LWT - Food Science and Technology 59 (2014) 234–246

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Novel isolates of lactobacilli from fermented Portuguese olive as potential probiotics

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ARTICLE INFO

Article history:
Received 17 September 2013
Received in revised form
22 February 2014
Accepted 2 March 2014
Available online 19 March 2014

Keywords:
Table olive fermentation
Lactobacillus spp.
Digestion resistance
Antibiotic resistance
Oleuropein degradation

ABSTRACT

The purpose of this work was to screen for and characterize the potential probiotic features of strains of lactic acid bacteria isolated from Galega cultivar fermented olives, to eventually develop an improved probiotic food from plant origin. From 156 isolated strains, 10 were acid – and bile salt tolerant, and exhibited survival rates up to 48%, following simulated digestion. All strains exhibited auto- (4–12%) and co-aggregation features (≥30%), as well as hydrophobicity (5–20%) and exopolysaccharide-producing abilities, while no strain possessed haemolytic capacity or ability to hydrolyse mucin. Antibiotic resistance, oleuropein degradation, proteolytic activity and antimicrobial activity were strain-dependent features. Overall, 10 strains – belonging to *Lactobacillus plantarum* and *Lactobacillus paraplantarum*, appear to possess a probiotic value.

1. Introduction

Among the very many functional foods available commercially, those containing live bacteria (mainly bifidobacteria and certain lactobacilli) and able to provide a beneficial health effect deserve a special mention (Gregoret, Perezlindo, Vinderola, Reinheimer, & Binetti, 2013; Rauch & Lynch, 2012; Shah, 2007). These are currently traded under the label of probiotic, and their efficacy depends mainly on the ability of said probiotic strain to survive throughout the whole food processing chain (including storage), and to compete with metabolically active microorganisms either along the food chain or during passage through the gastrointestinal tract (Mansouripour, Esfandiari, & Nateghi1, 2013). WHO/FAO (2002) has indeed defined probiotic organisms as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host". Probiotics are thus considered as GRAS ingredients (Mattia & Merker, 2008), and its consumption reduces the viable number of pathogens while strengthening body natural defences (Bertazzoni-Minelli & Benini, 2008; Larsen, Michaelsen, Pærregaard, Vogensen, & Jakobsen, 2009; Madureira et al., 2008; Savard et al., 2011); hence, they help boost the immune system, and consequently lower the risk of gastrointestinal diseases, cancer, diabetes and high serum cholesterol levels, while improving digestion itself (Kumar et al., 2012; de Vrese & Schrezenmeir, 2008).

Dairy products play a predominant role as carriers of probiotics. In addition to yogurts and fermented milks that are still the main vehicles for incorporation of probiotic cultures, new products are being introduced in the international market, such as milk-based desserts, powdered milk for newborn infants, ice cream, butter and various types of cheese (Granato et al., 2010). However, allergies attributed to dairy products, lactose intolerance and cholesterol content are major drawbacks related to the use of fermented dairy products for a representative percentage of consumers (Prado, Parada, Pandey, & Soccol, 2008). Therefore, probiotic food products manufactured via fermentation of cereals and fruits and vegetables have received increasing attention from

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http://dx.doi.org/10.1016/j.lwt.2014.03.003
0023-6438/

the scientific world, as well as consumers (Gupta and Abu-Ghannam, 2012). Unfortunately, only a few probiotic cultures isolated from human or animal sources – and used with success in dairy products, exhibit acceptable adaptation to plant matrices. Therefore, screening LAB strains of plant origin for potential probiotic features may help overcome such technological challenge (Peres, Peres, Hernández-Mendonza, & Malcata, 2012). In this respect, approaches to probiotic fortification of table olives have been recently assessed (Lavermicocca, 2006; Lavermicocca et al. 2005; de Bellis, Valerio, Sisto, Lonigro Stella, & Lavermicocca, 2010). Nevertheless, scarce scientific data have been produced relating to this matrix or LAB wild strains sufficient to validate their hypothesized health-promoting capacity. Hence, the goal of this work was to obtain potentially probiotic LAB from fermented Portuguese olives, in order to eventually produce a tailor-made starter culture that may be deliberately (and safely) introduced in brines, at the onset of fermentation but expected to prevail along the whole chain – thus ensuring proper evolution of fermentation, while inhibiting growth of undesirable microorganisms once in the gastrointestinal tract.

2. Materials and methods

2.1. Bacteria and Caco-2 cells

Ten *Lactobacillus* strains (Table 1) from our own culture collection, identified as *Lactobacillus plantarum* and *Lactobacillus paraplantarum,* were selected for this study according to their acid and bile salt tolerance. *Lactobacillus casei* Shirota (ACA-DC 6002), kindly provided by Laboratory of Microbiology and Biotechnology of Food at the Agricultural University of Athens (Iera Odos, Greece), was used as probiotic reference strain.

Enteropathogenic strains used were: *Escherichia coli* ATCC 8739, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Salmonella* Typhimurium ATCC 14028, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212 and *Helicobacter pylori* (ATCC 700392), all from our own collection.

The Caco-2 cell line ACC169 (DSMZ collection, Germany) used in the adhesion assay was provided by Animal Cell Technology Team (IBET, Oeiras, Portugal).

2.2. Probiotic features assessment

2.2.1. Acid and bile salt tolerance

The modified method of Tambekar and Bhutada (2010) was followed to determine acid and bile salt tolerance of selected strains. Briefly, 156 LAB strains, isolated from fermented table olives of Portuguese cultivars, were grown overnight in 5 mL of MRS broth at 37 °C. Afterwards, an aliquot (5 μL) of each culture was spotted onto the surface of MRS agar plates – previously adjusted to pH 3.5 using hydrochloric acid (1 M), and incubated at 37 °C for 48 h.

In a parallel experiment, overnight cultures were also blobbing (5 μL) onto MRS agar plates added with 0.3% of bile salt (w/v) (Oxoid, Hampshire, UK), and then incubated at 37 °C for 48 h.

Those strains showing visible growth after incubation were considered either acid- or bile salt-tolerant, so they were selected for further experimentation.

Table 1
Lactobacillus strains identified via PCR of recA gene, with species-specific primers (Torriani et al., 2001).

Fermentation process	Origin	Identified species	Code
Homemade	Beja	*Lactobacillus paraplantarum*	B13
	Santarém	*L. paraplantarum*	K
	Beja	*Lactobacillus plantarum*	B95
	Ladoeiro	*L. paraplantarum*	O1
Industrial	Campo Maior	*L. plantarum*	17.2b
	Envendos	*L. plantarum*	69B
	Envendos	*L. plantarum*	607
	Envendos	*L. plantarum*	P
	Envendos	*L. plantarum*	FF28
	Campo Maior	*L. plantarum*	33

2.2.2. Haemolytic activity

The CAMP (Christie, Atkins, Munch-Petersen) test was performed on Columbia Agar + 5% Horse blood (Biomérieux, Marcy l'Etoile, France) for haemolytic activity. Fresh cultures of selected *Lactobacillus* strains were streaked on blood agar plates and incubated at 37 °C for 24 h. Plates were then examined for the halo of haemolysis (zone of clearance). A positive control of *S. aureus* ATCC 6538 strain and a negative control of *L. casei* Shirota (LCS) were used.

2.2.3. Mucin degradation

In order to unfold mucin degradation by the selected strains, the LAB were cultured on Petri dishes containing swine gastric mucin (HGM type III, from Sigma Chemical, St Louis MO, USA); porcine mucin is similar in structure and chemical properties to its human gastric counterpart (Fumiaki et al., 2010), despite minor modifications. Agar plates were thus prepared using basal medium supplemented with 1.5% agar (Becton Dickinson and Company, Sparks, USA), 0.3% partial purified hog gastric mucin (w/v) and 1% glucose (w/v). Then, an aliquot (2 μL) of 16 h-old bacterial cultures was inoculated by spotting onto the surface of the agar plates. After incubation (37 °C, 72 h), plates were stained for 30 min with 0.1% amide black (w/v) (Merck) prepared in 3.5 M acetic acid, and washed with 1.2 M acetic acid. A mucin lysis zone (i.e. discoloured halo) around the colony of the positive control culture (*E. coli* ATCC 8739 and *S.* Typhimurium ATCC 14028) eventually appeared; *L. casei* Shirota was included in the plates as negative control.

2.2.4. Esterase- and β-glucosidase genes, and oleuropein degradation

Genes coding for esterase and β-glucosidase enzymes were detected in *Lactobacillus* strains following the colony PCR reaction described by Mtshali, Divol, van Rensburg, and du Toit (2010), with adaptations. Briefly, one colony from each *Lactobacillus* strains was added to a tube containing 50 μL of the PCR mix. The reactions mixtures were subjected to PCR using a thermocycler (VWR, DOPPIO thermal cycler 732-1210, USA), and an aliquot of each reaction was analysed by agarose gel electrophoresis. Gels were run for 120 min. DNA fragments were visualized by UV using a Chem-iDoc™ XRS system (Bio-Rad Laboratories, Hercules CA, USA) and analysed with image acquisition software (image Lab™).

Tolerance to oleuropein (Extrasynthese, France) was determined according to Ghabbour et al. (2011). Those strains developing colonies on said medium were considered tolerant to oleuropein, and used to monitor oleuropein biodegradation using X-Gluc as substrate (Ciafardini, Marsilio, Lanza, & Pozzi, 1994). Colonies of those strains producing β-glucosidase acquired a blue colour.

Additionally, the oleuropein-tolerant LAB strains were tested for their ability to metabolize oleuropein according to Ghabbour et al. (2011). Confirmation of oleuropein biodegradation was assessed by HPLC-DAD-ED analysis of the extracts of modified MRS broth containing oleuropein as sole carbon source, by 7 d of incubation, using a Surveyor equipment with a diode array (Thermo Finnigan-Surveyor, San Jose CA, USA) and an electrochemical detector