International Journal of Food Microbiology 171 (2014) 68–76

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Performance of two potential probiotic *Lactobacillus* strains from the olive microbiota as starters in the fermentation of heat shocked green olives

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ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form 2 October 2013
Accepted 1 November 2013
Available online 16 November 2013

Keywords:
Green olives
Olive fermentation
Probiotic bacteria
L. plantarum
L. pentosus

ABSTRACT

The performance of two potential probiotic *Lactobacillus* strains from olive microbiota, namely *L. pentosus* B281 and *L. plantarum* B282 was assessed as starter cultures in Spanish-style fermentation of heat shocked green olives cv. Halkidiki. Two different initial salt levels were studied, 10% (w/v) and 8% (w/v) NaCl, and the brines were inoculated with (a) *L. pentosus* B281, (b) *L. plantarum* B282, and (c) a mixture of both strains. A spontaneous fermentation was also taken into account as control treatment. Prior to brining, olives were heat shocked at 80 °C for 10 min to reduce the level of the indigenous microbiota on olive drupes and facilitate the dominance of the inoculated cultures. Microbiological, physicochemical and sensory analyses were conducted throughout fermentation. The composition of LAB population and the evolution of added inocula were assessed by Pulsed Field Gel Electrophoresis (PFGE). The final population of LAB was maintained above 6 log cycles in olive flesh. Both *L. pentosus* B281 and *L. plantarum* B282 were able to dominate over indigenous LAB, albeit strain B281 exhibited higher recovery percentages (100 or 94.7% for B281 and 58.8% or 55.0% for B282 in 10% or 8% NaCl, respectively). *L. pentosus* B281 also dominated over *L. plantarum* B282, when the two strains were co-inoculated in olive fermentations. The sensory assessment showed higher preference for inoculated fermentations of *L. pentosus* and *L. plantarum* separately in 8% NaCl, followed by the *L. plantarum* in 10% NaCl. The present study showed that probiotic strains *L. pentosus* B281 and *L. plantarum* B282, may offer a great potential for use as functional starter cultures in olive fermentation and deliver a promising probiotic food to the consumer.

1. Introduction

Fermentation is one of the oldest food-processing/preservation technologies known to mankind which is considered as an important determining factor to control microbial growth, improve digestibility and nutritional value of food, and enhance food safety (Nout and Rombouts, 1992; Tassou et al., 2010). The most important industrial preparations are the Spanish or Sevillian style for green olives, the Californian style for black oxidized olives and the Greek style for naturally black olives (Sánchez Gómez et al., 2006). Spanish-style green olive fermentation is the most economically important olive processing (Aponte et al., 2012). Traditionally, this technique consists of a treatment with alkaline lye to hydrolyse the bitter glucoside oleuropein, followed by a washing step to remove the excess alkali. Brine is then added and a spontaneous fermentation, carried out mainly by lactic acid bacteria (LAB), takes place (Garrido Fernandez et al., 1995).

However, the indigenous microbiota of the fruits varies as a function of the quality of the raw material, harvesting conditions and post-harvest treatments and may thus lead to variations in the sensory and organoleptic characteristics of the final product (Garrido Fernandez et al., 1997). Inoculation of the brine with an appropriate starter culture of LAB reduces the probability of spoilage and helps to achieve an improved and more predictable fermentation process (Panagou and Tassou, 2006). The preparation of improved commercial starter cultures specifically for table olives has been reported previously (Roig and Hernandez, 1991) with main focus on Spanish-style processing (Panagou et al., 2008). Depending on the geographical location or the olive production process, different LAB strains can be used as starter cultures. However, the majority of LAB preparations consist of *Lactobacillus plantarum*, *Lactobacillus pentosus* or both (Hurtado et al., 2010).

Moreover, a heat shock treatment that has been used in some studies prior to the brining has been reported to improve olive fermentability without modifying the course of fermentation (Balatsouras et al., 1983; Chorianopoulos et al., 2005; Etchells et al., 1966). This has been attributed to the partial eradication of the undesirable microbiota from the surface of heat shocked olives and the increased permeability of the plasma membrane of the fruit tissue cells (Balatsouras et al., 1983).

Among the traditional fermented foods, table olives could be a promising probiotic food through the use of functional probiotic starter

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.11.003

cultures. Functional starter cultures contribute to microbial safety and offer organoleptic, technological, nutritional or health advantages. In contrast to well-adapted industrial starters, wild-type strains that naturally dominate traditional fermentations tend to have higher metabolic capacities, which can beneficially affect product quality, for instance with regard to aroma formation and/or food safety. Natural selection is likely to have forced such strains to be more competitive by endowing them with ecological advantages (Ayad et al., 2002; Maldonado et al., 2002). A noteworthy attempt was made by Lavermicocca et al. (2005), to use table olives as a vehicle to incorporate probiotic bacterial species, namely *L. rhamnosus*, *L. paracasei*, *Bifidobacterium bifidum*, and *B. longum*, by inoculating them in the brines of fermented olives and investigating their survival for a period of 3 months. However, the first reported studies on olives fermented with probiotic starter cultures were performed by Saravanos et al. (2008) and De Bellis et al. (2010), where probiotic LAB of dairy origin (*L. plantarum* ACA-DC 146 and *L. paracasei* ACA-DC 4037) or a probiotic *L. paracasei* IMPC2.1 strain isolated from human intestine were used. Recent studies show that wild LAB strains originating from olives and retain probiotic potential may be also used as starters in olive fermentation (Argyri et al., 2013; Arroyo-López et al., 2012; Bautista-Gallego et al., 2013). Thus, higher survival of the inoculated strains due to their ecological advantages for natural dominance can be provided given the required final population needed (10^6–10^7 CFU/g) (Boylston et al., 2004; Oliveira et al., 2001) and provide a final product with acceptable organoleptic characteristics.

The purpose of this work was to assess the performance of two *Lactobacillus* strains, namely *L. pentosus* B281 and *L. plantarum* B282, as monocultures or co-cultures, previously found to possess potential probiotic activity in vitro (Argyri et al., 2013), as starter cultures in Spanish-style fermentation of cv. Halkidiki green olives. Prior to brining olives were heat shocked at 80 °C for 10 min to reduce most of the competitive microbiota and enhance fermentability (Etchells et al., 1966; Balatsouras et al., 1983). Two initial salt levels were used in an attempt to assess the starter performance at lower salt levels than the ordinary used by the table olive industry today. Their successful use as starters will lead to their testing in olive fermentations without the heat treatment. On the other hand the industries that usually pasteurize the final product, could use the heat treatment before the probiotic starter inoculation since the probiotic olives should not be pasteurized.

2. Materials and methods

2.1. Olive treatment and fermentation procedures

2.1.1. Olive treatment

Green olives cv. Halkidiki were harvested in mid-September and transported to the laboratory within 24 h. A total of 50 kg of fruits were initially subjected to a washing step with tap water to remove any impurities and subsequently immersed in a water bath applying a heat shock for 10 min at 80 °C. The purpose of the heat shock treatment was to reduce the level of the indigenous microbiota on olive drupes, facilitate the dominance of the inoculated cultures, and enhance the fermentability of the olives. Subsequently, the fruits were debittered using 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. 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. Moreover, the above procedure was followed without the heat shock step, in order to determine the microbial load of olives without this specific treatment at the time of the immersion of olives in brines.

2.1.2. Bacterial strains and inoculum preparation

Two strains of lactic acid bacteria previously characterized for their in vitro probiotic potential (Argyri et al., 2013), were employed in the fermentations, namely *L. pentosus* B281 and *L. plantarum* B282. The strains were activated from a stock culture stored at −80 °C in MRS broth, and subcultured into 10 ml MRS broth supplied with 4.5% (w/v) NaCl to allow adaptation of starter cultures to the saline environment of the brine. Cells were then harvested by centrifugation (5000 *g*, 10 min, 4 °C) and washed twice with sterile 10 ml Ringer solution (BR0052G, Oxoid). An appropriate inoculum was added in the fermentation vessels after 24 h of brining to achieve an initial population of approximately 7–8 log CFU/ml.

2.1.3. Olive fermentations

Fermentation took place in 5 L total capacity screw-capped PVC fermentation vessels containing 3 kg of olives and 2 L of freshly prepared brine (brine/olive ratio: 1.5/1). Two different initial NaCl concentrations were assayed namely, 8% and 10% (w/v). At the onset of fermentation the brines were acidified with 0.1% (v/v) lactic acid. Eight (8) fermentation cases were investigated (4 fermentation processes × 2 different salt contents): (i) spontaneous fermentation (control), (ii) inoculated fermentation with *L. pentosus* B281, (iii) inoculated fermentation with *L. plantarum* B282, and (iv) mixed inoculum of the two strains, with all cases evaluated in 8 and 10% salt. Duplicate vessels for each trial were studied. Fermentations were undertaken at room temperature (20–22 °C) for a period of 110 days.

2.2. Microbiological analyses

The enumeration of microorganisms was performed on both olive and brine samples. Specifically, brine samples (1 ml) were aseptically transferred to 9 ml sterile 1/4 Ringer's solution (BR0052G, Oxoid). In the case of olive fruit sampling, 10 g of olive flesh were aseptically added in 90 ml sterile 1/4 Ringer's solution and homogenized in a stomacher (Stomacher 400 Circulator, Seward) for 60 s at room temperature. The resulting suspensions were serially diluted in the same diluent and 1 or 0.1 ml samples of the appropriate dilutions were poured or spread on the following agar media: de Man–Rogosa–Sharp (MRS) medium (CM 0361, Oxoid) for LAB, adjusted in pH 5.7 and supplemented with 0.05% (w/v) cycloheximide (Sigma), overlaid with the same medium and incubated at 30 °C for 48–72 h; rose bengal chloramphenicol agar base (LAB 36 supplemented with selective supplement X009, LAB M), for yeasts/molds incubated at 25 °C for 48–72 h; violet red bile glucose agar (CM 0485, Oxoid) for Enterobacteriaceae overlaid with the same medium and incubated at 37 °C for 24 h; *Pseudomonas* agar base (CM559 supplemented with selective supplement CFC SR0103, OXOID), for *Pseudomonas* spp. incubated at 25 °C for 48 h.

2.3. Bacterial isolation

LAB were isolated from the highest dilution of MRS medium from the different sampling points. From each of the aforementioned samplings 20% of the colonies (i.e., 10–20 colonies) were randomly selected and purified (Harrigan, 1998). Pure cultures were stored at −80 °C in MRS 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 336 isolates were picked at 3 different sampling times in the inoculated processes (i.e., after inoculation, in the middle and at the end of fermentation) and at 2 sampling times for the control process (i.e., in the beginning and at the end of fermentation).

2.4. Strain differentiation and characterization

2.4.1. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis was applied to monitor the succession of inoculated starters during fermentation, according to Doulgeraki et al. (2010). The restriction enzyme *Apa*I (10U) (New England Biolabs, Ipswich, MA, USA) was applied according to the recommendations of the