regularly every 3–4 days during the first 3.5 months. Once LAB reached counts over 10^7 ml^{-1} the samples were taken every 35–40 days. Temperature of the vessel was recorded throughout the process. The pH of samples was determined using a GLP21 pH meter (Crison, Barcelona, Spain). The samples obtained from a single industrial fermentation were taken in duplicate for each depth.

2.2. Cell viability determination

For microbial analysis, brine samples were diluted when necessary in a saline aqueous solution (0.8%, NaCl, w/v) and plated on different culture media using the Whitley Automatic Spiral Plater (DW Scientific, W. Yorkshire, UK). The selective enumeration of LAB was done in MRS (Difco, Franklin Lakes, NJ, USA) agar modified by the addition of 0.6% (w/v) D,L-malic acid, 0.5% (w/v) fructose, nystatin (100 mg/l) and sodium azide (100 mg/l) at pH 5. Yeasts were enumerated in YEPD (Difco) agar supplemented with 100 mg/l chloramphenicol. Violet Red Bile Glucose Agar (Panreac, Barcelona, Spain) was used for the enumeration of enterobacteria and Cetrimide Agar (Panreac) for pseudomonads. Rose Bengal chloramphenicol agar (Panreac) was used to evaluate the effect of moulds in the total count of yeast. MRS plates were incubated for 48 h at 27 °C under a 10% CO_2 atmosphere. The other plates were incubated at 30 °C for 48 h. The ProtoCOL SR/HR software (version 1.27.1664; Synoptics Ltd., Cambridge, UK) was used to count the spirally diluted plates.

2.3. Microbial species identification

2.3.1. Enterobacteriaceae

Ten isolated colonies were randomly picked out from the VRBG agar plates for each sample, when they were present. They were identified as enterobacteriaceae using the Api 20E kit (BioMérieux S.A., Marcy-l'Etoile, France). Complete identification of enterobacteria was achieved by use of the tests in *Bergey's Manual of Determinative Bacteriology* (Holt, Krieg, Sneath, Staley, & Williams, 1994).

2.3.2. Yeasts

Thirty isolated yeast colonies were randomly picked out from the YEPD agar plates for each sample. A small-scale DNA extraction from fresh colonies (Ruiz-Barba, Maldonado, & Jiménez-Díaz, 2005) was carried out. One microliter of the extraction was used for specific yeast PCR reaction, followed by RFLP analysis: a fragment of the 5.8S rDNA including the lateral internal transcribed spacers (ITS) was amplified by PCR and digested with the enzymes *Cfo*I, *Hae*III and *Hin*fI (New England Biolabs, Ipswich, MA, USA) as described by Esteve-Zarzoso, Belloch, Uruburu, and Querol (1999). Restriction patterns and PCR products were compared using a database (www.yeast-id.com, CSIC, Valencia, Spain).

2.3.3. Lactic acid bacteria

Thirty isolated lactic acid bacteria colonies were randomly picked out from the modified MRS agar plates for each fermentation time. Two microliters of DNA from the micro-extraction (Ruiz-Barba et al., 2005) were used to perform LAB RFLP-PCR analysis (Rodas, Ferrer, & Pardo, 2003). A fragment of the sequence of 16S rDNA was amplified and digested using *Bfa*I and *Mse*I (New England Biolabs). Formamide 1% (Sigma–Aldrich, Steinheim, Germany) was added to the PCR reaction mix to improve the specificity (Sarkar, Kapelner, & Sommer, 1990). The identification was based on the comparison of the isolate band profiles with the patterns obtained from type culture strains. This methodology did not allow us to distinguish between *L. plantarum* and *L. pentosus*. Those strains that showed these *L. plantarum–L. pentosus* profiles were confirmed with the *recA* gene multiplex PCR (Torriani, Felis, & Dellaglio, 2001).

3. Results

The production of *Arbequina* table olives is a traditional process that has not been previously studied. This work has shown how the different microbial populations succeed each other throughout the spontaneous process of fermentation and maturation of *Arbequina* olives.

3.1. Population dynamics

Enterobacteriaceae reached populations between 10^5 and 10^7 cfu/ml at the beginning of the process (first 5 days) in surface samples (Fig. 1c). In the deepest sample enterobacteriaceae were only enumerated the first day. The disappearance of the enterobacteriaceae population was parallel with pH decrease and hypoxia conditions (increasing of depth). No more enterobacteriaceae were detected except at the end of the maturation process (days 190–240), when the population was *ca.* 10^5 cfu/ml near the surface. During this period the temperature of vat increased from 17 to 24 °C. No pseudomonad colony was found during the process.

Yeasts increased rapidly on the surface and progressively extended to deeper levels contributing to a pH decrease from 5.6 to 4.5 (Fig. 1b). Their numbers were around 10^5 cfu/ml in the first 20-cm of the vat, whereas they reached this number at 1 and 2 m depth after 10 and 60 days, respectively, after the start of the fermentation. Yeasts began to decrease at 60–70 days of fermentation until day 194. The last sample was characterised by a new increase in yeast counts at all depths corresponding to an increase of temperature. During the process a few moulds appeared in YEPD and Rose Bengale plates.

First LAB colonies (*ca.* 10 cfu/ml) appeared about day 15 of fermentation in the first meter of the vat (Fig. 1a). From days 20 to 30, noticeable growth was seen to reach populations of *ca.* 10^7 cfu/ml at day 40. However, a delay on LAB development was observed from depth to surface. From days 60 to 240, populations became equal at all depths. The last sample (day 240) was characterised by a higher decrease in LAB counts. During the predominance of LAB population, the pH was stable until the end of the process, especially on deeper samples (Fig. 1d). However, on superficial brine we observed a concomitant increase of both pH and enterobacteria and yeast populations.

3.2. Enterobacteriaceae identification

Eighty percent of the isolates of enterobacteriaceae were identified as *Proteus vulgaris* and 20% as *Providencia rettgeri* using the