Food and Chemical Toxicology 115 (2018) 491–498

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Effects of selected bacterial cultures on safety and sensory traits of Nocellara Etnea olives produced at large factory scale

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ARTICLE INFO

Keywords:
Table olives
Food safety
Lactobacillus
Biogenic amines

ABSTRACT

This work investigates the effects of different combinations of selected lactic acid bacteria strains on *Lactobacillus* species occurrence, on safety and on sensory traits of natural green table olives, produced at large factory scale. Olives belonging to Nocellara Etnea cv were processed in a 6% NaCl brine and inoculated with six different bacterial cultures, using selected strains belonging to *Lactobacillus plantarum, Lactobacillus paracasei* and *Lactobacillus pentosus* species.

The fermentation process was strongly influenced by the added starters and the identification of lactic acid bacteria isolated throughout the process confirms that *L. pentosus* dominated all fermentations, followed by *L. plantarum*, whereas *L. casei* was never detected. Pathogens were never found, while histamine and tyrosine were detected in control and in two experimental samples. The samples with the lowest final pH values showed a safer profile and the most appreciated sensory traits.

The present study highlights that selected starters promote prevalence of *L. pentosus* over the autochthonous microbiota throughout the whole process of Nocellara Etnea olives.

1. Introduction

Vegetables play an important role in human nutrition and health at large, for providing minerals, micronutrients, vitamins, antioxidants, phytosterols and dietary fibre. Table olives are one of the most important components of the Mediterranean diet, with an estimated world production of 2,736,500 tons, for 2016/17 crop year, and with a world consumption forecast to increase by 5% (IOC, 2016).

The most widespread ways to produce table olives result in green or Spanish olives, black ripe or Californian-style olives, and naturally black or turning colour olives (Bautista-Gallego et al., 2010; Garrido-Fernández et al., 1997; Tassou et al., 2002).

As other Mediterranean countries, Sicily has a long tradition in table olive production, which is mainly performed at olive farming level (Randazzo et al., 2012). After picking, olives are directly placed into brine (with an initial salt concentration of 8%) and left to ferment until they lose their bitterness, at least partially (Bautista-Gallego et al., 2010; Panagou et al., 2008a). The obtained "natural green olives" are unique for their fruity aroma, colour and residual bitterness (Garrido-Fernández et al., 1997). However, fermentation is a non-controlled process, in which cultivars, physico-chemical conditions (salt content, pH, aerobic/anaerobic conditions, temperature) and autochthonous microbial population influence the quality of the final product (Tassou et al., 2002; Panagou et al., 2008a). Recent studies on natural green olives (Tofalo et al., 2012; De Angelis et al., 2015; Randazzo et al., 2017), highlighted the importance of starter cultures in reducing the fermentation time and in inhibiting the risk of pathogens growth. Up to now, no official microbiological criteria for natural table olives are currently available, although the Standard 66–1981 (Rev. 1–1987) of the Codex Alimentarius (Anonymous, 1987) recommends the minimum hygienic requirements. Furthermore, as for other fermented foods, the accumulation of biogenic amines (BA) in table olives represents a health issue. Among BAs, histamine (HI) and tyramine (TY) have been widely studied for their implication in food-borne intoxications (Leuschner et al., 2013). In 2011, the EFSA BIOHAZ Panel concluded that the BA accumulation in fermented foods is a very complex process and that it is not possible to assess a quantitative risk for HI and TY due to insufficient available information (EFSA, 2011). In order to standardize the process and to obtain replicable, high quality and safe products, suitable starter cultures, mainly constituted by LAB strains

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https://doi.org/10.1016/j.fct.2018.03.045
Received 27 November 2017; Received in revised form 8 March 2018; Accepted 30 March 2018
Available online 03 April 2018
0278-6915/

and/or mixed cultures of LABs and yeasts, were proposed.

With the aim to standardize the Nocellara Etnea table olives fermentation and to obtain a high quality and safe products, selected starter cultures, differently mixed, were inoculated into olive brines, and their effects on microbial composition and on safety and sensory traits were investigated.

2. Materials and methods

2.1. Olive samples and fermentation procedures

Nocellara Etnea olives were kindly provided by three local companies, situated in Catania region (Sicily), and processed at Oleificio Consoli (Adrano, Catania, Italy). After harvesting (September-October 2015), about 3000 kg olives were subjected to quality control to remove damaged fruits, washed with tap water and directly immersed into 200 L total capacity screw-capped PVC vessels, containing approximately 120 kg of olives and 130 L of fresh brine at 6% (w/v) of NaCl. Brines were inoculated using six selected bacterial cultures (S1-6), as previously reported (Randazzo et al., 2017). Fermentation was carried out at room temperature and followed for 180 days. The salt concentration in brine was maintained constant by weekly additions of coarse salt. Fresh brine was periodically supplied, and the fruits were kept submerged in the brine by means of a perforated cap. Twenty-one vessels were obtained, eighteen as inoculated and three as un-inoculated (control) samples.

2.2. Bacterial strains and preparation of culture inoculum

Olives brines were inoculated with six different bacterial cultures obtained by mixing three strains belonging to *Lactobacillus* genus: *Lactobacillus plantarum* UT2.1 and *Lactobacillus paracasei* N24, belonging to the microbial collection of Di3A and *Lactobacillus pentosus* TH969, belonging to microbial collection of Veneta Agricoltura - Istituto per la Qualità e le Tecnologie Agro-alimentari (Thiene, Italy). The strains were selected for growing at different NaCl concentrations (from 6.0 to 9.0% w/v), at different pH values (from 3.0 to 5.0), and at different temperatures; for β-glucosidase and antimicrobial activities; for the inability to produce HI and TY (unpublished data). The six starter cultures were obtained as follow: S1: *L. plantarum* UT2.1 + *L. paracasei* N24 + *L. pentosus* TH969 (1:1:1); S2: *L. plantarum* UT2.1; S3: *L. paracasei* N24 + *L. pentosus* TH969 (1:1); S4: *L. plantarum* UT2.1 + *L. pentosus* TH969 (1:1); S5: *L. plantarum* UT 2.1 + *L. paracasei* N24 (1:1); S6: *L. pentosus* TH969, as previously reported (Randazzo et al., 2017). In details, single strains were overnight grown at 32 °C in Man-Rogosa-Sharpe broth (MRS, Oxoid, Italy) and re-cultured in the same medium supplemented with 4.5% (w/v) of NaCl. The strains were inoculated to obtain a final concentration of 8 log colony forming units (cfu) per mL. Samples not inoculated were used as control.

2.3. Physico-chemical analyses

Brine samples were analysed for salt concentration, by titrating brine samples (5 mL), using a standardized solution of silver nitrate (0.1 N) and potassium chromate (5% w/v) as indicator (Garrido Fernández et al., 1997). The pH of brines was detected by pHmeter (H19017, Microprocessor, Hanna Instruments). All analyses were performed in duplicate.

2.4. Microbiological analysis

Brine samples (10 mL) were analysed at 1, 15, 30, 60, 90 and 180 days. At each sampling time, brines were serially diluted, using sterile quarter-strength Ringer's solution (QRS), and plated in duplicate on the following agar media and conditions: Plate Count Agar (PCA, Sigma, Milan, Italy), incubated at 30 °C for 72 h, for total mesophilic bacteria; MRS, anaerobically incubated at 32 °C for 48 h, for LAB count; Sabouraud Dextrose Agar (SDA, Oxoid, Milan, Italy) supplemented with chloramphenicol (0.05 g/L) and incubated at 25 °C for 4 days for yeast count; Violet Red Bile Glucose Agar (VRBGA, Difco, Italy), aerobically incubated at 37 °C for 24 h, for Enterobacteriaceae count; Mannitol Salt Agar (MSA, Oxoid), incubated at 32 °C for 72 h, for staphylococci enumeration. Results were expressed as $\log_{10}$ cfu/mL.

2.5. Isolation and technological characterization of LAB strains

Isolation of LAB was performed from MRS plates of brine samples taken at 15, 30, 60, 90 and 180 days of fermentation. Ten single colonies were selected from each sampling time and 346 colonies were obtained. Each colony was purified, checked for catalase activity and Gram reaction, and microscopically examined prior to be stored in liquid culture using 20% glycerol at −80 °C. Among isolates, 200 presumptive LAB were submitted to phenotypic identification. The isolates were characterized for the ability to grow at 10 and 45 °C, at pH 3 and 10, in presence of 10 and 12% NaCl. The ability to produce HI and TY was tested following the method described by Bover-Cid and Holzapfel (1999), both under aerobic and anaerobic conditions. The β-glucosidase activity was performed following the screening method reported by Rosi et al. (1994). The antagonistic activity against bacterial pathogens was evaluated according to Argyri et al. (2013), against *Escherichia coli* strain ATCC 25922, *Staphylococcus aureus* ATCC 6538, and *Listeria monocytogenes,* belonging to the microbial collection of Di3A, previously isolated from table olives (Caggia et al., 2004).

2.6. Genotypic identification of LAB isolates

In order to evaluate the occurrence of inoculated species during fermentation, 150 presumptive LAB isolates, representative of the obtained clusters, were submitted to genotypic identification. Cell cultures (1.5 mL) in the late exponential growth phase were centrifuged at 8000 rpm for 10 min and the cell pellets were washed with 1mL of TE-buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0) and re-suspended into 0.5 mL of TE-buffer. The suspension was poured in a 2-mL screw-capped tube containing 0.3g of sterile zirconium beads (diameter, 0.1 mm), homogenized in a bead-beater (Biospect Product) at 10000 rpm for 3 min and cooled on ice. The homogenate was centrifuged at 13000 rpm for 5 min and the supernatant fluid was stored at −20 °C until use. Concentration and purity of the isolated DNA were measured using a spectrophotometer (Thermo Scientific NanoDrop ND2000C). The DNA was resolved by electrophoresis on a 1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA), visualized after staining with 2μL of Gel Red Nucleic Acid Stain (Biotium, Italy) at constant voltage of 120V for 10 min.

2.6.1. PCR using Lactobacillus spp. specific primer sets

In order to identify the LAB isolates a preliminary identification at genus level was carried out using specific PCR primers, as reported by Zhang et al. (2012). The amplifications were performed in a total volume of 25 μL of mix, containing 10 μL of master mix (5prime-mastermix 2.5X), 1.5 μg of $MgCl_2$, 1.5 μg of $MgCl_2$, 1 μL of each LactoF (5′-TGGAAACAGRTGCTAATACCG -3′) and reverse primer LactoR (5'-GTCCATTGTGGAAGATTCCC-3′), 1 μL of DNA template, and filtered water. The amplification process consisted of an initial denaturation step at 94 °C for 2 min followed by 35 cycles of 1 min at 94 °C, 2 min at 55 °C and 1 min at 72 °C and a final step of extension at 72 °C for 30 min. After amplification, the PCR products were analyzed by electrophoresis in ethidium bromide-stained 1.5% agarose gels and visualized under UV light.

2.6.2. tuf gene multiplex PCR

In order to identify the isolates belonging to *L. casei* group (*L. paracasei/L. casei/L. rhamnosus*) specie-specific PCR primers were used