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Novel isolates of lactobacilli from fermented Portuguese olive as potential probiotics

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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 e 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 e 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 the scientific world, as well as consumers (Gupta and Abu- Ghannam, 2012). Unfortunately, only a few probiotic cultures isolated from human or animal sources e and used with success in dairy products, exhibit acceptable adaptation to plant matrices. Therefore, screening LAB strains of plant origin for potential pro- biotic 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 e 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 para- plantarum,* 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 ^{o}C. Afterwards, an aliquot (5 mL) of each culture was spotted onto the surface of MRS agar plates e previously adjusted to pH 3.5 using hydrochloric acid (1 M), and incubated at 37 ^{o}C for 48 h.

In a parallel experiment, overnight cultures were also blobbing (5 mL) onto MRS agar plates added with 0.3% of bile salt (w/v) (Oxoid, Hampshire, UK), and then incubated at 37 ^{o}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.

2.2.2. *Haemolytic activity*

The CAMP (Christie, Atkins, Munch-Petersen) test was per- formed 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 mL) 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 b-glucosidase genes, and oleuropein degradation*

Genes coding for esterase and b-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 mL 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 re- action was analysed by agarose gel electrophoresis. Gels were run for 120 min. DNA fragments were visualized by UV using a ChemiDoc™ 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 b-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 (Dionex, ED40) with a vitreous carbon electrode. Separations were performed at 35 ºC with a LiChrospher C18 (5 μm, 250 mm 4 mm *i.d.*) column (Merck), and a guard column of the same type. Aliquots were injected using a 20 μL-loop. Separations were carried out at a flow rate of 700 μL/min, and the mobile phase consisted of a mixture of eluent A, phosphoric acid (0.1%) and eluent B, phosphoric acid-acetonitrile-water (1:400:599, v/v/v), according to the following gradient: 0-15 min from 0 until 20% eluent B; 10 min with 20% eluent B; 25-70 min, from 20 until 70% eluent B; 70-75 min, with 70% eluent B; 75-85 min from 70 until 100% eluent B; and 85-90 min, with 100% eluent B.

DAD was used between 200 and 800 nm. Electrochemical detection was programmed for a linear variation from 1.0 V to 1.0 V in 1.00 s, followed by integration in a voltammeter system using a cyclic variation of potential. Measurements were taken at a frequency of 50 Hz with an analogical/digital converter. Data acquisition used the Chromquest version 4.0 (Thermo Finnigan-Surveyor) for the diode array detector and the software 4880 (Unicam) for DAD. Peak areas from hydroxytyrosol and oleuropein were quantified using electrochemical and DAD at 280 nm, respectively. For peak identification, retention times were compared with peaks produced by a standard solution; one sample was also spiked with g/L standards for confirmation.

2.2.5. *Proteolytic activity*

The proteolytic activity of selected strains was ascertained via qualitative and quantitative assays, by the methods described by van den Berg et al. (1993) and Pereira, Barreto-Crespo, and San- Romão (2001), respectively, with modifications.

A qualitative assay was performed in MRS agar plates supplemented with 10% (v/v) skim milk (Matinal e Lactogal©, UHT skim milk, Portugal); g/L an aliquot (3 ml) of the activated culture was spotted on the top of milk-MRS agar plates. The plates were then incubated at 37 ºC for 16 h. A precipitation zone surrounding colonies was taken as positive indicator of proteolysis.

Subsequently, azocasein (Sigma) was chosen for being a sensitive and convenient non-specific substrate, to quantify proteolytic activity in cell-free supernatants (CFS), by measuring release of red coloured azopeptides. The protein content of CFS was determined using Bradford reagent, according to manufacturer's instructions (Sigma). CFSs were obtained after centrifugation of 20 h MRS broth culture of LAB strains at 6000 × *g* at 4 ºC, for 10 min. The reaction mixture, containing 50 μl of

azocasein (5 mg/mL) prepared in 50 mM TriseHCI buffer (pH 8.0) containing 2 mM $CaCl_2$, and 50 μL of the corresponding CFS, was incubated at 37 °C, for 1 h. The reaction was quenched via addition of 100 μl of 5% (w/v) TCA solution. After 10 min at room temperature, the mixture was centrifuged at 10 000x*g* for 5 min. The supernatant (50 μl) was mixed with 75 μl of 0.5 M NaOH, and absorbance of the mixture (containing the low molecular weight, red coloured azopeptides) was measured at 450 nm against a blank.

One unit (U) of protease activity was defined as a 0.001 increase in absorbance of the assay solution (for azocasein) per min and per mL. Specific activity was expressed as U per mg of extracellular protein.

2.2.6. *Exopolysaccharide production*

Exopolysaccharide production was evaluated as per Mourad and Nour-Eddine (2006). Overnight cultures were streaked on the surface of plates containing ruthenium red milk [10%, skim milk powder (w/v) from Oxoid, 1% sucrose (w/v) from Scharlau, (Spain), 1% yeast extract (w/v) and 0.08 g/L ruthenium red from Sigma, 1.5% agar (w/v) from Merck]. After incubation at 37 °C for 24 h, non-ropy strains gave red colonies due to staining of the bacterial cell wall, while ropy strains appeared as white colonies.

2.2.7. *Bile salt hydrolase activity for bile salt deconjugation (BSH activity)*

Bile acid deconjugating ability of lactobacilli strains was detec- ted by spreading an overnight bacteria culture on MRS agar containing 0.5% taurodeoxycholic acid (w/v) (TDCA, Sigma) and compared to the control MRS plates, after anaerobic incubation in GasPak jars (Oxoid) at 37 °C for 24 h. Deconjugation of TDCA results in a white precipitate of deoxycholate in the vicinity of bacterial colonies (Roos et al., 2005).

2.2.8. *Auto-aggregation, co-aggregation and hydrophobicity*

Auto-aggregation of selected strains, as well as co-aggregation with *E. coli*, *S. aureus, S.* Typhimurium, *L. monocytogenes* and *H. pylori* were assessed as described elsewhere (el-Naggar, 2004; del Re, Sgorbati, Miglioli, & Palenzona, 2000; Taheri et al., 2009).

Both lactobacilli and pathogens were cultured under growth conditions defined previously. Bacterial cultures were then centrifuged (8214 *g*, 5 min, 10 °C); the supernatant of lactobacilli was retained in a different tube, washed twice in PBS (0.5 M, pH 7.0) and resuspended in the same buffer until an OD_{600nm} of 0.5 units was attained.

For auto-aggregation tests, 5 mL of *Lactobacillus* suspension in PBS was centrifuged; the pellet was harvested, and then resus- pended in the original broth. After incubation at 37 °C for 2 h, 1 mL was taken from the upper part of

the culture, and OD_{600nm} was recorded. Finally, the culture was shaken and the total OD_{600nm} was measured. The auto-aggregation (A %) was expressed as 1e (OD_{600nm} upper culture/OD_{600nm} total) 100.

For co-aggregation analysis, 0.5 mL of each *Lactobacillus* and pathogen suspension in PBS was mixed thoroughly in a test tube for 10 s using a vortex. The OD_{600nm} of the bacterial mixture was measured after incubation at 37 °C, for 4 h; 1 mL of either *Lactobacillus* or pathogen suspension was used as control. The percent co-aggregation was calculated as {[(PC+LC)/2-(*P+L*)]/(PC+LC)/2}x100, where PC and LC denote the optical densities in control tubes containing only pathogen or *Lactobacillus* after 4 h of incubation, respectively, and *P+L* denotes the optical density of the mixed culture after the same period.

The relative surface hydrophobicity of *Lactobacillus* strains was determined using microbial adhesion to hydrocarbons, as described by Marin et al. (1997). The bacterial cell suspension in PBS was dispensed as 3 mL-volumes into test tubes, and mixed with 0.4 mL of xylene by vortexing for 30 s. The two phases were allowed to separate for 15 min at room temperature. The aqueous phase was carefully removed, and its OD_{600nm} was recorded. The decrease in absorbance of the aqueous phase was taken as a measure of cell surface hydrophobicity, calculated as [(*A*0-*A*)/*A*0]x100, where *A*0 and *A* denote absorbance before and after extraction with xylene, respectively.

2.2.9. *Gastrointestinal transit tolerance*

The viability of selected lactobacilli was assessed after exposing the cells to a dynamic *in vitro* model intended to simulate gastric and gastrointestinal stress conditions (Fernández de Palencia, López, Corbí, Peláez, & Requena, 2008). Briefly, lactobacilli strains that had been propagated on 35 mL of MRS broth were harvested by centrifugation (3214x*g*, 10 min, 10 °C) and resuspended in the same volume of sterile electrolyte solution [6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$ and 1.2 g/L $NaHCO_3$, (w/v)], adjusted to pH 6.2 with 1 M $NaHCO_3$. An aliquot was withdrawn (control G1). To simulate *in vivo* saliva conditions, 5 mL of a sterile electrolyte solution containing lysozyme (final concentration of 0.01% w/v) was added to 35 mL of cell suspension, and an aliquot was taken without further incubation (G2). To simulate the gastric environment, 3 mL of electrolyte solution (pH 5.0) added with 0.3% (w/v) pepsin was incorporated into the cell suspension. Then, the pH curve in the stomach was reproduced by adding 1 M HCl to the cell suspension, at an initial pH of 5 that was gradually decreased to 4.1, 3.0, 2.1 and 1.8. To mimic normal gastric emptying, aliquots of the suspension were collected after sequential incubations of 20 min, at 37 °C and 50 rpm, at each pH value (G3eG7). To simulate the intestinal environment, samples G3eG5 were adjusted to pH 6.5 with 1 M $NaHCO_3$, mixed with 4 mL of a sterile electrolyte solution (5 g/L NaCl, 0.6 g/L

KCl and 0.3 g/L $CaCl_2$ w/v), added with 0.45% (w/v) bile salts and 0.1% (w/v) pancreatin, and adjusted to pH 8.0. After 120 min of incubation at 37 °C and 50 rpm, to simulate the conditions prevailing in the duodenum, fractions of suspensions were collected (Gi3, Gi4, Gi5). Bacterial cell survival in all samples gathered into each stage of the dynamic model was estimated by pour-plating on MRS agar (Vinderola, Bailo, & Reinheimer, 2000). Strain survival percentage was calculated via colony counts (CFU/ mL) in G1 and Gi5 steps.

2.2.10. *Antimicrobial activity*

The antimicrobial activity assay was carried out according to Gu, Yang, Li, Chen, and Luo (2008) and Guo, Kim, Nam, Park, and Kim (2010), with modifications. *Lactobacillus* cultures previously grown in 10 mL of MRS broth (pH 6.2 and 37 °C, for 16 h) were removed by centrifugation. The cell-free supernatants (CFS) were concentrated by freeze-drying, and subsequently resuspended with 2 mL of PBS (pH 7) to exclude inhibition due to organic acids. The supernatants were then filter-sterilized (0.2 μm) and kept at -20 °C until use. In order to assess the antimicrobial activity of supernatants, one proceeded as follows: 16 h-old culture of each entero-pathogenic target bacteria was harvested by centrifugation, washed twice with PBS and resuspended to OD_{600nm} 0.5 (*ca.* $7x10^8$ CFU/ mL). Then, an aliquot (1% v/v) of adjusted suspension was inoculated in Mueller-Hinton (Oxoid) melted soft agar (0.8% agar), and poured over Petri dishes (10 mL per plate). Thereafter, six sterile 6 mm paper disks (Oxoid) were evenly spaced upon the agar seeded with the target bacteria, and 8 μL of the sterile supernatant to be tested was loaded on each disk. After setting, all plates were incubated at 37 °C for 24 h, without inversion. By the end of incubation, the diameters of the inhibition zones formed around the disks were measured in mm, using a dial caliper. LAB strains were classified as no (-), mild (+) and strong (++) strain inhibitors, according to the inhibition zones formed, i.e. no inhibition, be- tween 1 and 3 mm, and above 3 mm, respectively.

To confirm that inhibition in CFS was due to presence of a peptide-like compound, the heat stability at 100 °C for 5 min was assessed, as well as the stability to proteolytic action by 1 mg/mL proteinase K (Sigma) via incubation at 37 °C for 2 h (Albano et al., 2007). Positive controls as CFS were added to buffer without enzyme. Negative controls were heat-inactivated enzyme solutions (at the concentration above) submitted to enzyme reaction. At the end of the reaction, enzymes were heat-inactivated at 100 °C for 5 min, and immediately cooled in ice. All samples and blanks were assessed for bioactivity using an assay in microtiter plates (Bogovi-Matija'i'c & Rogelj, 2000).

2.2.11. *Screening for antibiotic resistant phenotypes*

The minimal inhibitory concentration (MIC) for each bacterial strain was determined by the broth microdilution protocol (D'Aimmo, Modesto, & Biavati, 2007; Wiegand, Hilpert, & Hancock, 2008). Antibiotics (i.e. chloramphenicol, tetracycline, kanamycin, gentamicin, streptomycin, erythromycin, ampicillin and vancomycin, all from Sigma) were chosen according to European Food Safety Authority recommendations (EFSA, 2012; Schwarz et al., 2010). To prepare the stock antibiotic solutions, each antibiotic was carefully weighed, dissolved in appropriate solvent, filter-sterilized (0.2 μm) and kept at -20 ^{o}C until use. The working solutions at specific concentrations were prepared daily. The antibiotic concentrations tested underwent serial 2-fold dilutions that ranged from an initial concentration of 1.024 mg/mL (0.256 mg/mL for ampicillin) to 0.002 mg/ mL. Overnight cell cultures were adjusted to an OD600nm of 0.8 with PBS, and used to inoculate (1% v/v) MRS broth containing each antibiotic at different concentrations (final volume of 200 μl per well of 96 micro-well plates). The plates were incubated at 37 ^{o}C for 24 h. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the isolate tested observed with the naked eye, as described by Wiegand et al. (2008). Resistance rates were calculated according to microbial cut-off values (μg/mL), as reported by Danielson and Wind (2003) and EFSA (2012).

2.2.12. *Caco-2 cell adhesion*

Caco-2 cells were routinely grown in RPMI-1640 medium + Gluta MAX™ (Gibco-BRL, Paisley, UK) supplemented with 10% (v/v) heat-inactivated (56 ^{o}C, 30 min) foetal bovine serum, and 1% (v/v) PenStrep (Gibco-BRL). The cells were incubated at 37 ^{o}C, under an atmosphere of 5% CO_2/90% air, with medium change every other day. Concentration of Caco-2 cells in the monolayer was determined by trypsinizing cells with 0.05% (v/v) Trypsin-EDTA (Gibco-BRL) solution at 37 ^{o}C for 5 min, and counting them in a heamocytometer. Aliquots of 2 mL containing $5x10^4$ cells/mL were seeded per well (9.5 cm^2), in a Tissue Culture Plate. The cells were maintained for 2 weeks until a complete monolayer was obtained. Prior to applying the adhesion assay, 16 h-hold LAB cultures were centrifuged for 10 min at 7000x*g*, washed twice with PBS and then diluted in RPMI-1640 medium (without serum and anti- biotics) to a concentration of 10^8 CFU/mL. Just prior to use of Caco-2 monolayers, the growth medium in the tissue culture plates was withdrawn, cells were washed twice with 3 mL of PBS (pH 7.4), and incubated at 37 ^{o}C for 1 h with 2 mL of RPMI-1640 without serum and antibiotics. After incubation, 1 mL of bacterial RPMI suspension was added per well. Co-incubation was performed in the presence of 5%CO_2/95% air, at 37 ^{o}C for 2 h. After 2 h of

incubation, the medium was removed, and the monolayer was washed three times with sterile PBS (1 mL). The Caco-2 cells were detached by trypsinization using 0.05% Trypsin-EDTA solution, and incubated at room temperature for 15 min. Finally, the cell suspension was platted on MRS agar by serial dilution, to determine the adherent bacterial cells. The plates were incubated for 24-48 h at 37 ^{o}C, and colonies were duly counted. The efficiency of adhesion was expressed as the ratio (%) of *Lactobacillus* viable cells that remained adhered to the Caco-2 enterocytes and added bacteria per well.

2.2.13. *Statistical analysis*

Statistical analysis of the experimental data was performed via one-way analysis of variance (ANOVA), using Statistical Package for Social Sciences software (v. 20: SPSS Chicago IL, USA); to look for significant differences between experiments, Tukey's posthoc test was employed. In all statistical analyses, P=0.05 was taken as reference level of significance; differences between bacterial strains were thus considered as statistically significant when $P < 0.05$. For PCA treatment, NTSYS software v. 2 (Exeter software) was used.

3. Results and discussion

3.1. *Acid and bile salts tolerance*

Tolerance to digestive stress is one of the main factors limiting use of microorganisms as live probiotic agents; acid and bile salt tolerance are indeed considered essential properties required for LAB be able to survive in the gut (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000).

Therefore, the 156 strains previously identified as lactobacilli, as well as the reference LAB strain were screened *in vitro* for their tolerance to acid and bile salt stress. Comparing the effects of acid and bile salts on the strains tested, the 10 *Lactobacillus* strains exhibiting the best tolerance ability (results not shown) were selected; these were identified as *L. plantarum* and *L. paraplantarum,* and further assayed for their potential probiotic features.

3.2. *Haemolytic activity*

Safety is an attribute recommended by FAO/WHO (2002) guidelines concerning evaluation of probiotics; absence of haemolytic activity is a criterion of selection for probiotic strains, as long as it indicates that these bacteria are not virulent (de Vuyst, Foulquie, & Revets, 2003). This test was important because *Lacto-bacillus* spp. are in general recognized as safe commensals, and absence of

haemolysins guarantees that opportunistic virulence will not arise among isolates e which is a requirement for eventual large-scale food manufacture.

None of the strains examined in our work revealed haemolytic activity (i.e. lysis of red blood cells) when grown on blood agar medium; this suggests no harmful characteristics *in vitro.* This finding confirms results by Kalui, Mathara, Kutima, Kiiyukia, and Wongo (2009) and Mami, Henni, and Kihal (2008), who reported that other *L. plantarum* strains e isolated from fermented maize porridge and raw goat's milk, respectively, do not exhibit any haemolytic properties.

3.3. *Mucin degradation*

The mucus layer (mucin) coating the surface of the GIT plays an important role as part of the mucosal barrier system; any changes in mucus content and structure will indeed compromise the mucosal defence barrier functions. Hence, mucin degradation is a marker for safety (pathogenicity and local toxic) assessment of probiotic LAB strains, thus ensuring that they are safe for human consumption (Fumiaki et al., 2010).

To demonstrate the mucinolytic activity, a Petri dish assay was carried out; unlike observed with positive (pathogens) and negative controls (registered as dairy probiotic strains), none of our isolates was able to produce a clear lysis zone; hence, all strains were considered negative for this feature. No mucinolytic activity implies that our LAB strains were unable to degrade gastrointes- tinal mucin *in vitro*. Considering that extensive degradation of mucin facilitates translocation of bacteria to extraintestinal tissues (Sriphochanart & Skolpap, 2010), our results suggest that these novel probiotic candidates are likely non-invasive of, and non-toxic at the mucosal interface. These findings are consistent with other studies encompassing *Lactobacillus* (e.g. *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *lactis*) and bifidobacteria (*Bifidobacterium animalis* subsp. *lactis*, *Bifidobacte- rium longum*, *Bifidobacterium breve* and *Bifidobacterium infantis*) that were claimed to be probiotic strains (Abe et al., 2010; Fernández, Boris, & Barbes, 2005; Zhou, Gopal, & Hill, 2001).

3.4. *Esterase and,* b-*glucosidase genes and oleuropein degradation*

Genes from *Lactobacillus* strains coding for the different en- zymes tested were identified through PCR detection using enzyme- specific primers, and the amplified DNA fragments were subse- quently sequenced for comparative analysis. PCR screening with the designed primers conveyed single gene products of 1392 bp for b-glycosidase and 1020 bp for esterase, in all *Lactobacillus* strains. Presence of β-glycosidase and esterase-encoding genes suggests that they might be essential for strain survival during olive fermentation, unlike happens with

winemaking LAB strains (Mtshali et al., 2010). Our results suggest that those LAB could play a role in release of ferulic acid from dietary ingredients in the digestive tract e which may overcome diabetes symptoms by stimulating insulin secretion, besides their important role upon organoleptic quality of fermented olives (Adisakwattana, Moonsan, & Yibchok-Anun, 2008).

Although all strains tested were found to possess the genes to act on specific substrates, a better understanding is urged of how these genes are regulated under conditions prevailing throughout olive fermentation, and also to confirm whether the expressed enzymes are active in olives. To confirm gene expression and capacity to degrade oleuropein, assays of growth in the presence of oleuropein were performed, followed by confirmation of degradation via HPLC (Table 2).

The entire sets of LAB strains assessed showed β-glucosidase and esterase activities, and were able to degrade X-Gluc (data not shown). The tolerance to presence of oleuropein (1%, v/v) exhibited by all LAB strains may be explained by natural selection during spontaneous lactic fermentation of non debittered olives, as claimed by Rozès & Peres (1991, 1992). From a technological point of view, strain tolerance to oleuropein suggests their possible use as starters in controlled processes of fermentation parallelling the spontaneous (uncontrolled) process.

Under the chromatographic conditions used, calibration curves showed a good linearity within the range of 1-400 ppm for hydroxytyrosol ($r^2 > 0.999$), and 10-40 ppm ($r^2 > 0.996$) for oleuropein. Data depicted in Table 2 indicate that oleuropein degradation produces mainly hydroxytyrosol, identified by comparison of their UV spectra and retention time to the corresponding standard solutions. Accumulation of hydroxytyrosol has been reported elsewhere as the main degradation product of oleuropein (Ciafardini et al., 1994; Landete, Rodriguez, de las Rivas, & Munoz, 2007; Rodriguez et al., 2009), and thus clearly demonstrates the debittering capacity of our LAB strains.

Oleuropein, a bitter-tasting secoiridoid glycoside present in olive drupe *(Olea europaea* L.), is a heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol containing a molecule of glucose. Its hydrolysis yields elenolic acid glucoside and hydroxytyrosol (Ansaria, Kazemipourb, & Fathib, 2011). If intended for consumption as such, olive fruits must first undergo some form of debittering. All germane technologies are based on delicate microbial fermentations that convert sugars to secondary metabolites (i.e. lactic and acetic acids, and minor compounds), which provide the finished product with a progressive acidification and a particular flavour (Garrido-Fernández, Fernández Díez, & Adams, 1997). Authors have reported that strains of *L. plantarum* (Ciafardini et al., 1994) and *Lactobacillus pentosus* (Servili et al., 2006) are able to degrade oleuropein, and claimed that those LAB produce β-glucosidase during

olive brining that catalyses rupture of the glycoside bond of said oleuropein moiety (Ciafardini & Zullo, 2000). *L. plantarum* initially hydrolyses oleuropein via b-glucosidase action, with formation of an aglycone and glucose e and, at a second stage, this derivative gives rise to hydroxytyrosol and elenoic acid via an esterase-mediated action (Rodriguez et al., 2009). Accumulation of simple phenolic compounds is highly desirable because they in- crease safety, nutritional and shelf-life features of fermented olives (Ordoudi & Tsimidou, 2006; Rodriguez et al., 2009; Visioli, Bellomo, Montedoro, & Galli, 1995).

3.5. *Proteolytic activity*

A qualitative assessment of proteolysis was done via formation of halo zones around the colonies in MRS plates added with skim milk (10%, v/v); the LAB strains studied produced different-sized precipitated zones (data not shown). To confirm the proteolytic activities of those isolates, a quantitative assay (using azocasein as substrate) was performed on their CFS; proteolytic activity seems to be strain-specific (Fig. 1). The highest activity was exhibited by 69B, 607, 33, P and O1 strains, ranging in specific activity from 2.5 to 3.4 U mg^{-1} protein. However, such an activity was not significantly different ($P < 0.5$) from that of the control strain (LCS). On the other hand, B13, 17.2b, FF28 and B95 strains exhibited the lowest specific activity (<2.5 U mg^{-1} protein).

Within the *Lactobacillus* genus, most species can bring about proteolysis, but to different extents among species. In the case of *L. plantarum*, proteolytic activity has been associated with cell wall- bound proteinase (Hegazi & Abo-Elnaga, 1987; Kojic, Fira, Banina, & Topisirovic, 1991). Hence, our strains with highest activity likely produced more extracellular cell-bound proteinase than did the lowest activity group. Since LAB are characterized by their strong requirement of essential growth factors, production of extracellular cell-bound proteinase may depend on amino acid requirements of individual strains (Matthews et al., 2004).

The proteolytic activity of our strains is relevant for their potential probiotic activity, specifically regarding release of bioactive peptides (biopeptides) e either during food fermentation or food digestion (Pelaez & Requena, 2005). These bio-peptides are known to include some amino acid sequences that exert a specific bio- logical activity on the consumer (Aimutis, 2004).

3.6. *Exopolysaccharide production*

EPS are exocellular polymers present on the surface of many LAB; they are constituted by long-chain polysaccharides composed of branched, repeating units of sugars (or sugar derivatives) loosely attached to the cell surface, or secreted into

the environment (Ruas- Madiedo & de los Reyes-Gavilan, 2005). EPS production is a relevant feature for selection of probiotic LAB strains because of its positive health effect as prebiotic e i.e. upon growth and performance of probiotic strains (Ruas-Madiedo, Hugenholtz, & Zoon, 2002).

All isolates tested were able to produce EPS, as reported previously by van den Berg et al. (1993) regarding LAB strains from Portuguese olive fermentations. Those results point at involvement of EPS in their adhesion to intestinal mucus (Ruas-Madiedo, Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2006). Cell surface-associated macromolecules are indeed considered to play an important role on adhesion of *Lactobacillus* in the GIT; in *L. acidophilus* CRL639, adhesion to extracellular components has been attributed to different types of EPS (Lorca, Torino, Font, & Ljungh, 2002). Moreover, it has been suggested that EPS from other bacteria may act as protective agents against antimicrobial compounds, besides involvement in adhesion to surfaces and bio- film formation (Rozen, Steinberg, & Bachrach, 2004).

3.7. Bile salt deconjugation activity

One important transformation of bile acids is via deconjugation; their reaction is to occur before further modifications are possible, and is catalysed by bile salt hydrolase (BSH) enzymes. Bile salt deconjugation may therefore confer a nutritional advantage to hydrolytic strains, and is even thought to play a relevant role in the enterohepatic cycle taking place in the upper small intestine (Begley, Colin, & Gahan, 2006). LAB with active BSH (or products containing it) have been claimed to lower cholesterol levels via interaction with host bile salt metabolism; enzymes able to deconjugate bile salts to amino acids and cholesterol reduce the corresponding toxicity. Molecules of cholesterol are converted to bile acids to replace those lost during excretion, thus leading to reduced levels of serum cholesterol. This mechanism could be used in the control of cholesterol levels in the blood by conversion of deconjugated bile acids into secondary bile acids carried out by probiotics (Kumar et al., 2012).

The ability of probiotic strains to hydrolyse bile salts has often been included among the criteria for probiotic strain selection. However, none of our *Lactobacillus* showed BSH activity (data not shown). In fact, BSH activity has not yet been detected in bacteria isolated from environments devoid of bile salts e as is the case of olive brines (Begley et al., 2006). These results are consistent with those by Bauptista-Gallego et al. (2013), who reported lack of this activity in *Lactobacillus* isolated from spontaneously fermented green olive brines. Conversely, bile salt hydrolase activity (or partial activity) has been found in some table olive related-*Lactobacillus* strains (Abriouel et al., 2012; Argyri et al., 2013).

3.8. *Auto-aggregation, co-aggregation and cell surface hydrophobicity*

Interaction of probiotic organisms with the natural gut microbiota is a key to potential success of the organism in terms of colonization and long-term persistence e thus constituting an important host-defence mechanism against enteropathogenic strains (del Re et al., 2000).

Auto-aggregation determines the ability of a bacterial strain to interact with itself, in a nonspecific way, which is known as a prerequisite for colonization and infection of the gastrointestinal tract by pathogens through adhesive ability (del Re et al., 2000); whereas co-aggregation determines the capacity to form biofilms that protect the host by preventing colonization by pathogens. This realization reinforces the importance of therapeutic manipulation of intestinal microbiota (Dunne et al., 2001). Cell surface hydro- phobicity measures the capacity to adhere to hydrocarbons, and holds a strong relationship with ability to adhere to the epithelium along the digestive tract (Kos et al., 2003).

All strains evaluated showed self-aggregation e but were lower than those indicated as acceptable for probiotic strains (>40%), as referred by del Re, Busetto, Vignola, Sgorbati, and Palenzona (1998), even though above the control LCS (Fig. 2). These results differ from those reported by Todorov et al. (2008) for other lactobacilli, thus showing strain-dependence. The percents recorded could be related to cell surface components specific of the original strain (Ecmekçi et al., 2009; Klayraung, Viernstein, Sirithunyalug, & Okonogi, 2008).

The interactions between microorganisms and host cells are non-specific, yet there is a good correlation between surface hydrophobicity and ability to adhere to the intestinal mucosa in the case of probiotic strains. Although hydrophobicity may assist in adhesion, it is not a prerequisite for strong adhesion to human in- testinal cells (Todorov et al., 2008). Hydrophobicity varies among even genetically closely related species, and among strains within the same species (Schar-Zammaretti & Ubbink, 2003). Previous studies on the physical chemistry of microbial cell surfaces have unfolded the presence of glycoproteinaceous material that causes higher hydrophobicity, whereas hydrophilic surfaces are associated with presence of polysaccharides (Kos et al., 2003). The hydro- phobic characteristics of the surface of our lactobacilli were studied on the basis of their adhesion to the hydrocarbon phase in a solution containing xylene (Fig. 3) e and hydrophobicity phenotypes were duly identified. The highest hydrophobic features were revealed by FF28, 17.2b, P and B13 strains (≥20% of cells adhered to hydrocarbon), whereas intermediate ones were recorded for K, 607, 33 and O1 strains (15 and 20%) e similar to the control (LCS). Conversely, strains B95 and 69B entailed the lowest adhesion capacity (≤10%), lower than the control.

Analysis of these results, one concludes that there is no correlation between

auto-aggregation and hydrophobicity, also found by others using xylene (Zárate, Morata de Ambrosini, Chaia, & González, 2002) e but not by Darilmaz, Beyatli, and Yuksekdah (2012). However, the values obtained for the strains tested (except FF28 strain) were higher than those found for LCS strains, in agreement with previously reported values (Kiely & Olson, 2000). The lowest hydrophobicity values obtained unfold the influence of food source on the tendency for bacterial adhesion onto gut epithelial cells in the human intestine (Klayraung et al., 2008).

Food-associated lactobacilli that co-aggregate with pathogens are of special interest for potential applications, and co-aggregation appears to be a mechanism of adhesion preventing pathogens from colonizing the host tissue and thus contributing to the probiotic properties ascribed to LAB (del Re et al., 2000). Results of co- aggregation are expressed as percent reduction, after 4 h, in absorbance of a mixed suspension when compared with the individual suspension (Fig. 4). In our study, all strains proved able to co- aggregate with five enteropathogens (*S. aureus*, *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes* and *H. pylori*), yet this ability is a strain-dependent feature e as previous demonstrated by Collado, Meriluoto, and Salminen (2008). B95 strain demonstrated a marked co-aggregation with all pathogens (≥40%), but *S. aureus* exhibited the highest co-aggregation ability e with all LAB strains tested, and to a high level (≥30%). The lowest levels were observed toward *L. monocytogenes* (10-20%), whereas the behaviour of *H. pylori* was similar for all LAB strains.

Overall, our results suggest a wide variability among strains of *Lactobacillus* genus obtained from our olive fermentation broths, bearing properties consistent with likely adhesion to the intestine.

3.9. *Gastrointestinal transit tolerance*

Probiotic bacteria delivered using food systems as vehicles have first to survive transit through the upper gastrointestinal tract, and then persist in the gut e so as to provide therapeutic functions on the host (del Re et al., 2000). This study aimed to assess the transit tolerance of potential probiotic strains in human upper gastrointestinal tract where the microbiota encounter low pH and gastric enzymes in the stomach, followed by exposure to bile and pancreatic enzymes in the duodenum. Probiotics thus need to tolerate bile salts for colonization, survival and metabolic activity in the small intestine; otherwise they will not be able to exert a positive effect upon health and wellbeing of the host (Havenaar, Brink, & Huis In't Veld, 1992). According to Gilliland, Staley, and Bush (1984), 0.3% of bile is critical for screening of resistant strains. Our *in vitro* methodology that mimics *in vivo* human upper gastrointestinal transit was based on the work by Fernández de Palencia et al. (2008), with modifications. It considered three relevant factors during digestion: the effect of lysozyme; the influence of acid pH values, together with pepsin and

sequential gastric emptying at increasingly lower pH (reaching 1.8 units) and transit time of food through stomach (20 min for each pH value); and action of bile salts and pancreatin, coupled with sequential gastric delivery of bacteria to the intestine. Changes in total viable counts by the end of each stage of digestion were monitored. Good probiotic strains should withstand a pH of at least 3.0 (Fernandez, Boris, & Barbes, 2003).

Our results indicated that most gastric emptying will release a large number of viable probiotic cells into the intestine, and unfolded little differences between strains with regard to their sensitivity to gastric and intestinal secretions (Table 3). Our *Lacto- bacillus* strains showed ability to colonize the various compartments of the GIT for the duration of the study, and they demonstrated a small susceptibility through the three stages, with strain- and pH-dependencies pointing at an effective tolerance to stomach and small intestine environments. In general, they exhibited great resistance to gut enzymes, with high levels of viability compared to the initial inoculum (10^9 CFU/mL), and until step G5 no significant differences ($P > 0.05$) were observed. A significant impact on survival of strains occurred only at pH below 2 ($P > 0.05$) - which may thus be considered critical for selection of (potential) probiotics, as claimed by Zhou, Pan, Wang, and Li (2007).

The control used (LCS) showed a great sensitivity to more acidic conditions, with a reduction of viability to 10^3-10^4 CFU/mL. It has been proposed that damage of the bacterial cell envelope arising from a low pH could sensitize cells to bile action upon their membranes; above this value, significant numbers of viable bacteria reach the intestine from the gastric content e thus explaining the small decrease in viable cells (Callegari et al., 2006). Under intestinal conditions, statistical analysis indicated that the differences were strain-dependent ($P < 0.05$).

Strains 33, K, O1, 69B, 17.2b and B13 showed the higher survival percent (47.83, 29.55, 27.59, 25.00, 21.31%, and 12.89, respectively), compared with the other strains (FF28, 607, B95, and P) with 0.004, 0.074, 4.92, 6.53, respectively. The control (LCS) showed the best value, with 86.96% after *ca*. 180 min of dynamic gastrointestinal transit. The overall degree of survival in the gut relative to the initial population was higher than that observed by Bauptista-Galego et al. (2013).

3.10. *Antimicrobial activity*

Antimicrobial activity is an important feature related to pro- biotic performance. The role of lactobacilli within such dynamic ecosystems as the gastrointestinal tract, and in preventing colonization and infection caused by pathogenic organisms has been increasingly recognized (Soccol et al., 2010). This protective role against

gastrointestinal pathogens, as well as the underlying mechanisms have received special attention e and such an interaction has served as criterion for selecting new probiotics; mechanisms ascribed to probiotic action include competitive exclusion, production of antimicrobial compounds, modulation of immune response, alteration of intestinal bacterial metabolic activity, alteration of ecology and inhibition of bacterial translocation (Soccol et al., 2010).

Screening of the antagonistic activity of our lactobacilli was assessed against ten target pathogens by the disk diffusion method. The CFS displayed varying zones of inhibition, thus revealing different responses by pathogens (results not shown). In general, *E. coli* and *S.* Typhimurium were the most sensitive bacteria, with inhibition zones above 3 mm. These data are in agreement with those by Obadina, Oyewole, Sanni, and Tomlins (2006), who re- ported that *L. plantarum,* isolated from cassava fermentation, exhibited high but varying degree of inhibition of pathogens *S.* Typhimurium*, E. coli* and *S. aureus.*

Moreover, microtiter plate assay showed that CFS presented high levels of antimicrobial activity against enteropathogens (60e 86 % reduction of cell viability). In general terms, interactions were strain-specific, and the most effective strains were, in a ranking order, K > FF28 > 69B > O1 > B13 > B95 > 33; all of them exhibited significantly ($P < 0.05$) higher antimicrobial level (≥83% reduction of cell viability) than probiotic reference strain (LCS).

Statistical analysis showed that CFS from strains 17.2b, 33 and LCS were significantly ($P < 0.05$) more effective toward Gram- negative (*ca*. 84 % reduction of cell viability) than against Gram- positive target bacteria (*ca*. 78 % reduction of cell viability). For the remaining strains, no significant difference was observed.

On the other hand, when the CFS were adjusted to pH 7 and subjected to proteinase K treatment, most pathogens showed a significantly ($P < 0.05$) higher growth than those CFS untreated, as shown in Table 4. These results indicate that the compound(s) enrolled in antimicrobial activity of our lactic strains seem to be of a proteinaceous nature.

Conversely, addition of protease and pH adjustment did not produce a significant effect ($P < 0.05$) upon inhibitory activity of CFS from strain 607. Hence, the antimicrobial activity observed might be attributed to substances resistant to proteinase K cleavage and/or to non bacteriocin-like compounds (Bilkova, Kinova-Sepova, Bukovsky, & Bezakova, 2011). However, more studies are needed to clarify the nature of the antimicrobial compounds involved.

All these results suggest that these LAB can help reduce the incidence of enteropathogens tested, either acting directly on certain foods - e.g. when included in olive brines, or after accommodation in the intestine of the host following ingestion.

3.11. *Antibiotic resistance*

Screening for antibiotic resistance was via determination of MIC e as the lowest concentration of antimicrobial agent that inhibits naked eye-visible growth (Wiegand et al., 2008). Microbial cut-off values were according to EFSA (2012); for vancomycin and streptomycin, the criterion was that described by Danielson & Wind (2003).

The results tabulated in Table 5 indicate that isolated bacteria are more resistant to antibiotics of the amino-glucoside group (i.e. kanamycin, gentamycin and streptomycin) and glycopeptide group (i.e. vancomycin). Our results corroborate previous reports indicating that LAB are resistant to amino-glucoside antibiotics (Coppola at al., 2005; Flórez, Delgado, & Baltasar, 2005; Zhou, Pillidgec, Gopalc, & Gilla, 2005); according Rodriguez-Alonso, Fernández-Otero, Centeno, and Garaball (2009), this may be due to hampering protein formation in invading bacteria. Resistance to such antibiotics is usually intrinsic and non transmissible, so the corresponding genes will not be transferred to pathogens (Ammor, Belén, & Mayo, 2007). On the other hand, antibiotic-resistant pro- biotic strains may benefit patients with unbalanced intestinal microbiota, or greatly reduced in viable numbers due to administration of a variety of antimicrobial agents (Salminen et al., 1998). Also the plant origin of our strains implies that no contact with antibiotics has occurred, and may explain the resistance as an intrinsic characteristic e unlike the isolates from humans and animals.

Vancomycin is a glycopeptide that inhibits bacterial cell wall synthesis by interfering with glycopeptide synthesis, but raises a rather important concern because it is one of the latest antibiotics broadly efficacious in treating clinical infections caused by other- wise multidrug-resistant pathogens (Woodford, Johnson, Morrison, & Speller, 1995). All strains showed resistance to vancomycin. Although Gram-positive bacteria (including LAB) are especially vulnerable to vancomycin (Reynolds, 1989), *Lactobacilli, Pediococci* and *Leuconostoc* spp. have been reported to possess a high natural resistance to vancomycin e due to presence of D-Ala-D-lactate in their peptidoglycan, instead of the regular dipeptide D-Ala-D-Ala (Ammor et al., 2008; Ashraf & Shah, 2011); this property is useful to discriminate them from other Gram-positive bacteria (Hamilton- Miller & Shah, 1998). However, the high frequency of occurrence of vancomycin resistance among lactobacilli is distinct from the inducible, transferable mechanism observed in enterococci (Klein et al., 2000). In our case, all strains tested were resistant to that antibiotic, in agreement with the expected native resistance of strains (Salminen et al., 1998).

All strains were classified as sensitive to erythromycin, which belongs to the macrolide group e as referred by Ammor et al. (2007) for *Lactobacillus* species that are susceptible chiefly to antibiotics inhibiting synthesis of proteins. Our results indicated that resistance (3) and susceptibility (8) to tetracycline seems

to be species-dependent, with *L. plantarum* being susceptible; the resistance showed by *L. paraplantarum* strains was also mentioned previously (Inkao & Yajime, 2012; Rabia & Shah, 2011). This class of antibiotics bind to ribosomes, blocks protein synthesis (bacterio- static) and are effective against Gram-positive microorganisms (Liasi et al., 2009). For ampicillin and chloramphenicol, all strains tested were classified as susceptible, except LCS strain that was resistant to chloramphenicol.

The natural resistance of strains to multiple classes of antibiotics is probably due to cell wall structure and membrane impermeability, complemented in some cases by their efflux mechanisms (Ammor et al., 2007). This feature might represent a competitive advantage, especially when a probiotic product is administered with antimicrobials for treatment of an infectious disease e thereby reducing likelihood of disbiosis, and rapidly rebalancing normal microbiota.

3.12. *Caco-2 cell adhesion*

Adhesion of probiotic strains to such intestinal cells as Caco- 2 cells is believed to play a critical role upon increasing the possi- bility of colonization of the GIT and survival in such a hostile environment. It has previously been claimed (Darilmaz, Aslım, Suludere, & Akca, 2011) that digestive conditions may affect bac- terial adhesion, under regular conditions, in a strain-dependent manner. The original impetus to screen for good adhesive strains was the hypothesis that adherent strains would easily colonize the intestine, in particular the small one (Ouwehand & Salminen, 2003). The ability of LAB to adhere to mucosal surfaces prevents their rapid removal by gut contraction, and subsequent peristaltic flow of digest, and may accordingly confer a competitive advantage. Quantitative binding of *Lactobacillus* was investigated on Caco-2 cell lines by colony count using MRS agar after trypsinization e since it permits enumeration of bacteria attached to the cells. All test cultures adhered to Caco-2 cell lines, albeit to different extents (Fig. 5). The adhesion ability ranged from 3.4 to 15.2%, with 33, 17.2b and 607 strains showing the highest levels of adherence (15.2, 12.9 and 9.7%, respectively) when compared to the control strain e whereas O1 and K strains showed the lowest ability (3.4 and 3.6%, respectively), similar to LCS (control) with 4.0%.

Several studies involving *Lactobacilli* strains and Caco-2 cells have been previously published, but slightly higher adhesion levels were reported. Lehto and Salminen (1997) reported adhesion of *Lactobacillus* GG and *L. rhamnosus* LC-705 to Caco-2 culture as 10e 12%. In a related study, Tuomola and Salminen (1998) screened 12 *Lactobacillus* strains for adhesion to Caco-2 cell line, and concluded that the most adhesive strains were *L. casei* (Fyos®), *L. acidophilus* 1 (LC1®), *L. rhamnosus* LC-705 and *Lactobacillus* GG (ATCC 53103) - with values in the range 9-14%. However, the adhesion levels obtained in our work are not strictly comparable because of topical

differences in assay procedures.

It should be emphasized that most models used to assess adhesion *in vitro* of probiotics represent simplifications of *in vivo* conditions, so great care it to be taken when extrapolating from *in vitro* observations to *in vivo* environments. Transient adhesion and colonization of probiotics is thus to be determined *in vivo*, although a clear correlation between *in vitro* and *in vivo* adhesion has been reported for six strains of propionic bacteria using experimental animals (Zárate et al., 2002).

3.13. *Statistical analysis*

In order to compare the experimental data obtained for such probiotic parameters as auto-aggregation (AA), hydrophobicity (H), co-aggregation with pathogens (CoA) and simulated digestion (GS), a multivariate analysis (Principal Component Analysis) was per- formed with the 11 LAB strains. The first three components could explain 91.5% of the total variance. A tridimensional plot is depicted in Fig. 6, where the distribution of LAB strains and the contribution of the various parameters to discrimination between samples are apparent.

The discrimination of samples along PC1 is due mainly to CoAE, CoAa, CoAs, CoAh and CoAl variables; samples are discriminated along PC2 by H and AA values, whereas discrimination along PC3 is due to GS. The control strain (LCS) displays lower values for CoAs, CoAe and CoAl, while GS has higher values.

In summary, strains did not show a great variability in terms of GS; only strains B95 and 69B could be told apart from the other strains, and the variables contributing mainly to that behaviour are GS, CoA and AA.

4. Conclusions

Our research effort demonstrated that LAB strains isolated from Portuguese olive fermentations are potential candidates for a probiotic culture, and consequently for future development of novel health-promoting foods from plant origin. Concerning safety and adherence, their characteristics measured may be advantageous toward successful colonization of, and competition in the GIT. Resistance of our probiotic strains to antibiotics may be used with both preventive and therapeutic purposes, in attempts to control intestinal infection. However, further testing to clarify the nature of antibiotic resistance is in order. All strains passed most *in vitro* tests recommended by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2002); therefore, they may readily undergo complementary *in vivo* studies to support claims prior to eventual marketing.

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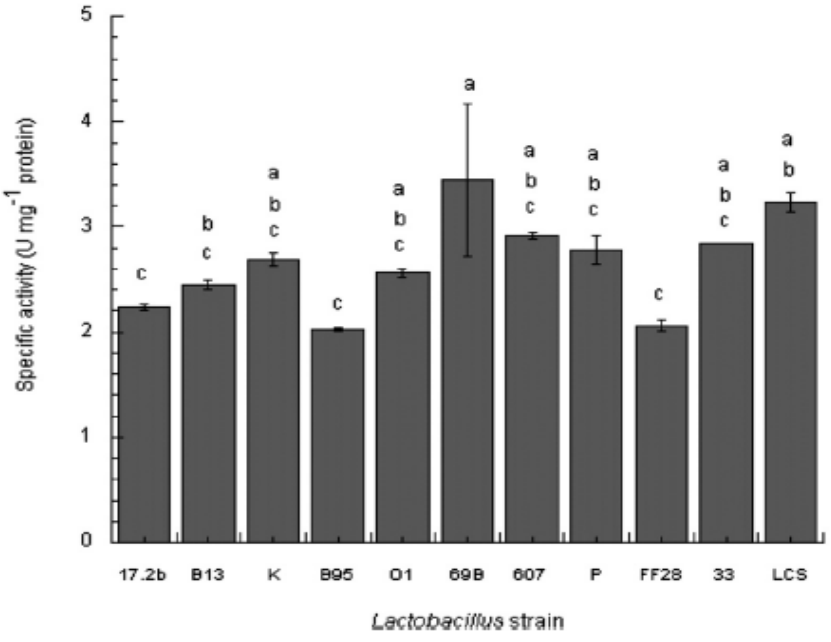

Fig. 1. Specific proteolytic activity (U mg^{-1} protein) of cell-free supernatants of *Lactobacillus* strains, determined by azocasein method (mean ± standard deviation). Bars with no common letters differ significantly ($P \leq 0.05$) from each other.

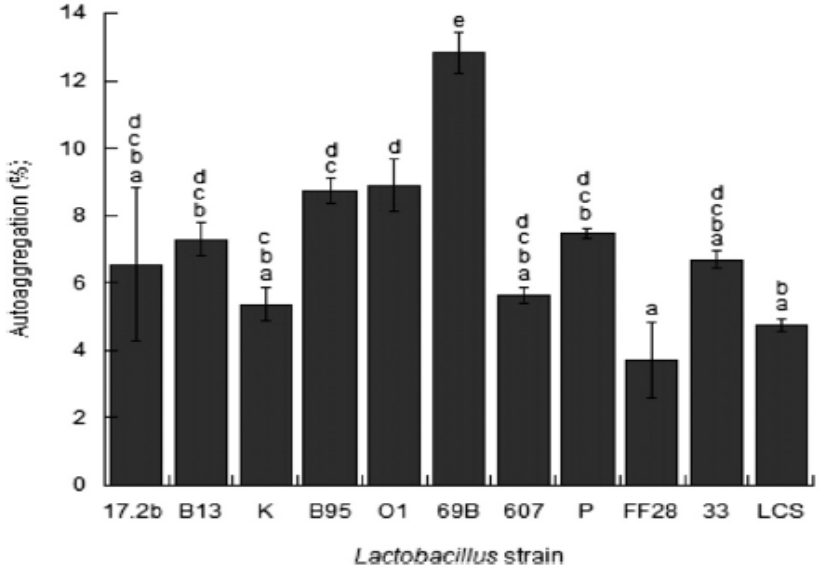

Fig. 2. Percent of auto-aggregation (%) of *Lactobacillus* strains (mean ± standard deviation, $n = 3$). Bars with no common letters differ significantly ($P \leq 0.05$) from each other.

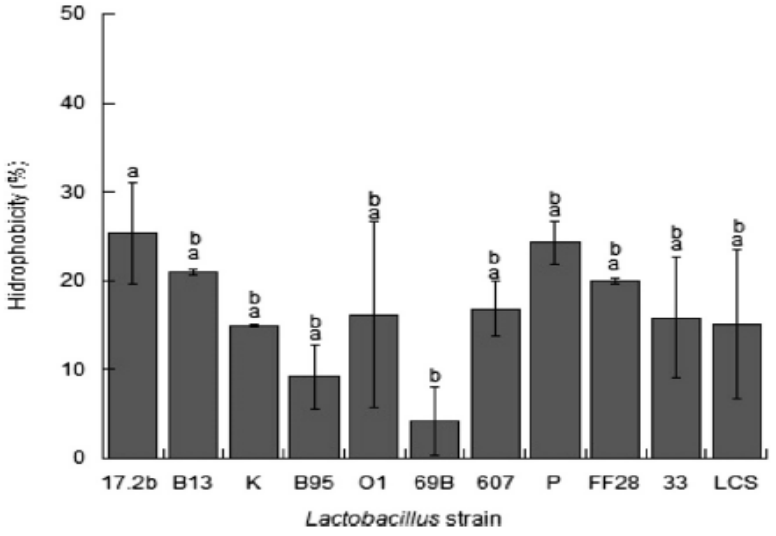

Fig. 3. Percent of surface hydrophobicity (%) of *Lactobacillus* strains assessed. Mean (±standard deviation, $n = 3$). Bars with no common letters differ significantly ($P \leq 0.05$) from each other.

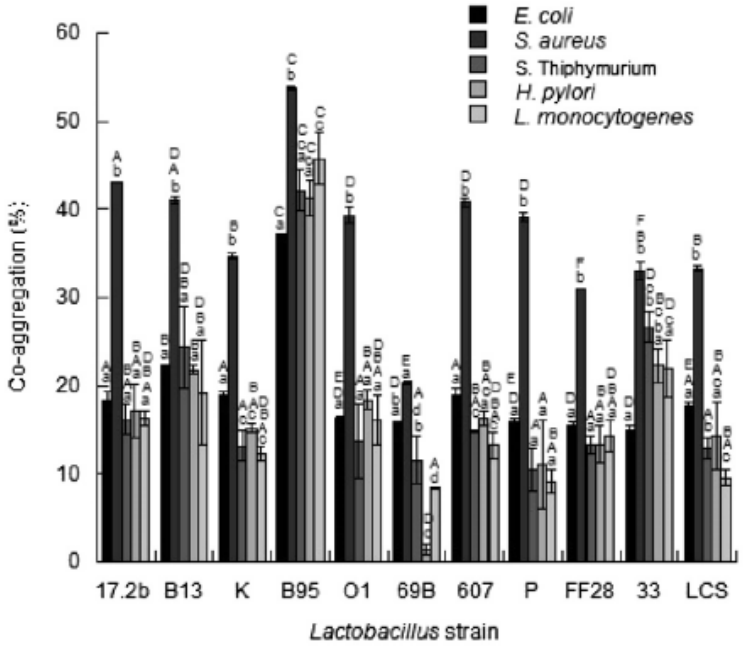

Fig. 4. Percent of co-aggregation (%) of *Lactobacillus* strains with potential gut pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Helicobacter pylori* and *Listeria monocytogenes*) after 5 h incubation at room temperature in PBS at pH 7.2. (Mean ± standard deviation, $n = 3$). Bars with no common letter differ significantly ($P \leq 0.05$) from each other; lower case letters pertain to differences between pathogens for each *Lactobacillus* strain, and capital letters pertain to differences of each pathogen between *Lactobacillus* strains.

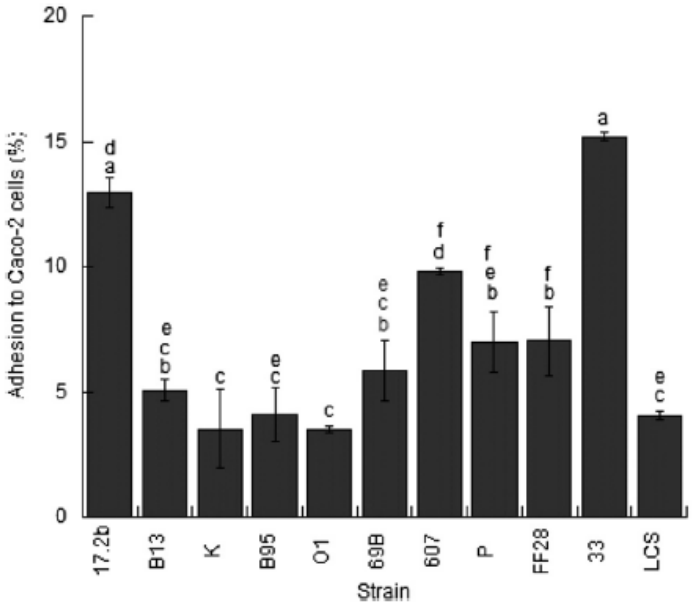

Fig. 5. Efficiency of adhesion expressed as ratio (%) of lactobacilli viable cells that remained adhered to Caco-2 enterocytes (mean ± standard deviation, $n = 3$). LCS e positive control. Bars with no common letter differ significantly ($P \leq 0.05$) from each other.

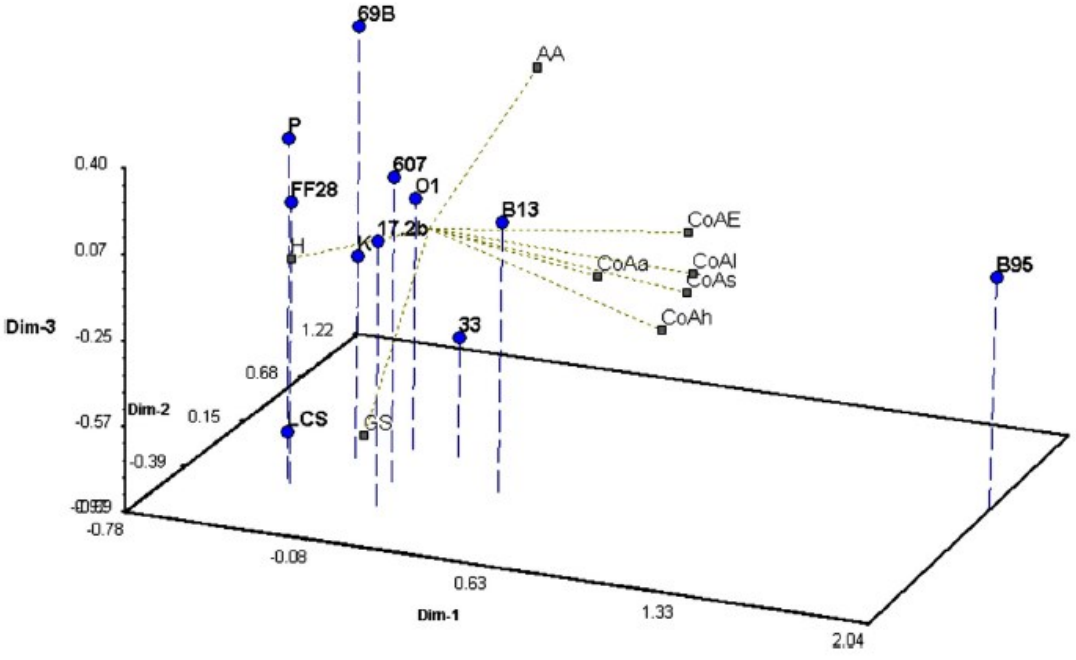

Fig. 6. Biplo principal component analysis, expressed as biplot mapping onto the first two principal components.

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

Table 2

Hydroxyityrosol and oleuropein contents via diode array detection (280 nm).

	Sample	Hydroxytyrosol [Mg/l]	Oleuropein [Mg/l]
Control	Medium without Oleuropein	–	–
	Medium with Oleuropein	86.1	12,065.5
Strain	17.2b	518.0	1255
	B13	471.8	1286
	K	485.1	1286
	B95	466.0	1290
	O1	565.6	1280
	69B	469.2	1314
	607	541.4	1239
	P	529.2	1273
	FF28	413.2	1171
	33	476.0	1171
	LCS	489.3	1204

Table 3

Viable numbers [Log10 (CFU/mL)] at various stages (G1 to Gi5) within dynamic *in vitro* model, and survival rates (%) of *Lactobacillus* strains after 2.4 h of exposure to simulated gastric fluid and intestinal fluid in sequential digestion, via plate counting. Equal lower case subscript letters indicate no significant differences between digestion steps for each strain; equal capital subscript letters indicate no significant differences between strains, in each digestion step e both according to Tukey's test ($P > 0.05$).

Strains	G1	G2	G3	G4	G5	G6	G7	Gi3	Gi4	Gi5	Survival (%)
17.2b	9.77 ± 0.14 a,A,B	9.84 ± 0.17 a,A,B,C	9.85 ± 0.11 a,A	9.89 ± 0.09 a,A,B	9.83 ± 0.09 a,A	6.00 ± 0.00 c,A,B	4.02 ± 0.17 d,A,B	9.67 ± 0.02 a,A	9.54 ± 0.07 a,b,A	9.13 ± 0.02 b,A,B	21.31
B13	9.63 ± 0.63 a,A,B	9.37 ± 0.56 a,C,D	9.35 ± 0.59 a,A,B,C	9.18 ± 0.78 a,A,B,C	9.09 ± 0.84 a,A,B	9.27 ± 0.02 a,A	6.56 ± 0.02 b,A	9.18 ± 0.08 a,B	9.11 ± 0.04 a,A,B	8.99 ± 0.04 a,A,B	12.89
K	9.64 ± 0.11 a,A,B	9.49 ± 0.11 a,B,C	9.54 ± 0.08 a,A,B	9.58 ± 0.12 a,A,B	9.62 ± 0.07 a,A	7.22 ± 0.01 d,A	3.82 ± 0.10 e,A,B	8.68 ± 0.03 c,D,E	9.43 ± 0.08 a,b,A	9.11 ± 0.11 a,A,B	29.54
B95	10.13 ± 0.11 a,A	9.83 ± 0.07 b,A,B,C	9.78 ± 0.13 b,A	9.69 ± 0.13 b,A,B	10.19 ± 0.07 a,A	7.45 ± 0.02 d,A	6.01 ± 0.00 e,A	8.82 ± 0.02 c,C,D	8.65 ± 0.06 c,A,B,C	8.81 ± 0.03 c,A,B	4.92
O1	9.93 ± 0.10 a,A	9.70 ± 0.08 a,b,A,B,C	9.69 ± 0.09 a,b,A,B	9.83 ± 0.11 a,A	9.61 ± 0.04 a,b,A	6.57 ± 0.22 d,A,B	5.55 ± 0.11 e,A,B	8.97 ± 0.08 c,B,C,D	8.92 ± 0.14 c,A,B,C	9.38 ± 0.03 b,A	27.58
69B	9.82 ± 0.11 a,A,B	9.64 ± 0.07 b,A,B,C	9.67 ± 0.10 a,b,A,B	9.60 ± 0.03 a,b,A,B	9.60 ± 0.02 b,A	6.83 ± 0.01 d,A	4.75 ± 0.01 e,A,B	9.02 ± 0.04 c,B,C	9.16 ± 0.01 c,A	9.21 ± 0.01 c,A	25.00
607	9.37 ± 0.06 a,B	9.45 ± 0.14 a,B,C	8.73 ± 0.08 b,C	8.45 ± 0.51 b,c,C	7.96 ± 0.37 c,B	6.16 ± 0.05 d,A,B	3.75 ± 0.38 d,A,B	6.88 ± 0.02 d,F	6.74 ± 0.01 d,D	6.24 ± 0.02 d,C	0.074
P	9.86 ± 0.08 a,b,A,B	9.89 ± 0.06 a,A,B	9.79 ± 0.09 a,b,A	9.63 ± 0.01 a,b,A,B	9.51 ± 0.06 b,A	5.79 ± 0.08 e,A,B	4.59 ± 0.16 f,A,B	8.39 ± 0.06 d,E	8.98 ± 0.01 c,A,B,C	8.62 ± 0.31 c,d,A,B	6.52
FF28	10.15 ± 0.14 a,A	10.10 ± 0.10 a,A	10.07 ± 0.11 a,A	9.99 ± 0.00 a,A	9.92 ± 0.04 a,A	6.88 ± 0.09 c,A	5.88 ± 0.09 d,A	8.42 ± 0.17 b,E	8.14 ± 0.02 b,C	5.74 ± 0.06 d,C	0.004
33	9.38 ± 0.02 a,D	9.34 ± 0.08 a,C,B	8.67 ± 0.69 a,b,C	8.11 ± 0.33 b,C	9.05 ± 0.02 a,b,A,B	5.99 ± 0.02 c,A,B	3.54 ± 0.09 d,A,B	8.74 ± 0.01 a,b,C,D	8.89 ± 0.01 a,b,A,B,C	8.45 ± 0.12 a,b,A,B	47.82
LCS	8.59 ± 0.14 a,C	8.86 ± 0.14 a,D	8.85 ± 0.14 a,B,C	8.94 ± 0.08 a,B,C	9.17 ± 0.42 a,A	1.89 ± 3.28 b,B	1.64 ± 2.84 b,B	8.79 ± 0.10 a,C,D	8.26 ± 0.57 a,B,C	8.24 ± 0.61 a,B	86.95

Table 4

Antagonistic activity of cell-free supernatant (CFS) of *Lactobacillus* strains against Gram-positive and -negative pathogens via plating on MRS broth in microplates. Strain survival (%) was via absorbance at 600 nm (initial and final OD growth), with and without proteinase K (mean ± standard deviation, n ¼ 3). Different lower case subscript letters for each CFS indicate that treatment with proteinase K was significantly different ($P < 0.05$) compared to treatment without proteinase K, for each pathogen.

LAB (CFS)	Proteinase K	Pathogen strain survival (%)						
		Listeria monocytogenes ATCC 7644	*Pseudomonas aeruginosa* ATCC 9027	*Bacillus subtilis* ATCC 6538	*Escherichia coli* ATCC 8739	*Salmonella* Typhimurium ATCC 14028	*Staphylococcus aureus* ATCC 6538	*Enterococcus faecalis* ATCC 29212
17.2b	With	55.19 ± 5.22^a	50.45 ± 8.67^a	61.64 ± 8.37^a	62.01 ± 10.17^a	57.57 ± 8.10^a	56.36 ± 5.39^a	55.50 ± 6.08^a
	Without	27.02 ± 1.15^b	15.67 ± 1.96^b	25.98 ± 2.48^b	23.76 ± 4.73^b	19.34 ± 2.31^b	31.05 ± 3.77^b	25.22 ± 0.52^b
B13	With	36.86 ± 0.60^a	30.08 ± 0.48^a	42.14 ± 6.28^a	41.58 ± 0.65^a	44.48 ± 9.10^a	40.93 ± 0.52^a	36.88 ± 1.69^a
	Without	14.24 ± 0.97^b	12.19 ± 1.98^b	15.89 ± 0.33^b	17.89 ± 0.57^b	16.49 ± 0.00^b	18.42 ± 1.26^b	15.38 ± 0.65^b
K	With	37.54 ± 3.21^a	35.69 ± 8.43^a	40.55 ± 8.58^a	41.19 ± 1.68^a	45.74 ± 8.88^a	42.49 ± 8.15^a	44.63 ± 3.04^a
	Without	13.92 ± 0.15^b	10.88 ± 0.12^a	13.91 ± 0.29^b	16.97 ± 1.77^b	15.23 ± 1.32^b	15.86 ± 1.27^b	14.32 ± 0.25^b
B95	With	32.73 ± 4.54^a	34.17 ± 4.91^a	37.44 ± 2.80^a	35.98 ± 8.64^a	44.03 ± 8.46^a	35.47 ± 3.02^a	38.53 ± 1.48^a
	Without	18.94 ± 3.29^a	12.00 ± 0.74^b	17.81 ± 1.58^b	16.64 ± 0.59^a	16.11 ± 0.61^b	18.77 ± 2.21^b	15.37 ± 1.43^b
O1	With	40.63 ± 4.15^a	34.32 ± 6.91^a	43.50 ± 4.90^a	38.23 ± 2.47^a	40.06 ± 4.29^a	40.99 ± 4.22^a	40.01 ± 5.03^a
	Without	14.15 ± 0.48^b	12.29 ± 1.19^b	15.35 ± 0.12^b	16.77 ± 1.65^b	16.10 ± 0.21^b	16.78 ± 0.02^b	14.87 ± 0.06^b
69B	With	40.31 ± 5.71^a	37.42 ± 7.02^a	47.31 ± 4.21^a	43.29 ± 1.26^a	48.90 ± 4.78^a	47.98 ± 7.93^a	39.27 ± 3.60^a
	Without	13.89 ± 0.47^b	11.20 ± 0.36^b	16.09 ± 0.69^b	16.65 ± 1.36^b	16.09 ± 0.20^b	17.02 ± 2.26^b	15.65 ± 1.10^b
607	With	37.13 ± 6.21^a	21.01 ± 1.98^a	35.39 ± 1.91^a	39.56 ± 2.83^a	41.45 ± 0.89^a	45.39 ± 6.10^a	33.91 ± 4.15^a
	Without	23.73 ± 2.92^a	15.74 ± 0.18^a	22.02 ± 7.60^a	30.51 ± 4.22^a	20.65 ± 1.70^b	29.63 ± 3.49^a	25.64 ± 1.71^a
P	With	70.88 ± 3.49^a	82.58 ± 1.53^a	84.49 ± 3.86^a	83.76 ± 4.80^a	97.19 ± 2.94^a	85.12 ± 3.41^a	84.16 ± 1.31^a
	Without	37.72 ± 2.53^b	31.77 ± 4.72^b	42.18 ± 5.29^b	46.51 ± 3.01^b	39.28 ± 4.08^b	54.00 ± 5.97^b	42.25 ± 3.58^b
FF28	With	32.39 ± 4.15^a	31.97 ± 0.88^a	40.41 ± 4.88^a	35.87 ± 0.50^a	39.96 ± 1.54^a	36.94 ± 1.40^a	35.49 ± 0.64^a
	Without	14.86 ± 0.30^b	12.26 ± 1.10^b	15.70 ± 0.51^b	15.69 ± 1.88^b	15.65 ± 0.00^b	16.22 ± 1.47^b	14.76 ± 1.85^b
33	With	42.09 ± 8.45^a	35.89 ± 8.20^a	48.99 ± 2.32^a	39.51 ± 4.07^a	50.61 ± 3.36^a	46.28 ± 5.31^a	51.39 ± 3.54^a
	Without	18.74 ± 1.44^a	12.17 ± 0.02^a	15.49 ± 0.50^b	16.70 ± 0.77^b	15.80 ± 0.60^b	20.35 ± 5.93^b	17.64 ± 0.53^b
LCS	With	41.97 ± 7.05^a	37.58 ± 0.05^a	40.65 ± 1.85^a	39.45 ± 6.69^a	44.77 ± 2.54^a	40.73 ± 5.02^a	36.82 ± 0.72^a
	Without	22.53 ± 1.20^a	11.52 ± 0.81^b	17.14 ± 0.14^b	18.06 ± 0.30^b	16.69 ± 0.62^b	22.67 ± 2.65^b	18.06 ± 0.24^b

Table 5
Antibiotic susceptibility (in minimum inhibitory concentration, MIC) of tested isolates of *Lactobacillus* spp

Strain	MICs* of antibiotic agents (μg/mL)							
	Erythromycin	Chloramphenicol	Tetracycline	Gentamicin	Kanamycin	Streptomycin	Ampicillin	Vancomycin
17.2 b	$<2^S$	4^S	8^S	512^R	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
B13	$<2^S$	4^S	16^R	$\geq 1024^R$	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
K	$<2^S$	4^S	32^R	512^R	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
B95	$<2^S$	4^S	16^S	$\geq 1024^R$	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
O1	$<2^S$	4^S	16^R	$\geq 1024^R$	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
69B	$<2^S$	4^S	16^S	512^R	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
607	$<2^S$	4^S	16^S	512^R	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
P	$<2^S$	4^S	16^S	512^R	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
FF28	$<2^S$	4^S	8^S	512^R	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
33	$<2^S$	4^S	16^S	512^R	$\geq 1024^R$	$\geq 1024^R$	4^S	$\geq 1024^R$
LCS	$<2^S$	16^R	16^R	$\geq 1024^R$	$\geq 1024^R$	1024^R	4^S	$\geq 1024^R$

Resistance rates calculated according to cut-off values [Danielsen & Wind (2003) for vancomycin and streptomycin, and EFSA (2012)].
*MIC: minimum inhibitory concentration.
S – Susceptible; R – Resistant.