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Lactobacillus pentosus dominates spontaneous fermentation of Italian table olives

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ABSTRACT

Culture-dependent and -independent approaches were applied to identify the bacterial species involved in Italian table olive fermentation. Bacterial identification showed that *Lactobacillus pentosus* was the dominant species although the presence of *Lactobacillus plantarum*, *Lactobacillus casei*, *Enterococcus durans*, *Lactobacillus fermentum* and *Lactobacillus helveticus* was observed. Rep-PCR allowed to obtain strain-specific profiles and to establish a correlation with table olive environment. PCR-DGGE (Denaturing Gradient Gel Electrophoresis) confirmed the heterogeneity of bacterial community structure in fermented table olives as well as the prevalence of *L. pentosus*. The strains were characterized on the basis of technological properties (NaCl tolerance, β-glucosidase activity and the ability to grow in synthetic brine and in presence of 1 g/100 mL oleuropein). *L. pentosus* showed a high capacity of adaptation to the different conditions characterizing the olive ecosystem. This species showed the highest percentage of strains able to grow in presence of 10 g/100 mL NaCl, oleuropein and in the synthetic brine. Moreover, all the strains belonging to *L. pentosus* and *L. plantarum* species showed a β-glucosidase activity. This study allowed both to identify the main species and strains associated to Italian table olives and to obtain a lactic acid bacteria collection to apply as starter culture in the process of olive fermentation.

1. Introduction

Italy, besides Spain and Greece, has an important tradition in table olive production, but despite the economic importance of this sector table olive processing remains craft-based (Hurtado, Reguant, Bordons, & Rozès, 2012; Panagou, Schillinger, Franz, & Nychas, 2008; Randazzo, Ribbera, Pitino, Romeo, & Caggia, 2012). The final product derives from empirical manipulations of the fruits and the environmental conditions in order to achieve an attractive and durable product (Domínguez-Manzano et al., 2012). One of the most widespread method used to process table olives is the Greek-style (IOC, 2004): olives are harvested when they are fully ripe and are directly put in a brine containing 8–10 g/100 mL NaCl where they undergo to a spontaneous fermentation. Besides this method, spontaneous or "natural" fermentation is applied, at present, to different type of olives, even processing fruits at various phases of ripening. This kind of olives are characterized by a greater microbial diversity compared to treated processed olives (e.g. by the Spanish-style system) because the natural process does not include the lye treatment (Abriouel, Benomar, Lucas, & Gálvez, 2011; Hurtado et al., 2012). Also their taste is completely different from that of lye-treated fruits (Panagou, Tassou, & Katsaboxakis, 2003) mainly due to the residual bitterness they retain. In terms of nutritional characteristics, they have a higher content of polyphenols, protein and thiamin a result of which they are highly appreciated by consumers in the Mediterranean area (Panagou et al., 2003). This process is strongly influenced by the cultivar, the industrial and agricultural practices and is driven by lactic acid bacteria (LAB) and yeasts. Yeasts apart from improving the sensorial properties of the product have been shown to produce vitamins, amino acids and purines essential for *Lactobacillus* species growth (Arroyo-López, Querol, Bautista-Gallego, & Garrido-Fernández, 2008; Dominguez-Manzano et al., 2012). On the other side LAB release lactic acid which induce a decrease of the pH which inhibits the growth of pathogens and spoilage microorganisms (Hurtado et al., 2012).

Despite the fact that olive industry is perceived to be highly traditional there is a growing interest in the use of selected LAB strains as starters for olive production. An ideal starter culture for table olive fermentation should show conventional characteristics

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(homofermentative metabolism, high acidification rate and fast consumption of fermentable substrates, flavour development, oleuropein-splitting capability, minimum nutritional requirements and a fast and predominant growth under fermentation) (Corsetti, Perpetuini, Schirone, Tofalo, & Suzzi, 2012; Delgado, Brito, Peres, Arroyo-Lopez, & Garrido-Fernández, 2005; Hurtado et al., 2012), but also probiotic traits since consumers are looking for healthier and safer foods (Lavermicocca et al., 2005; Silva et al., 2011; Sisto & Lavermicocca, 2012). In this context the isolation and identification of LAB species that dominate the microbiota of table olives are one of the key points to be addressed for the development of new starter cultures so, several molecular methods, besides culture-dependent and -independent approaches, have recently been applied to table olives for more rapid and reliable identification of LAB (Botta & Cocolin, 2012).

The aim of the present work was to assess bacterial composition and diversity of six naturally fermented Italian table olive cultivars through traditional techniques and PCR-DGGE to better understand the species and the strains involved in spontaneous olive fermentation. In addition, all the LAB isolates were subjected to a technological characterization in order to select LAB with interesting properties to be used as starter cultures.

2. Materials and methods

2.1. Sample origin

Six different Italian cultivar of table olives (Itrana bianca, Itrana nera, Peranzana, Nocellara del Belice, Cellina di Nardò and Bella di Cerignola) obtained from six traditional producers located in three Italian regions (Apulia, Sicily and Lazio) were characterized from a microbiological and physico-chemical point of view in a previous study. In particular, each factory sent two samples. All table olive analyzed samples were naturally fermented and collected at the end of the fermentation process, when they were ready for the market (Tofalo, Schirone, Perpetuini, Angelozzi, et al., 2012).

2.2. Lactic acid bacteria isolation

Brine samples (25 mL) were diluted in a solution of NaCl 0.85 g/100 mL, serially diluted and plated on MRS (Oxoid, Milan, Italy) plates supplemented with cycloheximide (Sigma–Aldrich Srl, Milan, Italy) 100 mg/L, for LAB isolation. Plates were incubated under microaerophilic conditions at 30 °C for 48–72 h. Colonies were randomly collected from the plates at the highest dilution in order to increase the probability to pick up strains belonging to the dominant species. The random colony selection from the highest dilution plates allowed us to collect the most frequent and predominant species present in each sample, as reported by Tofalo et al. (2009), Pulvirenti, Solieri, Gullo, De Vero, and Giudici (2004) and Solieri, Landi, De Vero, and Giudici (2006). All the isolates were stored in liquid cultures with 20 mL/100 mL glycerol (Sigma–Aldrich Srl, Milan, Italy) at −80 °C and belong to the Culture Collection of the Faculty of BioScience and Technology for Food, Agriculture and Environment (University of Teramo).

2.3. LAB identification

Gram positive, catalase negative, rod and coccal-shaped bacteria were identified by a polyphasic approach. In particular, phenotypic identification was carried out using the API 50 CH test strips for rod shaped isolates and rapid ID 32 STREP test for the coccal-shaped isolates. The data were elaborated by the BioMerieux software (BioMerieux, Nurtingen, Germany).

For the genotypic identification DNA was isolated according to de Los Reyes Gavilán, Limsowtin, Tailliez, Séchaud, and Accolas (1992) from 2 mL samples of overnight cultures grown in MRS at 30 °C. A *rec*A gene multiplex PCR assay was performed for the identification of *Lactobacillus paraplantarum*, *Lactobacillus plantarum* and *Lactobacillus pentosus*. Amplification was carried out in a thermocycler (MyCycler, Bio-Rad Laboratories, Milan, Italy) as described by Torriani, Felis, and Dellaglio (2001) and Bringel et al. (2005). PCR products were separated in 2 g/100 mL agarose gels in 1× TAE (0.04 mol/L Tris–acetate, 0.001 mol/L EDTA, pH 8.2) buffer and the fragment sizes were evaluated by comparison with the 1 kb DNA Plus Ladder (Invitrogen, Milan, Italy). Gels were stained with ethidium bromide 0.5 μg/mL, and documented by the Gel Doc 2000 EQ System (Bio-Rad). All isolates in which the amplification failed were selected for 16S rRNA sequence analysis. Amplification was carried out according to Bringel et al. (2005), using Lac16S-for (5′-AATGAGAGTTTGATCCTGGCT-3′) and Lac16S-rev (5′-GAGGTGATCCAGCCGCAGGTT-3′) primers. The amplified fragment was then purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences AB, Uppsala, Sweden), according to the manufacturer's instructions and after drying was delivered to BMR Genomics (Padua University, Padua, Italy) for sequencing. The obtained sequences were compared to those available in the GenBank database (http://www.ncbi.nml.nih.gov/BLAST) and those of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) to determine the closest known relative species on the basis of 16S rRNA gene homology.

2.4. $(GTG)_5$ rep-PCR typing

Fingerprints of genomic DNA were obtained amplifying repetitive bacterial DNA elements (rep-PCR). PCR amplification was carried out using $(GTG)_5$ primer as described by Gevers, Huys, and Swings (2001). Amplification was performed on a MyCycler (Bio-Rad) with an initial denaturation at 94 °C for 5 min followed by 30 cycles consisting of 1 min at 94 °C, 1 min at 40 °C, 8 min at 72 °C and a final extension of 15 min at 72 °C. PCR products were separated on a 1.5 g/100 mL agarose gel in 1× TAE buffer. 1 kb Plus DNA (Invitrogen) was used as a marker. After electrophoresis, the gels were stained and photographed under UV transillumination as reported above. The repeatability of rep-PCR fingerprints was determined by triplicate loading of independent triplicate reaction mixtures prepared with the same strain. Conversion, normalization, and further analysis of the rep-PCR patterns were carried out with Fingerprinting II Informatix™ software program. Similarities among profiles were calculated by clustering the Pearson's correlation matrix using the Unweighted Pair-Group Method with Average (UPGMA) algorithm.

2.5. PCR-DGGE analysis

For the PCR-DGGE analyses, DNA was extracted directly from olive brine using the Power Soil DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, CA, USA). Ten mL of each brine sample, in duplicate, was centrifuged to collect more cells. The DNA was then extracted according to manufacturer's instructions with some modifications as reported by Tofalo, Schirone, Perpetuini, Suzzi, and Corsetti (2012).

A nested PCR was performed using Lac16S-for and Lac16S-rev, as previously described. Then PCR product was used to amplify the V3 region using primers HDA1-GC (5′-(*GC*) AC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) according to Ogier, Son, Gruss, Tailliez, and Delacroix-Buchet (2002) with some modifications as reported below. PCR products were separated by DGGE by using the D-code apparatus (Bio-Rad).