Food Microbiology 56 (2016) 35–44

Contents lists available at ScienceDirect

Food Microbiology

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

Biofilm formation on Conservolea natural black olives during single and combined inoculation with a functional *Lactobacillus pentosus* starter culture

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ARTICLE INFO

Article history:
Received 20 June 2015
Received in revised form
18 November 2015
Accepted 6 December 2015
Available online 15 December 2015

Keywords:
Biofilm
Black olives
Functional foods
Lactobacillus pentosus
Pichia membranifaciens

ABSTRACT

The potential of biofilm formation of multifunctional starters *Lactobacillus pentosus* B281 and *Pichia membranifaciens* M3A during inoculated fermentation of Conservolea natural black olives according to Greek-style processing was investigated. Olives were directly brined in 8% (w/v) NaCl following three fermentation procedures namely, i) spontaneous fermentation, ii) inoculated fermentation with *L. pentosus* B281, and iii) co-inoculated fermentation with *L. pentosus* B281 and *P. membranifaciens* M3A. Lactic acid bacteria (LAB) and yeasts were monitored on olives by plate counting for a period of 153 days, whereas the survival of the inoculated strains was confirmed by Pulsed Field Gel Electrophoresis (PFGE) and Restriction Fragment Length Polymorphism (RFLP) analysis. Inoculated fermentation with *L. pentosus* B281 with/without the presence of the yeast resulted in higher acidification of the brine compared to the spontaneous process where no indigenous LAB could be enumerated. The population of LAB on olives ranged between 5.5 and 6.5 log CFU/g and it was maintained at higher levels compared to yeasts (3.5–4.5 log CFU/g) throughout the process. PFGE analysis revealed that *L. pentosus* B281 could successfully colonize the surface of black olives presenting high recovery rate (100%) at the end of fermentation in contrast to *P. membranifaciens* M3A that was successfully recovered (42%) only after 72 days of the process. The obtained results provide interesting perspectives for the production of natural black olives with functional properties.

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1. Introduction

It has long been established that microorganisms in natural environments tend to adhere to any solid surface immersed in a liquid medium and assemble in a complex multispecies consortium often embedded in a extracellular polymeric matrix (EPS) produced by the microorganisms known as biofilm (Hall-Stoodley et al., 2004; Wimpenny, 2009). During table olive preparation the drupes are originally immersed in brine that is gradually enriched by nutrients from the olive mesocarp serving as substrate for microorganisms to initiate fermentation in order to obtain a microbiologically safe final product with enhanced sensory characteristics (Garrido-Fernández et al., 1997). The presence of aggregates of LAB and yeasts colonizing the surface of natural black olives was first described by Nychas et al. (2002) whereas biofilm development between LAB-yeasts species on Spanish-style green olives has been recently reported (Arroyo-López et al., 2012; Domínguez-Manzano et al., 2012; Benítez-Cabello et al., 2015). The most representative LAB species during table olive processing are *Lactobacillus pentosus, Lactobacillus plantarum* and to a lesser extent *Lactobacillus paraplantarum* (review: Hurtado et al., 2012) whereas diverse yeast species belonging to the genera *Candida*, *Issatchenkia*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, and *Wickerhamomyces* have been reported (Arroyo-López et al., 2008).

On the other hand, a number of studies focussing on the development of new functional probiotic foods has been carried out in the last years indicating that plant based products could be successfully employed as carriers of microorganisms with probiotic potential (Sun-Waterhouse, 2011; Gupta and Abu-Ghannam, 2012). Towards this aim, Lavermicocca et al. (2005) used selected strains of lactobacilli and bifidobacteria as probiotic cultures in fermented

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http://dx.doi.org/10.1016/j.fm.2015.12.002
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olives with promising results in terms of adhesion and colonization on olive epidermis, survival rate and good recovery during storage. In another study (De Bellis et al., 2010) the strain *Lactobacillus paracasei* IMPC2.1, isolated from the human intestine, was used as probiotic starter culture to control the fermentation of Bella di Cerignola green olives resulting in a final product with functional appeal. Since the main source of probiotic isolates is the human gastrointestinal tract, the exploitation of fruits and vegetables, including table olives has been suggested as means of selection of autochthonous LAB strains with functional properties (Di Cagno et al., 2013). In this sense, a number of LAB originating from the table olive environment has been screened for functional properties (Bevilacqua et al., 2011; Abriouel et al., 2012; Argyri et al., 2013; Bautista-Gallego et al., 2013) and their application as starters in table olive fermentation has indicated promising results (Arroyo-López et al., 2012; Argyri et al., 2014; Blana et al., 2014; Rodríguez-Gómez et al., 2013, 2014). The exploitation of such species possessing functional and/or probiotic potential together with their ability to colonize and establish biofilms on olives could make this agricultural commodity a carrier of health promoting properties for consumers. However, the above works focused on green olive fermentation, whereas the applicability of multifunctional starter cultures in natural black olive fermentation remains mostly unexplored. In a recent work (Tufariello et al., 2015) selected yeast and LAB isolates with technological and safety characteristics (β-glucosidase activity, absence of amino acids decarboxylation activity, presence of protease and lipase activity) were used as starter cultures in a sequential fermentation strategy of Italian and Greece black table olive varieties with promising results in the control and standardization of the fermentation process, improvement of sensory characteristics and minimization of fermentation time compared with commercial products.

In the present study, the ability of multifunctional starter cultures namely, *L. pentosus* B281 and *Pichia membranifaciens* M3A was investigated during Greek-style processing of Conservolea natural black olives. The probiotic potential of the former microorganism has been previously elucidated (Argyri et al., 2013) whereas the selected yeast is part of the dominant yeast biota in black olive fermentation (Nisiotou et al., 2010) and its technological properties have been recently reported (Bonatsou et al., 2015). The *in situ* predisposition of the selected microorganisms to colonize the surface of olives and form biofilm communities was recently reported using black oxidized olives as a model system (Grounta and Panagou, 2014). However, as in this trade preparation of olives there is absence of indigenous microbiota, it was deemed necessary to extend the obtained results in natural black olive fermentation where the selected multifunctional cultures would have to compete with the interfering indigenous microbiota in order to colonize the olives and form biofilms.

2. Materials and methods

2.1. Microorganisms and preparation of inocula

The LAB *L. pentosus* B281 and the yeast *P. membranifaciens* M3A used in this study belong to the culture collection of the Laboratory of Microbiology and Biotechnology of Foods (LMBF), Agricultural University of Athens, and have been previously isolated from the brines of industrially fermented olives (Doulgeraki et al., 2012, 2013). The selected LAB strain exhibited *in vitro* probiotic potential including survival in low pH, resistance to bile salts, partial bile salt hydrolase activity, absence of β-haemolytic activity, adherence to *Caco-2* cells, antibiotic resistance to kanamycin, gentamycin, and erythromycin (Argyri et al., 2013). Moreover, the selected yeast species presented resistance to salt, survival in simulated gastric digestion, β-glucosidase and esterase activity (Bonatsou et al., 2015). The LAB and yeast were maintained as stock cultures in de Man- Rogosa-Sharpe broth (MRS, Lab M, Heywood, UK) and YEPD (1% yeast extract, 2% peptone, 2% glucose) broth, respectively, supplemented with 20% glycerol and stored at −80 °C until use. The LAB strain was revived by adding 10 μL of the stock culture in 10 mL MRS broth and incubating at 30 °C for 24 h. Working cultures were obtained by adding 500 μL of the revived culture in 50 mL MRS broth containing 4.5% (w/v) NaCl and incubating at 30 °C for 24 h. The yeast was revived by adding 10 μL of the stock culture in 10 mL YEPD broth and incubating at 25 °C for 48 h with agitation. Working cultures were obtained by adding 500 μL of the revived yeast culture in 50 mL YEPD broth and incubating at 25 °C for 24 h with agitation. LAB and yeast cells were centrifuged at 5000 g for 10 min at 4 °C and the resulting pellet was resuspended in 50 mL quarter-strength Ringer's solution resulting in a final concentration of *ca.* 9.0 log CFU/mL and 7.0 log CFU/mL for *L. pentosus* B281 and *P. membranifaciens* M3A, respectively, as assessed by plate counting.

2.2. Fermentation procedures and inoculation of the brine

The olives were of the Conservolea variety (*Olea europaea media rotunda*) kindly supplied by Konstantopoulos S.A table olive industry. The drupes were harvested in mid-December, subjected to quality control at the processor's facilities to remove defective olives and transported to the laboratory within 24 h. On arrival, the olives were transferred in 14 L plastic vessels containing 7.0 kg of olives and 5.0 L of freshly prepared 8% (w/v) NaCl brine solution. The brine was acidified at the onset of fermentation with 0.5% (v/v) vinegar (*ca.*6%, v/v, acetic acid). Inoculation was carried out after 24 h of brining using a 50-mL aliquot of each working culture in order to achieve an inoculum level in the brine of 7.0 log CFU/mL and 5.0 log CFU/ml for the LAB and yeast starter, respectively. Overall, three fermentation treatments were investigated namely, (i) spontaneous fermentation (control), (ii) inoculated fermentation with *L. pentosus* B281, and (iii) co-inoculated fermentation with *L. pentosus* B281 and *P. membranifaciens* M3A. During the period of fermentation the salt level was adjusted to 8% by periodical additions of coarse salt in the brine. All fermentations were performed in duplicate and the vessels were maintained at room temperature for a period of 153 days.

2.3. Microbiological analysis

Olive samples were analysed at regular time intervals (16 sampling points per treatment) to allow for the determination of the population dynamics on the surface of olives. It needs to be noted that the population dynamics in natural black olive fermentation of cv. Conservolea has been routinely monitored in the brine medium (Nychas et al., 2002; Tassou et al., 2002; Panagou et al., 2008; Bleve et al., 2015; Tufariello et al., 2015). However, in this work the focus was given on the disposition of the selected starters to colonize the olive surface and form biofilms and thus it was deemed necessary to monitor the changes in the microbial population directly on the olive drupes. For this reason, three olives (*ca.* 20–22 g total weight) were randomly sampled from each fermentation vessel and rinsed twice with 10 mL sterile Ringer's solution to remove the loosely attached cells (Giaouris et al., 2013) and then depitted with sterile scalpel and forceps under aseptic conditions. Subsequently, the drupes were transferred into a stomacher bag, decimally diluted with sterile quarter-strength Ringer's solution and homogenized in a stomacher device for 2 min (Lab Blender 400, Seward Medical, London, UK) at room temperature. Serial decimal dilutions were prepared in Ringer's solution and 1 or 0.1 mL from the appropriate dilution were poured

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