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Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation

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ABSTRACT

The present work presents a successful attempt to achieve an enhanced and more predictable fermentation process in Spanish-style green olive technology by selection and use of autochthonous starter cultures. During the first phase of this work, two Spanish-like fermentations of green table olives of cultivar (cv) "Nocellara del Belice", coming from irrigated and not irrigated fields, were monitored, in order to highlight the best agricultural conditions for drupe production and to isolate lactic acid bacteria strains with relevant technological properties. Among 88 identified isolates, one *Lactobacillus pentosus* strain showed remarkable biochemical features and high acidification rate in synthetic brine. In the second phase, the selected strain was used as starter culture in three different trials to establish the best conditions for its use. Microbial counting, as well as starter tracking by M13 RAPD-PCR, reflected the optimal adaptation of the strain to the environment. Spontaneous fermentation needed a 14-day long lag phase to reach the same population as the inoculated trials. Moreover, sensory traits of table olives obtained with adjunct culture showed better characteristics compared to those processed in the other trials, in particular concerning the presence of off-odours.

1. Introduction

Table olives are probably the most popular fermented vegetable in the Western world with almost two million tons produced every year, 38% of which is produced in Spain, Italy and Greece (IOOC, 2008). The most important industrial preparations are the Spanish or Sevillian preparation for green olives, the Californian preparation for black oxidized olives and the Greek preparation for naturally black olives (Garrido-Fernández et al., 1997).

Spanish-style green olive fermentation is the most economically important olive processing. Traditionally, this technique consists of a treatment with alkaline lye (1.8–2.5%, w/v NaOH) to hydrolyse the bitter glucoside oleuropein, followed by a washing step to remove the excess alkali. Brine (10–13% (w/v) NaCl) is then added and a spontaneous fermentation, carried out mainly by lactic acid bacteria (LAB), takes place (Garrido-Fernández et al., 1995). As a consequence of the alkaline treatment, during the first days, the pH values of brine are not favourable for the growth of LAB, in particular lactobacilli, whereas potential spoilage microorganisms, such as Enterobacteriaceae and butyric-acid-forming clostridia, can proliferate and spoil the product (Garrido-Fernández et al., 1995).

To improve fermentation and produce consistent and high quality final products, a process control is necessary. The main driving forces of the fermentation are the availability of fermentable substrates, salt content, pH, aerobic/anaerobic conditions and temperature control (Tassou et al., 2002).

Brine inoculation with an appropriate starter culture of LAB helps to achieve an enhanced and more predictable fermentation process. In most cases, inoculation is carried out with wild strains of LAB isolated from previous fermentations, a process known as back-slopping. However, such lactic cultures exhibit a diversity of metabolic activities, even among strains of the same species, including differences in growth rate, adaptation to a particular substrate, antimicrobial properties, flavour and quality attributes as well as competitive growth behaviour in mixed cultures (Holzapfel, 1997), resulting in non-consistent and low quality final products.

To avoid these drawbacks, modern table olive industry is directed towards the use of pure starter cultures to achieve an improved and more predictable fermentation process. The selection of starters is based on diverse criteria including homo- and

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hetero-fermentation, acid production, salt tolerance, flavour development, temperature range, oleuropein-splitting ability and bacteriocin-production Ruiz-Barba and Jiménez-Díaz, 1995; Durán Quintana et al., 1999; Delgado et al., 2005. However, the use of starter cultures is still not very common in European vegetable fermentations, although commercial preparations are already available on the market.

The present work presents a successful attempt to achieve the onset of lactic acid fermentation as soon as possible (thus reducing the likelihood of spoilage) through microbial and chemico-physical monitoring of two fermentations of green table olives of cultivar (cv) "Nocellara del Belice", technological and molecular characterization of LAB isolates and monitoring of the effects of inoculation of the selected strains on the microbiological and sensory profile of table olives in comparison with a spontaneous fermentation process.

2. Methods

2.1. Experimental plan

Green olives from the cultivar "Nocellara del Belice" were harvested when the maturity stage was suitable for processing (October 2007) and transformed according to the traditional Sevillian technology. Fruits were collected from trees trained into two different agronomic conditions: irrigation (IR) and not irrigation (NI). The orchard was located in Castelvetrano (Trapani, Sicily, Italy).

According to the Sevillian technology, olives were supplemented with lye (1.3% of NaOH) to remove bitterness and, after about 9 h, six sequential washing with complete water substitution were performed; finally vessels were filled in with concentrated brine (9% of NaCl). Each experimental production was performed in triplicate (500 kg). Room temperature was daily registered. Fermentations were carried out in a factory located at Castelvetrano (Gruppo Curaba, TP, Sicily).

2.2. Carpological and physico-chemical analysis

Drupe quality evolution was monitored by means of carpological analysis up to 120 days of fermentation (determination of fruit and pit weight, longitudinal and transversal diameter). Lactic acid fermentation was followed by monitoring pH, total titratable acidity (TTA) and soluble solid content (SSC) of brines. The olives (30 fruits randomly sampled from the whole bulk) were analyzed at 0, 1, 6, 11, 18, 25, 34, 51, 61, 68, 88, 102 and 144 days in brine. SSC was measured by using an Atago Palette PR-32 digital refractometer (Atago Co., Ltd., Tokyo, Japan). TTA was determined by titrating up to pH 8.3 with 0.2 N NaOH and expressed as percent (w/v) of lactic acid according to Sánchez et al. (2001). Values of pH and TTA were determined with a Crison compact titrator, (Crison Instruments, SA, Alella, Barcelona, Spain).

2.3. Microbiological analysis

Microbiological analysis was performed on olives and brines at 6, 11, 18, 25, 44, 61, 88 and 131 days of fermentation. Aliquots of 10 g of olives and 25 mL of the corresponding brines were diluted with 0.9% NaCl solution (315 mL), homogenized with a Stomacher Lab-Blender 400 (Seward Medical, London, United Kingdom) for 2 min, serially diluted and plated in triplicate for both microbial enumeration and isolation. Media and incubation conditions were as follows: Plate Count Agar (PCA) at 30 °C for 48 h for mesophilic bacteria as well as for spore-forming bacteria after treatment of cell suspensions at 80 °C for 10 min; M17 agar with lactose (LM17) at 37 °C for 48 h and MRS agar with 0.17 g L^{-1} of cycloheximide (Sigma) (CMRS), at 30 °C for 4 days in anaerobiosis (Anaerogen kit, Oxoid, Basingstoke, United Kingdom), for LAB; Violet Red Bile Glucose Agar (VRBGA) at 37 °C for 18–24 h for Enterobacteriaceae; Baird-Parker agar base with Egg Yolk Tellurite Emulsion (BP) at 37 °C for 48 h for staphylococci; Dichloran Rose-Bengal Chloramphenicol agar base with the addition of Chloramphenicol Selective Supplement (DRBC) at 25 °C for 3–4 days for yeasts and moulds; *Pseudomonas* agar base with CFC Supplement (PCFC) at 30 °C for 48 h for *Pseudomonas* spp. All media and the supplements used were provided by Oxoid.

2.4. Isolation and identification of microorganisms

A total of 88 colonies were randomly picked from CMRS agar plates inoculated with the highest sample dilutions to analyze LAB. Each colony was purified by repeated streaking on MRS after incubation at 30 °C for 48 h. All isolates were stored in MRS broth (Oxoid) with 20% (v/v) glycerol at −25 °C. Working cultures were prepared in 10 mL of MRS broth incubated for 24 h at 30 °C. Prior to identification, strains from CMRS agar were analyzed by rep-PCR (Repetitive-Polymerase Chain Reaction) with GTG5 (5′-GTG GTG GTG GTG GTG-3′) primer according to the procedure detailed by De Angelis et al. (2007) and RAPD-PCR (Random Amplified Polymorphic DNA) with M13 (5′-GAG GGT GGC GGT TCT-3′) primer (Huey and Hall, 1989) according to the protocol described by Rossetti and Giraffa (2005). DNA from pure cultures was extracted following a protocol developed in this study. Cells were grown in 10 mL of MRS up to the exponential phase, washed in STE (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8.00) and suspended in 250 μL of lysis solution consisting of ET (50 mM Tris–HCl, 5 mM EDTA, pH 8.00) containing 1.6 mg mL^{-1} of lysozyme, 40 U mL^{-1} of mutanolysin and 0.4 mg mL^{-1} RNase (overnight incubation at 37 °C). Then, 25 μL of 25% (v/v) sodium dodecyl sulphate and 2 μL of pronase (20 mg mL^{-1}) were added, and incubation proceeded at 37 °C for 1 h. A quantity of sodium acetate (5 M) equal to the volume of the water phase was added and, after cooling at 0 °C, DNA was precipitated with 0.7 volume of isopropanol. After centrifugation (14,000×g for 10 min at 4 °C), the pellet was washed with 70% (v/v) ethanol and left to dry. The DNA was dissolved in 50 μL of rehydratation buffer (Promega, Madison, WI, USA) (55 °C for 1 h). All reagents, where not differently detailed, were provided by Sigma. PCR products were separated by electrophoresis (2 h at 130 V) on 1.5% (w/v) agarose gel (Invitrogen, Cergy-Pontoise, France) and the DNA was detected by UV transillumination after staining with ethidium bromide (0.5 μg mL^{-1}). Gels were analyzed using the Bionumerics software, version 5.1 (Applied Maths, Kortrijk, Belgium). RAPD-PCR patterns were grouped by means of cluster analysis with the Pearson's product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA). The profiles generated by both primers were concatenated by means of Bionumerics software to obtain a single dendrogram.

The representative strains of the obtained pattern groups were identified by restriction analysis of a 499 bp DNA fragment belonging to the *hsp*60 gene according to the procedure described by Blaiotta et al. (2008) by using primers LB308F and LB806RM. Restrictions were performed by digesting 35 μL of PCR product with 20 U of *Alu*I and *Taq*I (Promega) in a total volume of 50 μL at 37 °C for 5 h. Digested fragments were separated by agarose (2% w/v) gel electrophoresis at 100 V for 3 h. Identifications were further confirmed by partial sequencing of *hsp*60 gene. The amplified fragments were excised from agarose gel 1.5% (w/v) and purified by Qiaquick Gel Extraction Kit (Qiagen, Milan, Italy) according to the supplier's instructions. DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) by using the