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Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives

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ABSTRACT

The performance of two strains of lactic acid bacteria (LAB), namely *Lactobacillus pentosus* B281 and *Lactobacillus plantarum* B282, previously isolated from industrially fermented table olives and screened *in vitro* for probiotic potential, was investigated as starter cultures in Spanish style fermentation of cv. Halkidiki green olives. Fermentation was undertaken at room temperature in two different initial salt concentrations (8% and 10%, w/v, NaCl) in the brines. The strains were inoculated as single and combined cultures and the dynamics of their population on the surface of olives was monitored for a period of 114 days. The survival of inoculated strains on olives was determined using Pulsed Field Gel Electrophoresis (PFGE). Both probiotic strains successfully colonized the olive surface at populations ranged from 6.0 to 7.0 log CFU/g throughout fermentation. PFGE analysis revealed that *L. pentosus* B281 presented higher colonization in both salt levels at the end of fermentation (81.2% and 93.3% in 8% and 10% NaCl brines, respectively). For *L. plantarum* B282 a high survival rate (83.3%) was observed in 8% NaCl brines, but in 10% NaCl the strain could not colonize the surface of olives. *L. pentosus* B281 also dominated over *L. plantarum* B282 in inoculated fermentations when the two strains were used as combined culture. The biochemical profile (pH, organic acids, volatile compounds) attained during fermentation and the sensory analysis of the final product indicated a typical lactic acid fermentation process of green olives.

1. Introduction

Table olives are one of the major agricultural products that are consumed fermented. The primary purpose of table olive fermentation is to achieve a preservation effect and enhance the sensory attributes of the processed product (Sánchez Gómez et al., 2006). Diverse microbial groups are involved in olive fermentation determining the quality and sensory properties of the final product but it is generally accepted that LAB and yeasts are the most relevant microorganisms dominating the process (Arroyo-López et al., 2008; Hurtado et al., 2012). LAB influence fermentation in a variety of ways, the most important being the production of lactic acid from fermentable substrates resulting in pH decrease with a concurrent increase in acidity, ensuring thus the microbiological stability during storage even at ambient temperature for extended periods of time. Today, pure starter cultures of LAB are available in the market and used in several vegetable fermentations (Leroy and De Vuyst, 2004; Di Cagno et al., 2013) but their use in table olive processing is still limited. For the selection of LAB as starter cultures in table olive processing certain technological properties must be assured including fast and predominant growth, antimicrobial activity, high acidification rate and fast consumption of fermentable substrates, utilization of non-digestible a-galactosidase sugars, tolerance to bile salts and acidic pH, screening of enzymes with biotechnological potential (Abriouel et al., 2012).

Probiotic food products are in general fermented foods containing an amount of viable and active microorganisms large enough to reach the intestine and exert an equilibrating action on the intestinal microflora (FAO/WHO, 2002). Intake of probiotics is considered to stimulate the growth of beneficial microorganisms, reduce the amount of pathogens and help boost the immune system, lowering thus the risk of gastro-intestinal diseases (Cross, 2002; Reid, 2008).

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Throughout the past two decades, probiotic health-promoting microorganisms have been increasingly included into commercial products in a response to the consumer demand for healthy foods that improve overall health, intestinal function, and digestion (Menrad, 2003). Most of these microorganisms are LAB and, among them, lactobacilli represent one of the fundamental microbial groups which have been introduced in a wide range of food products. Until recently, probiotic foods have been restricted almost exclusively to dairy products (Saad et al., 2013). However, these foods cannot be consumed by certain groups of the population who suffer from lactose intolerance or need a diet based on non-milk derived products. Thus, in the last years, fermented foods of plant origin have been increasingly considered as vectors for incorporation of probiotic cultures following the well-established procedure of vegetable fermentation (Gupta and Abu-Ghannam, 2012). In this way, fruits and vegetables already containing high levels of beneficial substances (e.g., antioxidants, vitamins, dietary fibres, minerals) can be reinforced with probiotic bacteria that can bring about additional health promoting features (Soccol et al., 2010; Peres et al., 2012). Recent research has focused on the exploitation of the microstructure of the olive surface as a carrier of probiotic strains of LAB confirming for the first time the suitability of the olive surface for this purpose (Lavermicocca et al., 2005; Saravanos et al., 2008; De Bellis et al., 2010; Arroyo-López et al., 2012; Rodriguez-Gómez et al., 2013). A probiotic potential is expected to greatly enhance the already important nutritional value of table olives and convey a favourable economic impact, especially knowing that such products originate in the less developed regions of the EU. However, depending on the geographical location, the cultivar, and the olive production process followed in each country, different LAB strains with diverse technological traits may prevail in the process and can be thus used as starter cultures. It is thus necessary to provide more evidence about the suitability of these strains to dominate the process and survive in high numbers in the final product.

The purpose of the current study was to assess the performance of two *Lactobacillus* strains, namely *Lactobacillus pentosus* B281 and *Lactobacillus plantarum* B282 as starter cultures in Spanish-style green olive fermentation in terms of microbiological and biochemical profiles attained during the process. Both strains have been previously isolated from naturally fermented olives (Doulgeraki et al., 2013) and selected for their *in vitro* probiotic potential (Argyri et al., 2013). The survival of the inoculated strains during the course of fermentation was determined using molecular techniques. In addition, two initial salt levels in the brines were used in an attempt to assess the starter performance at lower salt levels than the ordinary used by the table olive industry today in order to produce a low salt probiotic final product. Finally, the organoleptic attributes of fermented olives were assessed by a trained sensory panel to ensure the acceptability of the final product.

2. Materials and methods

2.1. Olive cultivar and treatment

Green olives cv. Halkidiki were harvested in mid-September (season 2010–2011), subjected to quality control at the processor's installations to remove defective drupes and size graded to an average size of 111–120 fruits/kg. The olives were kindly provided by Konstantopoulos S.A., a table olive industry located in Northern Greece and processed in our laboratory according to the Spanish style method employed by the industry. A total amount of 110 kg of olives were initially subjected to a washing step with tap water to remove any impurities and subsequently immersed in a 1.9% (w/v) NaOH solution for 10–12 h at room temperature (20–22 °C) until the alkali penetrated approximately 2/3 of the flesh as measured from the epidermis to the pit. Debittering took place in the same vessels used for fermentation (see Section 2.3) using 4.6 L of water and 87.4 g NaOH/vessel. A washing step was followed, replacing the NaOH solution with tap water. The process included two water changes at 4 and 8 h to remove the residual lye from the olive flesh.

2.2. Bacterial strains, preparation of inocula and inoculation

Two strains of LAB, namely *L. pentosus* B281 and *L. plantarum* B282, isolated previously from industrially fermented olives (Doulgeraki et al., 2013) and characterized for their *in vitro* probiotic potential (Argyri et al., 2013) were employed in the fermentations. Stock cultures were maintained in vials of treated beads in a cryoprotective fluid (Protect Bacterial Preservers, Lancashire, UK) at −80 °C until use. The cultures were revived by adding one bead of the frozen culture of each strain in 10 mL MRS broth medium supplemented with 4.5% (w/v) NaCl to allow adaptation of starter cultures to the saline environment of the brine and incubated at 30 °C for 24 h. Working cultures were prepared by adding 50 μL of each strain into 50 mL MRS broth supplemented with 4.5% (w/v) NaCl and incubated at 30 °C for 24 h (*ca.* 9.0–9.5 log CFU/mL). Bacterial cells were centrifuged twice at 5.000 *g* for 15 min at 4 °C using a Heraeus Multifuge 1S-R centrifuge (Thermo Electron Corporation, Langenselbold, Germany), and the pellet was resuspended in 2.5 and 8.0 mL sterile ¼ Ringer's solution for *L. pentosus* B281 and *L. plantarum* B282, respectively to a final concentration of *ca.* 10.0 log CFU/mL. A 23-mL aliquot of the working culture was added in the fermentation vessels after 24 h of brining to achieve an initial population of *ca.* 7.0–8.0 log CFU/mL in the brine. In the case of combined cultures, equal volumes of each strain were mixed together and 23 mL of the resulting cocktail were added in the vessels. The cell concentration of the composite inocula in the brine was also *ca.* 7.0–8.0 log CFU/mL as in the case of monoculture.

2.3. Fermentation procedures

Fermentation was undertaken in 14 L total capacity screw-capped plastic vessels containing 6.8 kg of olives and 4.6 L of freshly prepared brine (brine/olive ratio: 1.48/1). Two different initial salt concentrations were prepared namely, 8% and 10% (w/v) in order to evaluate the performance of the inoculated starter cultures at a lower salt level than those routinely employed by the Greek table olive industry today. At the onset of fermentation the brines were acidified with 0.1% (v/v) lactic acid (95%, Sigma) and 0.014% HCl following the industrial practice. Overall, eight initial fermentation processes were investigated namely, (i) spontaneous fermentation (control) in 8 and 10% salt brines, (ii) inoculated fermentation with *L. pentosus* B281 in 8 and 10% salt brines, (iii) inoculated fermentation with *L. plantarum* B282 in 8 and 10% salt brines, and (iv) combined inoculum of the two strains in 8 and 10% salt brines. All treatments were performed in duplicate (i.e., two fermentation vessels per treatment). Fermentation took place at room temperature (*ca.* 20–22 °C) for a period of 114 days.

2.4. Microbiological analysis

Olive samples were analysed after 24 h of brining and at regular time intervals during the experiment (22 sampling points per treatment). Olives were recovered from the brine, the pit was aseptically removed with the aid of a sterile blade and olive flesh (25 g) was aseptically cut, added in 225 mL sterile ¼ Ringer's solution and homogenized in a stomacher (LabBlender, Seward Medical, London, UK) for 60 s at room temperature. The resulting suspension was serially diluted in the same diluent and 1 or 0.1 mL samples of the appropriate dilutions were mixed or spread on the