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Research note

Molecular characterization of lactic acid bacteria isolated from industrially fermented Greek table olives

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ABSTRACT

A total of 145 lactic acid bacteria (LAB) isolates have been recovered from fermented table olives and brine and characterized at strain level with molecular tools. Pulsed-Field Gel Electrophoresis (PFGE) of *Apa*I macrorestriction fragments was applied for strain differentiation. Species differentiation was based either on Denaturing Gradient Gel Electrophoresis (PCR-DGGE) (black olives) or on restriction analysis of the amplified 16S rRNA gene (PCR-ARDRA) (brine and green olives). Species identification was based on sequence analysis of 16S rRNA gene. When the data were insufficient to resolve the species level of the isolates, specific multiplex PCR assays targeting the *rec*A or *tuf* genes were employed. From 145 LAB isolates, 71 different strains were recovered from fermented olive and brine samples; 17 strains were assigned to *Leuconostoc mesenteroides*, 51 were grouped in *Lactobacillus plantarum* group (including 13 *L. plantarum*, 37 *Lactobacillus pentosus*, 1 *Lactobacillus paraplantarum*), 2 *Lactobacillus paracasei* subsp. *paracasei* and 1 *Leuconostoc pseudomesenteroides*. *L. plantarum* was recovered mainly from green olive fermentation, whereas in black olives the main species identified were *L. pentosus* and *Ln. mesenteroides*). These observations reveal that olives are a highly diverse ecosystem regarding the presence of LAB, which may affect the quality of the final fermented product.

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1. Introduction

The cultivation of table olives is widespread throughout the Mediterranean basin and holds a major role not only through its contribution to rural economy but also from the cultural and environmental point of view. The estimated world production of table olives for the period 2011/12 amounted to 2.6 million tonnes approximately and has shown an increasing trend over the last years (IOC, 2012). Today, the market offers a wide variety of table olive elaborations that are able to satisfy all consumer tastes, even the most sophisticated ones. However, there are three basic commercial preparations of table olives that have attracted the interest of the scientific community, namely Spanish style green olives, naturally black olives (Greek style) and black ripe olives (Californian style) for which processing conditions are well established in the literature (Sánchez Gómez, García García, & Rejano Navarro, 2006). A basic step in table olive processing, especially for Spanish style green olives and natural black olives, is fermentation. This process is carried out primarily by the spontaneous microbiota of lactic acid bacteria encountered on olive drupes and in the brine environment aiming at the biotransformation of fermentable substrates (mainly reducing sugars) in olives to organic acids (primarily lactic acid and acetic acid) that are released into the fermentation brine. The net effect is an accumulation of acids and a lowering of brine pH which in combination with the salt content of brine ensures the microbiological safety of the product and at the same time provides the desirable sensory attributes of the final product (Garrido Fernández, Fernández Díez, & Adams, 1997). Moreover, LAB produce small amounts of ethanol and other volatile compounds that make a significant contribution to the final flavor of table olives (for a review on lactic acid bacteria and table olive fermentation see Hurtado, Reguant, Bordons, & Rozès, 2012). It is generally accepted that the table olive industry is highly traditional and even in our days table olive processing remains craft and empirical despite its economic importance. However, new identification and typing techniques for LAB have been continuously developed and implemented to identify the ecological diversity of this microbial group in table olive processing using both culture dependent and independent techniques (Abriouel, Benomar, Lucas, & Gálvez, 2011; Ercolini, Villani, Aponte,

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& Mauriello, 2006; Hurtado et al., 2011; Hurtado, Reguant, Esteve Zarzoso, Bordons, & Rozès, 2008; Panagou, Schillinger, Franz, & Nychas, 2008; Randazzo, Restuccia, Daniele Romano, & Caggia, 2004).

The aim of the present research note is to report on the characterization of LAB isolates from industrially fermented Spanish-style green olives and natural black olives in Greece using molecular identification techniques. Until now such information came only from small-scale experimental fermentations and there was a lack of LAB characterization from industrial installations. The results obtained in this work will be employed in further research focusing on the investigation of the technological properties of the isolated strains (e.g. probiotic potential, bacteriocin production) for the selection of potential starter cultures with improved characteristics in table olive fermentation.

2. Materials and methods

2.1. Isolation of LAB

Brine and olive samples from Spanish-style green olives of cv. Conservolea and Halkidiki, as well as natural black olives from cv. Conservolea and Kalamata were collected from six processing plants in different regions of Greece (Peloponnese, Sterea Ellada). Olive samples were taken directly from fermentation vessels at the end of fermentation process and transported to the laboratory with minimum delay for further analysis.

Olive samples (25 g) and brine (1 ml) were aseptically transferred to 225 ml or 9 ml of sterile quarter strength Ringer's solution, respectively. Decimal dilutions were prepared in the same Ringer's solution and duplicate 1 ml samples of the appropriate dilutions were mixed on de Man–Rogosa–Sharp medium (MRS; Merck 1.10660, Darmstadt, Germany), overlaid with 10 ml of the same medium and incubated at 25 °C for 48–72 h, to quantify the population of LAB. All plates were examined visually for typical colony types and morphological characteristics that were associated with the growth medium. Moreover, the selectivity of the MRS medium was routinely checked by Gram staining and microscopic examination of smears prepared from randomly selected colonies.

LAB were isolated from the highest dilution of MRS growth medium. From each of the aforementioned samplings 10% of the colonies (i.e., 10–15 colonies) were randomly selected and purified. Pure cultures were stored at −80 °C in MRS broth medium supplemented with 20% (v/v) glycerol (Serva, Heidelberg, Germany). Before experimental use each isolate was subcultured twice, while the purity of the culture was always checked. A total of 145 pure cultures were finally picked from both olives and brine and subjected to molecular analysis.

2.2. Pulsed Field Gel Electrophoresis (PFGE)

Pulsed Field Gel Electrophoresis was performed in order to determine the LAB heterogeneity at strain level. In brief, genomic DNA extraction was performed from all isolates as reported elsewhere (Doulgeraki, Paramithiotis, Kagkli, & Nychas, 2010). The restriction enzyme *Apa*I (10U) (New England Biolabs, Ipswich, MA, USA) was applied according to the recommendations of the manufacturer. Restriction fragments were separated in 1% PFGE grade agarose gel in 0.5 mM Tris–Borate buffer on CHEF-DRII (Bio-Rad, Hercules, CA, USA) equipment with the following running parameters: 6 V cm^{-1}, 1 s initial switching time, 10 s final switching time, and 16 h of total run at 14 °C. Gels were then stained with ethidium bromide (0.5 μg mL^{-1}) in water for 1 h and distained for 2 h before being photographed using a GelDoc system. Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Bionumerics software, version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

2.3. Identification and characterization of strains

Following PFGE differentiation, the different isolates were subjected to PCR-DGGE –(33 isolates from black olives) (Cocolin et al., 2004) and PCR-ARDRA (38 isolates from brine and green olives) (Rodas, Ferrer, & Pardo, 2003), respectively for species differentiation. The identification of a representative number of the different patterns detected in the later methods was achieved by sequence analysis of V1–V6 region of 16S rRNA gene (Doulgeraki et al., 2010). In the case where the acquired data were insufficient to resolve the species level of the bacteria isolates, for the differentiation of *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* or *Lactobacillus casei* and *Lactobacillus paracasei*, specific multiplex PCR assays targeting the *rec*A gene (Torriani, Felis, & Dellaglio, 2001) or *tuf* gene (Ventura, Canchaya, Meylan, Klaenhammer, & Zink, 2003) were employed. In all cases, DNA was extracted according to Doulgeraki, Paramithiotis, and Nychas (2011). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are JX129193 to JX129206.

3. Results and discussion

The microbiological analysis of olive samples resulted in average LAB populations of 6.03 (±1.03) and 5.73 (±0.35) log cfu g^{-1} for black and green olives, respectively, whereas in the brines the respective average values were 6.15 (±0.57) and 6.48 (±0.83) log cfu ml^{-1}. The application of PFGE-*Apa*I macrorestriction analysis to a total of 145 isolates resulted in 71 different fingerprints (Fig. 1). In total 33 (black olives) and 38 (brine and green olives) different fingerprints were subjected to PCR-DGGE and PCR-ARDRA which resulted in 3 and 2 different patterns, respectively. The results from sequence analysis of a representative number of isolates revealed that the three different patterns detected in PCR-DGGE were assigned to *Leuconostoc mesenteroides* (17 fingerprints), *Leuconostoc pseudomesenteroides* (1 fingerprint) and *L. plantarum* group (15 fingerprints). Additionally, the two patterns detected in PCR-ARDRA were assigned to *L. plantarum* group (36 fingerprints) and *L. casei* group (2 fingerprints). For the differentiation of isolates assigned to *L. plantarum* group and *L. casei* group, multiplex PCR assays targeting the *rec*A and *tuf* genes, respectively were employed, resulting in 13 *L. plantarum,* 37 *L. pentosus,* 1 *L. paraplantarum* and 2 *L. paracasei* subsp. *paracasei*.

Table 1 summarizes the prevalence of the different genera detected in the different samples. The aforementioned species have been previously associated with the microbiota of spontaneous fermentation of table olives. More accurately, *L. plantarum* and *L. pentosus* have been reported as the predominant species in the fermentation of table olives (Abriouel et al., 2011; Asehraou, Peres, Faid, & Brito, 2002; Chamkha, Sayadia, Brub, & Godon, 2008; De Bellis, Valerio, Sisto, Lonigro, & Lavermicocca, 2010; Hurtado et al., 2008; Mourad & Nour-Eddine, 2006; Ruiz-Barba & Jiménez-Díaz, 1995). It has to be noted that *L. pentosus* was the most common species detected with a great diversity at strain level. Similarly to these results, *L. pentosus* has been reported as the most frequently isolated species and found to show a high strain diversity throughout fermentation of green olives (De Bellis et al., 2010), black cv. Conservolea olives (Doulgeraki, Hondrodimou, Iliopoulos, & Panagou, 2012) and brine of green olives (Hurtado et al., 2008). It needs to be noted that *L. plantarum* was considered to be the dominant LAB species in table olive fermentation (Garrido Fernández et al., 1997). However, when *L. pentosus* was first described as a new species (Zanoni, Farrow, Phillipps, & Collins,

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