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Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses

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ABSTRACT

Ninety-eight *Lactobacillus plantarum* strains isolated from Italian and Argentinean cheeses were evaluated for probiotic potential. After a preliminary subtractive screening based on the presence of *msa* and *bsh* genes, 27 strains were characterized. In general, the selected strains showed high resistance to lysozyme, good adaptation to simulated gastric juice, and a moderate to low bile tolerance. The capacity to agglutinate yeast cells in a mannose-specific manner, as well as the cell surface hydrophobicity was found to be variable among strains. Very high β-galactosidase activity was shown by a considerable number of the tested strains, whereas variable prebiotic utilization ability was observed. Only tetracycline resistance was observed in two highly resistant strains which harbored the *tet*M gene, whereas none of the strains showed β-glucuronidase activity or was capable of inhibiting pathogens. Three strains (Lp790, Lp813, and Lp998) were tested by *in vivo* trials. A considerable heterogeneity was found among a number of *L. plantarum* strains screened in this study, leading to the design of multiple cultures to cooperatively link strains showing the widest range of useful traits. Among the selected strains, Lp790, Lp813, and Lp998 showed the best probiotic potential and would be promising candidates for inclusion as starter cultures for the manufacture of probiotic fermented foods.

1. Introduction

Probiotics are non-pathogenic microorganisms that, when ingested in adequate amounts, exert a positive influence on their host's health (FAO/WHO, 2006). A variety of microorganisms, typically food grade lactic acid bacteria (LAB), have been evaluated for their probiotic potential and are applied as adjunct cultures in various types of food products or in therapeutic preparations (Rodgers, 2008). Within the genus *Lactobacillus*, *Lactobacillus plantarum* is a member of the facultatively heterofermentative group of lactobacilli. It is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including dairy, meat, fish, and many vegetable or plant fermentations. *L. plantarum* strains have also been found in many cheese varieties. Moreover, strains of *L. plantarum* have proven ability to survive gastric transit and colonize the intestinal tract of humans and other mammals (De Vries et al., 2006; Mathara et al., 2008; Georgieva et al., 2009). Various special therapeutic or prophylactic properties have been associated with *L. plantarum*, such as reduced incidence of diarrhea in daycare centers, reduced pain and constipation associated with irritable bowel syndrome, reduced bloating, flatulence, ability to displace enteropathogens from Caco-2 cells, and capacity to exert positive effect on immunity in HIV^+ children (Parvez et al., 2006; Candela et al., 2008).

The available genome sequence of *L. plantarum* WCSF1, which has been sequenced and annotated (Kleerebezem et al., 2003), generated a major advantage for molecular investigation of this bacterium's behavior in the GIT and its potential probiotic features.

Although the selection criteria for probiotic strains for humans should include a human origin, currently many non-starter LAB (NSLAB) such as *L. paracasei* and *L. plantarum*, which constitute the majority of NSLAB found in most ripened cheese varieties (Pisano et al., 2008), are used in commercial probiotic products. Among industrially used probiotics, selected *L. plantarum* strains are available in the market (De Vries et al., 2006). The search for strains which show resistance to biological barriers of the human GIT, and which possess physiological characteristics compatible with probiotic properties among LAB isolated from food, may eventually lead to the finding of new probiotic strains for functional food products (Bude-Ugarte et al., 2006).

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As part of the selection of new probiotic candidates, 98 *L. plantarum* strains, isolated within the numerically predominant cultivable species from Italian and Argentinean cheeses and genotypically biodiverse, were subjected to a series of *in vitro* (following FAO/WHO guidelines, 2006) and *in vivo* analyses to assess their probiotic properties.

2. Materials and methods

2.1. Bacterial strains, yeast cells, culture media, and growth conditions

Ninety-eight *L. plantarum* strains isolated from Italian and Argentinean cheeses were included in this study. The type strain ATCC 14917^T and the strain LMG 9211 were obtained from the American Type Culture Collection (Rockville, MD) and the Laboratory of Microbiology of Gent University (Gent, Belgium), respectively and were reactivated overnight at 30 °C in MRS (Merck, Darmstadt, Germany) broth. *Saccharomyces cerevisiae* was grown overnight in TSB (Tryptone Soya Broth, Oxoid, Basingstoke, England) medium under shaking conditions at 30 °C. Bacterial and yeast cells were maintained as frozen stocks at −80 °C in the presence of 150 ml/l glycerol as cryoprotective agent.

2.2. Preliminary selection by PCR amplification

The *L. plantarum* strains were tested by PCR to search for the presence of *bsh* and *msa* genes encoding for the bile salt hydrolase (BSH) and the mannose-specific adhesin (MSA), respectively. PCR primers were designed on the basis of the gene sequences of *bsh* (GenBank accession number AL935262.1) and *msa* (GenBank accession number AL935255.1) of *L. plantarum* WCFS1. The primers had the following sequences: 5′–CGTATCCAAGTGCTCATGGTTTAA–3′ (bsh for, nucleotide position 150568 to 150593 of the *bsh* gene), 5′–ATGTGTACTGCCATAACTTATCAATCTT–3′ (bsh rev, nucleotide position 151487 to 151460), 5′–GCTATTATGGGGATTACGTTG–3′ (msa for, nucleotide position 133061 to 133081 of the *msa* gene) and 5′–CTGTCTTGACAATAGCCATATA–3′ (msa rev, nucleotide position 134801 to 134781). The primers were designed to obtain PCR product lengths of 919 bp (for *bsh*) and 1740 bp (for *msa*). The strain LMG 9211 was used as positive control for both PCR amplifications.

PCR amplifications were performed in 20 μl volumes with 0.5 μM of each primer (Biotez, Berlin, Germany), 2.5 units/100 μl of AmpliTaq DNA polymerase (Applied Biosystems, Monza, Italy), 1.5 mM $MgCl_2$, 20 ng of total DNA, and 200 μM of each dNTP. DNA amplifications were performed by a Perkin Elmer thermal cycler (mod. 9700, Applied Biosystems) and were set as follows: 4 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 64 °C (bsh for/rev) or 52 °C (msa for/rev), and 1 min at 72 °C; the final extension step consisted of 7 min at 72 °C. The PCR products were separated on a 1% agarose gel in Tris-Acetate EDTA buffer (TAE: 40 mM Tris-Acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under UV light.

2.3. Lysozyme resistance

Cells of *L. plantarum* strains grown overnight in 10 ml MRS broth at 30 °C, were pelleted by centrifugation, washed twice with phosphate buffer (0.1 M, pH 7.0), and resuspended in 2 ml of Ringer solution (Sigma–Aldrich, Milan, Italy). To simulate the *in vivo* dilution by saliva, 10% of the bacterial suspensions were inoculated in a sterile electrolyte solution (SES; 0.22 g/l $CaCl_2$, 6.2 g/l NaCl, 2.2 g/l KCl, 1.2 g/l $NaHCO_3$) in the presence of 100 mg/l of lysozyme (Sigma–Aldrich) according to Vizoso-Pinto et al. (2006). Bacterial suspensions in SES without lysozyme were included as controls. Samples were incubated at 37 °C and microbial counts after 30 and 120 min were carried out on MRS agar (48 h; 30 °C). Survival rate was calculated as percentage of the CFU/ml after 30 and 120 min compared to the CFU/ml at time 0.

2.4. Bile resistance

The ability of the strains to grow in the presence of 0.3%, 0.5% and 1.0% of bile (w/v) was determined according to the method of Vinderola and Reinheimer (2003). The results were expressed as the percentage of growth compared to the control.

2.5. Tolerance to simulated gastric juice

Cells of *L. plantarum* strains grown overnight were pelleted by centrifugation, washed twice with phosphate buffer (0.1 M, pH 7.0), resuspended in SES and was immediately added to the same volume of "gastric" solution [0.6% (w/v) pepsin (Merck, Germany), 1% (w/v) NaCl]. Cell suspensions were immediately placed in a water bath (37 °C) and gradually acidified, under gentle agitation, from pH 5.0 to 2.2 in 90 min, according to Marteau et al. (1997) and Blanquet et al. (2004). Cell counts on MRS agar were performed at time 0, 30, 60, 70, 80, and 90 min. After 90 min, an aliquot of each cell suspension was taken, pelleted by centrifugation and the cells were resuspended in phosphate buffer (0.1 M, pH 8.0) containing 0.3% and 0.1% (w/v) of bile and pancreatin (Sigma–Aldrich), respectively. The cells were kept for further 60 min at 34 °C and then counted. Washed cells resuspended in phosphate buffer and subjected to the same conditions as treated samples were used as controls. Survival rate was calculated as percentage of the CFU/ml after 30, 60, 70, 80, 90 min and 150 min compared to the CFU/ml at time 0.

2.6. BSH activity

BSH activity of the cultures was detected using a plate screening procedure according to Nguyen et al. (2007) with minor modifications. Briefly, overnight cultures were spotted on MRS agar plates containing 0.37 g/l $CaCl_2$ and 0.5% sodium salt of taurodeoxycholic acid (TDCA) or 0.5% sodium salt of glycodeoxycholic acid (GDCA) (Sigma–Aldrich). Plates were anaerobically incubated at 37 °C for 72 h. The presence of halos around colonies or white opaque colonies indicated BSH activity. *Enterococcus faecium* LMG 16170 was used as BSH-positive strain. The inoculum of each strain in MRS without supplementation was included as negative control.

2.7. Yeast agglutination

This assay was conducted as described previously by Pretzer et al. (2005) with slight modifications. Bacterial strains were grown overnight, washed and suspended in 0.1 ml of phosphate buffer saline (PBS, 0.1 M, pH 7.2). Fifty μl of a fourfold dilution of the initial bacterial suspensions in PBS were transferred to microtiter plates. To each well, 50 μl buffer or buffer with methyl-α-D-mannopyranoside (final concentration 100 mM; Sigma–Aldrich) were added as well as 100 μl of 1% *S. cerevisiae* suspended cells in PBS. The microtiter plates were shaken for 30 min at 28 °C. The ability of each strain to induce visible yeast cell agglutination was evaluated by bright-light microscopy at 40-fold magnification. Agglutination with or without the addition of methyl-α-D-mannopyranoside was compared to evaluate the involvement of mannose in the adhesion mechanism.

2.8. Surface hydrophobicity

To evaluate the surface hydrophobicity, the ability of the cells to adhere to hydrocarbon was determined according to the method of Vinderola and Reinheimer (2003).

2.9. Antimicrobial activity

Escherichia coli V517 (INLAIN Collection), *Salmonella enteritidis* OMS-Ca (INLAIN Collection), *Listeria monocytogenes* ATCC 15313 and *Staphylococcus aureus* 76 (INLAIN Collection) were included as pathogens in inhibition assays. Overnight cultures grown in TSB (Britania, Buenos Aires, Argentina) or BHI (Brain Heart Infusion, Britania, for *Listeria*) broth were inoculated (2% v/v) in Tryptone Soya Agar (TSA) or BHI agar (melted and cooled to 45–50 °C). After vigorous homogenization, cell suspensions were poured in 160 mm Petri dishes. Six wells (10 mm) were made on each agar plate. Overnight cultures of *L. plantarum* strains were centrifuged and the supernatants were recovered. A sample of each supernatant was separated for use as non-treated control. The remaining supernatant was neutralized (NaOH pellets, Mallinckrodt, New York, USA), filter-sterilized (0.45 μm, Millipore, Cork, Ireland), and kept at −70 °C for further use. To different wells, 180 μl of each supernatant, neutralized or not, were added. Petri dishes were incubated 24 h at 37 °C and the diameters of the halos of inhibition were recorded (Vinderola et al., 2008).

2.10. Enzymatic activities

The o-nitrophenyl-ß-D-galactopyranoside (ONPG) substrate was used to determine the β-galactosidase activity as described by Vinderola and Reinheimer (2003). The β-glucuronidase activity was performed using the API-ZYM Kit (BioMérieux, Rome, Italy). Readings were made according to the manufacturer's indications.

2.11. Determination of antibiotic resistance

The minimum inhibitory concentration (MIC) of gentamicin, tetracycline, erythromycin and chloramphenicol (Sigma–Aldrich) was determined for each strain using a microdilution test according to Rossetti et al. (2009). PCR-based detection of genes responsible for resistance to gentamicin [*aac(6')-aph(2')-Ia*], tetracycline [*tet(L), tet(M), tet(S)*], erythromycin [*erm(B), erm(C)*], and chloramphenicol [cat_{pIP501}] was applied to strains suspected to carry acquired resistance. Primers and protocols were those described by Maietti et al. (2007).

2.12. Prebiotic utilization

Seven commercially available prebiotics were investigated in this study: lactulose, xylitol, and D+ raffinose (Sigma–Aldrich), inulin and oligofructose P95 (from Orafti, Tienen, Belgium), Litesse™ polydextrose (Danisco, Denmark), and Promitor™ soluble corn fiber (Tate & Lyle, London, UK). Glucose (Sigma–Aldrich) was included as positive control. All prebiotics and glucose were solubilized in distilled water and sterilized by filtration using a 0.22 μm syringe filter unit (Millipore, Milan, Italy). The strains were inoculated (1.5% inoculum) in 3-ml aliquots of MRS modified medium (containing 2% each of the seven prebiotics or glucose as the only carbon source) and grown anaerobically at 37 °C for 24 h. Utilization of prebiotics and glucose was evaluated by measuring the optical density at 560 nm (OD_{560}) of each culture. The growth cell rate in the presence of each prebiotic was calculated with the following formula: [((MRSp − MRSb)*100)/(MRSg − MRSb)], where MRSp is MRS with each prebiotic, MRSb is MRS without carbon source (negative control) and MRSg is MRS with glucose (positive control).

2.13. Mice feeding

A total of 55 6-week-old BALB/c female mice weighing 20.2 ± 0.4 g were obtained from the Centro de Experiment aciones Biológicas y Bioterio, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza, Santa Fe). The mice, which were housed in plastic cages in groups of five animals per cage, were kept in a controlled atmosphere (temperature 21 ± 1 °C; humidity 55 ± 2%) with a 12-h light/dark cycle. They were fed *ad libitum* with conventional balanced chow (22% protein, 6.5% raw fiber, 10.5% total minerals, 1.4% Ca, 0.7% P and 12.7% moisture and vitamins). The mice were maintained and treated according to the criteria outlined in the NIH Guide for the Care and Use of Laboratory Animals. Control groups received water or 1% (w/v) sterile skim milk (SSM; Difco, Becton Dickinson and Company, Sparks, MD, USA) in the drinking bottles whereas treated animals received cell suspensions of *L. plantarum* Lp790, Lp813, and Lp998 for 2, 5 or 7 consecutive days. For preparing cell suspensions, overnight cultures of each strain were centrifuged (4000 × *g*, 5 °C, 15 min) and washed twice with PBS (pH 7.4). Cells were resuspended in 1% SSM to a concentration of ca. 1.0×10^8 CFU/ml. Cell suspensions were renewed every day and agar plate counts were performed to verify that cells remained viable in the drinking bottles.

2.14. Bacterial translocation assay and phagocytosis assay

At the end of each feeding period, mice were anaesthetized, killed by cervical dislocation and the liver and spleen were aseptically removed. Before liver and spleen removal, peritoneal macrophages were collected from the peritoneal cavity and their phagocytic activity was determined according to Vinderola et al. (2005). Organs were homogenized in 5 ml 0.1% sterile peptone water and 1 ml of each homogenate was plated on MacConkey agar and LAPTg agar (Britania, Buenos Aires, Argentina). Plates were incubated under aerobic conditions for 48 h at 37 °C. At the end of the incubation period, plates were examined and the result was expressed as positive (presence of bacteria on plates) or negative (absence of bacteria on plates) bacterial translocation. Translocation was considered to have occurred when colonies were observed on agar plates, since liver and spleen are organs normally devoid of bacteria.

2.15. Histological studies of the gut

Small intestine was removed from treated and control animals after each feeding period and immediately and smoothly flushed with 5 ml of cold (5 °C) buffered formaldehyde fixing solution to avoid tissue damage by the action of autolytic enzymes. Intestines were processed for paraffin inclusion following the Sainte-Marie technique (Sainte-Marie, 1962). Serial paraffin sections (4 mm) were stained with haematoxilin-eosin followed by light microscopy examination.

2.16. Immunofluorescence test for IgA-producing cells enumeration

The number of IgA-producing cells was determined on histological slices of samples from the ileal region near Peyer's patches by direct immunofluorescence (Vinderola et al., 2005). Small intestine was removed for histological preparation as described above. The immunofluorescence test was performed using (α-chain specific) anti-mouse IgA FITC conjugate (Sigma–Aldrich). Deparaffinized histological samples were incubated with a 1:75 antibody

Table 1
Lactobacillus plantarum strains used for the *in vitro* study of functional characteristics.

Strains	Origin	Strains	Origin
Lp751	Pecorino cheese	Lp813	Spressa cheese
Lp752	Pecorino cheese	Lp814	Canestrato Pugliese cheese
Lp754	Pecorino cheese	Lp842	Monte Veronese cheese
Lp755	Pecorino cheese	Lp885	Pannerone cheese
Lp790	Morlacco cheese	Lp994	Argentinean cheese
Lp791	Canestrato Pugliese cheese	Lp995	Argentinean cheese
Lp793	Canestrato Pugliese cheese	Lp996	Argentinean cheese
Lp794	Monte Veronese cheese	Lp997	Argentinean cheese
Lp797	Morlacco cheese	Lp998	Argentinean cheese
Lp799	Monte Veronese cheese	Lp999	Argentinean cheese
Lp800	Morlacco milk	Lp1017	Monte Veronese cheese
Lp803	Canestrato Pugliese cheese	LMG 9211	LMG collection
Lp804	Monte Veronese cheese	ATCC 14917^T	Type strain
Lp805	Monte Veronese curd		

dilution in 0.1% bovine serum albumin (Sigma–Aldrich) - PBS solution for 30 min at 37 °C. Samples were then washed three times with PBS solution and examined using a fluorescent light microscope (30 fields by slide). Results were expressed as the number of IgA positive cells (fluorescent cells) per 10 fields (magnification 400×), and they are the means of three histological slices for each animal, and for each feeding period.

2.17. Statistical analysis

Statistical analysis of data was carried out using STATISTICA 6 (StatSoft Italia, Padova, Italy). Student-*t* test was done to determine a significant difference of viability between *L. plantarum* before and after simulated gastric juice treatment. Finally, analysis of variance (ANOVA) with the Duncan test was done to verify if there is a significant difference in *L. plantarum* bacteria treated with lysozyme, bile and in the expression of β-galactosidase activity and surface hydrophobicity. Data were analysed at the significance level of $P < 0.05$.

3. Results

3.1. Preliminary strain selection

Ninety-eight *L. plantarum* strains, identified on the basis of species-specific PCR for *L. plantarum* group described by Torriani et al. (2001), with different M13 PCR fingerprinting profiles (Rossetti and Giraffa, 2005) (data not shown) and coming from different Italian and Argentinean cheeses, were searched for the presence of *msa* and *bsh* genes, that are considered to be associated with probiotic features. Twenty-five (Table 1) out of the 98 strains tested showed the expected amplicons for both genes (data not shown). These 25 strains, as well as the *L. plantarum* type strain ATCC 14917^T and *L. plantarum* LMG 9211, were selected for further characterization.

3.2. In vitro resistance to lysozyme, bile and simulated gastric juice

The overall resistance of the strains to lysozyme, expressed as percentage of survival, ranged from a minimum value of 3.24% to a maximum value of 99.97% (Table 2). Fifteen strains showed higher lysozyme resistance with a survival rate greater than 68%. The strains Lp804, Lp805, Lp994 and the strain ATCC 14917^T, in particular, demonstrated a high lysozyme resistance, with a survival rate greater than 95% at all the incubation times.

The strains showed a capability to grow in the presence of bile, which was similar at all the concentrations tested. The overall average value of growth was about 40% (Table 2). Nine strains showed the highest bile resistance (percentage of growth compared to the control ≥ 50%). Minimum (12.21%) and maximum (95.98%) values, were observed only for strains Lp790 and ATCC 14917^T, respectively, being significantly different from the others.

Regarding the resistance of *L. plantarum* strains, in simulated gastric juice conditions (Table 3), results showed that no significant difference ($P \geq 0.05$) was observed within the first 60 min when pH decrease from 5.0 to 2.5. Only the type strain ATCC14917 was sensitive to these conditions with a population reduction of about 3 log CFU/ml. After 70 min (pH 2.4) and 80 min (pH 2.3), 17 and 8 strains, respectively, did not show a significant decrease of cell number. At the end of the test, when the simulated gastric juice reached pH 2.2 after 90 min, 9 strains showed survivor counts still higher than 6 log CFU/ml, but only 2 strains (Lp803 and Lp813) did not differ significantly from the beginning. When these 9 strains were put in conditions that simulated the stomach duodenum passage, i.e. they were resuspended in a solution at pH 8.0 with 0.3% bile and 0.1% pancreatin for a further 60 min at 37 °C, the survival rates for 6 of them did not change significantly, and the cell populations remained over 6 log CFU/ml.

3.3. BSH activity

All 27 strains demonstrated the ability to hydrolyze both sodium glycodeoxycholate and sodium taurodeoxycholate, as shown by halos around colonies after growth in MRS-GDCA and by the appearance of white opaque colonies after growth in MRS-TDCA, respectively (data not shown).

Table 2
Evaluation of four potential probiotic properties of the 27 *L. plantarum* strains of dairy origin.

Strains	Lysozyme resistance (% survival)	Bile resistance (% growth)	Cell hydrophobicity (% hydrophobicity)	β-galactosidase activity (Miller units)
Lp751	72.52^{c,d,e,f,g,h}	49.81^{g,h,i}	30.80^{m}	248.09^{b,c,d}
Lp752	43.07^{b,c}	54.27^{i}	21.68^{h,i,l}	269.50^{c,d}
Lp754	26.98^{a,b}	37.72^{d,e}	7.64^{b}	163.79^{a,b,c}
Lp755	51.36^{b,c}	44.71^{f,g}	17.03^{c,d,e,f,g}	106.06^{a,b,c}
Lp790	65.22^{c,d,e,f,g}	12.21^{a}	30.83^{m}	613.49^{e}
Lp791	68.12^{c,d,e,f,g,h}	56.83^{i}	24.28^{i,l}	160.07^{a,b,c}
Lp793	87.85^{e,f,g,h}	53.26^{i}	18.46^{e,f,g,h}	134.17^{a,b,c}
Lp794	68.44^{c,d,e,f,g,h}	55.35^{i}	6.76^{b}	121.14^{a,b,c}
Lp797	58.16^{c,d,e}	36.33^{d,e}	17.26^{c,d,e,f,g}	35.59^{a}
Lp799	47.37^{b,c}	53.79^{i}	19.88^{f,g,h}	12.69^{a}
Lp800	84.69^{d,e,f,g,h}	45.98^{f,g,h}	4.60^{a,b}	101.70^{a,b,c}
Lp803	72.64^{c,d,e,f,g,h}	33.12^{c,d,e}	20.43^{g,h,i}	45.97^{a}
Lp804	96.63^{g,h}	44.86^{f,g}	18.20^{d,e,f,g,h}	389.58^{d}
Lp805	95.60^{f,g,h}	44.57^{f,g}	21.09^{g,h,i}	359.44^{d}
Lp813	74.04^{c,d,e,f,g,h}	32.55^{c,d}	13.84^{c,d}	46.12^{a}
Lp814	3.24^{a}	36.61^{d,e}	7.96^{b}	82.69^{a,b,c}
Lp842	64.00^{c,d,e,f}	52.30^{h,i}	15.63^{c,d,e,f}	132.37^{a,b,c}
Lp885	54.45^{b,c,d}	50.93^{g,h,i}	8.45^{b}	104.93^{a,b,c}
Lp994	99.97^{h}	26.22^{b,c}	15.67^{c,d,e,f}	32.17^{a}
Lp995	56.20^{b,c,d,e}	27.42^{b,c}	8.94^{b}	60.23^{a,b}
Lp996	57.35^{c,d,e}	26.31^{b,c}	2.19^{a}	1062.14^{f}
Lp997	70.11^{c,d,e,f,g,h}	27.41^{b,c}	15.22^{c,d,e}	45.16^{a}
Lp998	70.37^{c,d,e,f,g,h}	25.10^{b}	25.27^{l}	65.27^{a,b}
Lp999	62.18^{c,d,e}	36.78^{d,e}	20.67^{g,h,i}	61.55^{a,b}
Lp1017	71.36^{c,d,e,f,g,h}	45.67^{f,g,h}	7.28^{b}	82.72^{a,b,c}
LMG9211	84.52^{d,e,f,g,h}	40.08^{e,f}	13.81^{c,d}	71.49^{a,b}
ATCC14917	95.98^{f,g,h}	65.83^{l}	13.53^{c}	576.97^{e}

Average values of 'Lysozyme resistance (% survival)' correspond to the percentage of survival after 30 and 120 min in the presence of 100 mg/l of lysozyme.
Average values 'Bile resistance (% growth)' correspond to the percentage of growth in the presence of 0.3%, 0.5% and 1% of bile.
Average values from three independent repetitions are presented.
For each determination, average values within the same column followed by common superscript letters do not differ significantly ($P < 0.05$).

Table 3
Effect of simulated gastric juice on *L. plantarum* strains during 150 min of gastric transit.

Strains	Population size (log mean CFU/ml ± SD) at different times						
	t_0	t_{30}	t_{60}	t_{70}	t_{80}	t_{90}	t_{150}
	pH 5.0	pH 3.8	pH 2.5	pH 2.4	pH 2.3	pH 2.2	pH 8.0 + 0.3% bile
Lp751	9.68 ± 0.10^{a}	9.72 ± 0.02^{a}	9.71 ± 0.02^{a}	9.46 ± 0.06^{a}	6.69 ± 0.12^{b}	4.54 ± 0.09^{c}	2.06 ± 0.08^{c}
Lp752	9.62 ± 0.03^{a}	9.77 ± 0.05^{a}	9.63 ± 0.05^{a}	8.81 ± 0.04^{b}	5.73 ± 0.04^{c}	4.36 ± 0.09^{d}	<2.00^{e}
Lp754	9.37 ± 0.06^{a}	9.33 ± 0.04^{a}	9.36 ± 0.05^{a}	9.25 ± 0.04^{a}	5.90 ± 0.08^{b}	<2.00^{c}	<2.00^{c}
Lp755	9.55 ± 0.04^{a}	9.64 ± 0.07^{a}	9.50 ± 0.04^{a}	8.72 ± 0.02^{b}	5.15 ± 0.21^{c}	4.24 ± 0.34^{c,d}	4.09 ± 0.12^{d}
Lp790	9.08 ± 0.05^{a}	9.16 ± 0.23^{a}	8.65 ± 0.09^{a}	8.17 ± 0.18^{a,b}	6.81 ± 0.01^{b,c}	6.22 ± 0.06^{c}	6.17 ± 0.21^{c}
Lp791	9.07 ± 0.03^{a}	9.16 ± 0.04^{a}	9.52 ± 0.10^{a}	7.21 ± 0.09^{b}	6.08 ± 0.02^{c}	4.07 ± 0.02^{d}	<2.00^{e}
Lp793	9.55 ± 0.11^{a}	9.52 ± 0.10^{a}	9.34 ± 0.05^{a}	6.34 ± 0.03^{b}	3.98 ± 0.07^{c}	1.50 ± 0.09^{d}	1.48 ± 0.07^{d}
Lp794	9.63 ± 0.04^{a}	9.63 ± 0.03^{a}	9.66 ± 0.16^{a}	9.52 ± 0.01^{a,b}	7.06 ± 0.31^{b,c}	5,06 ± 0.50^{c}	3.61 ± 0.13^{c}
Lp797	9.54 ± 0.04^{a}	9.56 ± 0.02^{a}	9.50 ± 0.03^{a}	9.51 ± 0.10^{a}	9.49 ± 0.09^{a}	8.87 ± 0.10^{b}	8.46 ± 0.02^{b}
Lp799	9.44 ± 0.01^{a}	9.44 ± 0.02^{a}	9.38 ± 0.10^{a}	7.18 ± 0.20^{b}	3.36 ± 0.05^{c}	2.18 ± 0.15^{d,e}	1.98 ± 0.03^{e}
Lp800	9.39 ± 0.04^{a}	9.44 ± 0.08^{a}	9.48 ± 0.04^{a}	9.54 ± 0.05^{a}	8.53 ± 0.14^{a}	6.85 ± 0.15^{b}	6.38 ± 0.25^{b}
Lp803	9.47 ± 0.08^{a}	9.50 ± 0.12^{a}	9.61 ± 0.19^{a}	9.53 ± 0.09^{a}	9.26 ± 0.20^{a}	6.91 ± 1.18^{a,b}	5.46 ± 0.45^{b}
Lp804	9.33 ± 0.06^{a}	9.35 ± 0.07^{a}	9.42 ± 0.04^{a}	7.82 ± 0.03^{b}	7.11 ± 0.12^{b}	5.01 ± 0.02^{c}	4.65 ± 0.07^{c}
Lp805	9.23 ± 0.07^{a}	9.13 ± 0.18^{a}	9.38 ± 0.08^{a}	8.89 ± 0.07^{a,b}	8.31 ± 0.05^{b,c}	7.04 ± 0.19^{c,d}	6.64 ± 0.10^{d}
Lp813	9.39 ± 0.03^{a}	9.42 ± 0.01^{a}	9.51 ± 0.01^{a}	9.42 ± 0.08^{a}	8.98 ± 0.03^{a}	8.17 ± 0.45^{a}	6.27 ± 0.51^{b}
Lp814	9.48 ± 0.06^{a}	9.55 ± 0.06^{a}	9.39 ± 0.02^{a}	5.90 ± 0.05^{b}	5.15 ± 0.04^{c}	4.68 ± 0.04^{d}	<2.00^{e}
Lp842	9.34 ± 0.06^{a}	9.44 ± 0.06^{a}	9.37 ± 0.07^{a}	6.13 ± 0.18^{b}	5.15 ± 0.21^{c,d}	4.03 ± 0.04^{d,e}	3.77 ± 0.10^{e}
Lp885	9.65 ± 0.05^{a}	9.58 ± 0.04^{a}	9.61 ± 0.03^{a}	9.52 ± 0.15^{a,b}	6.45 ± 0.21^{b,c,d}	5.09 ± 0.12^{c}	4.32 ± 0.13^{d}
Lp994	9.40 ± 0.06^{a}	9.54 ± 0.01^{a}	9.50 ± 0.06^{a}	9.48 ± 0.04^{a}	9.55 ± 0.04^{a}	7.48 ± 0.07^{b}	5.44 ± 0.08^{c}
Lp995	9.49 ± 0.05^{a}	9.56 ± 0.03^{a}	9.47 ± 0.03^{a}	9.20 ± 0.06^{a}	6.61 ± 0.02^{b}	3.13 ± 0.04^{c}	<2.00^{d}
Lp996	9.42 ± 0.06^{a}	9.54 ± 0.04^{a}	9.60 ± 0.03^{a}	9.42 ± 0.06^{a}	7.84 ± 0.09^{b}	4.63 ± 0.21^{c,d}	4.54 ± 0.04^{d}
Lp997	9.43 ± 0.18^{a}	9.60 ± 0.11^{a}	9.59 ± 0.02^{a}	9.45 ± 0.03^{a}	8.09 ± 0.12^{b,c}	5.06 ± 0.31^{c,d}	4.81 ± 0.10^{d}
Lp998	9.42 ± 0.10^{a}	9.58 ± 0.04^{a}	9.61 ± 0.06^{a}	9.57 ± 0.04^{a}	9.53 ± 0.08^{a}	6.92 ± 0.01^{b}	5.59 ± 0.02^{c}
Lp999	9.45 ± 0.18^{a}	9.55 ± 0.07^{a}	9.59 ± 0.01^{a}	9.39 ± 0.04^{a}	9.26 ± 0.08^{a}	6.45 ± 0.05^{b}	6.33 ± 0.01^{b}
Lp1017	9.60 ± 0.01^{a}	9.63 ± 0.02^{a}	9.61 ± 0.04^{a}	9.06 ± 0.20^{a}	6.95 ± 0.67^{a,b}	5.79 ± 0.30^{b,c}	4.54 ± 0.51^{c}
LMG2911	9.56 ± 0.05^{a}	9.57 ± 0.08^{a}	9.51 ± 0.03^{a}	8.20 ± 0.08^{b}	5.87 ± 0.15^{c}	2.50 ± 0.02^{d}	1.61 ± 0.07^{e}
ATCC14917	9.10 ± 0.12^{a}	8.98 ± 0.04^{a}	5.98 ± 0.09^{b,c}	3.45 ± 0.21^{c,d}	2.95 ± 0.07^{d}	2.81 ± 0.05^{d}	2.87 ± 0.24^{d}

Average values (±SD) from three independent repetitions are presented. For each strain, average values (±SD) within the same row followed by common superscript letters do not differ significantly ($P < 0.05$).

3.4. Agglutination properties and surface characteristics

Twenty-three *L. plantarum* strains were able to agglutinate *S. cerevisiae* cells. Strain Lp790 (Fig. 1B) and strain Lp793, in particular, showed the highest degree of agglutination while strains Lp754, Lp806, Lp842, and Lp1017 did not agglutinate. All the strains did not agglutinate *S. cerevisiae* cells after addition of methyl-D-mannopyranoside. Strains showed a variable hydrophobicity, with values that ranged from 2.19% to 30.83% (Table 2). Half of the strains showed a hydrophobicity percentage greater than the overall average value (i.e. 16%) and only 2 strains (Lp751 and Lp790) differed significantly from the other for the highest values (about 30%).

3.5. ß-galactosidase and β-glucuronidase activities

The average value of β-galactosidase activity expressed by all the strains was around 190 Miller units (Table 2). Three strains showed the highest activities: ATCC14917 and Lp790, with values of about 600 Miller units, and strain Lp996 that displayed a significantly higher activity of 1062.14 Miller units. No strain exhibited β-glucuronidase activity, as determined by the API-ZYM method (data not shown).

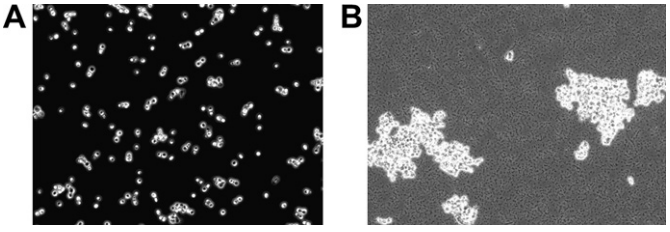

Fig. 1. Agglutination of *Lactobacillus plantarum* Lp790 with *Saccharomyces cerevisiae* (A, B) as visualized by bright-light microscopy. (A) *S. cerevisiae* with no Lp790 cells added (magnification × 40); (B) *S. cerevisiae* co-incubated with Lp790 (x 40).

3.6. Antagonism against pathogens

None of the L. plantarum tested was capable of inhibiting the pathogenic microorganisms used in this study (data not shown).

3.7. Antibiotic resistance

All 27 *L. plantarum* strains tested were susceptible to gentamicin, erythromycin, and chloramphenicol. Strains Lp804 and Lp805, which were resistant to tetracycline with MIC values of 512 μg/ml, were tested by PCR for the presence of *tet* resistance genes. After PCR amplification, the *tet(M)* gene was detected in both strains (data not shown).

3.8. Prebiotic utilization

All the strains showed the highest cell growth with lactulose (growth rate values nearing 100% with respect to glucose) (Fig. 2). Corn fiber was also fermented by all the strains but at a lower rate (about 30% on average), with only 3 strains (Lp994, Lp996 and ATCC 14917^{T}) able to use more than 50%. Raffinose was used at higher rates (≥50%) by 8 strains, with the type strain ATCC 14917^{T} showing the highest (100%) degree of utilization. Oligofructose and polidextrose were poorly fermented. The strains were not capable of using inulin and xylitol within 24 h (data not shown).

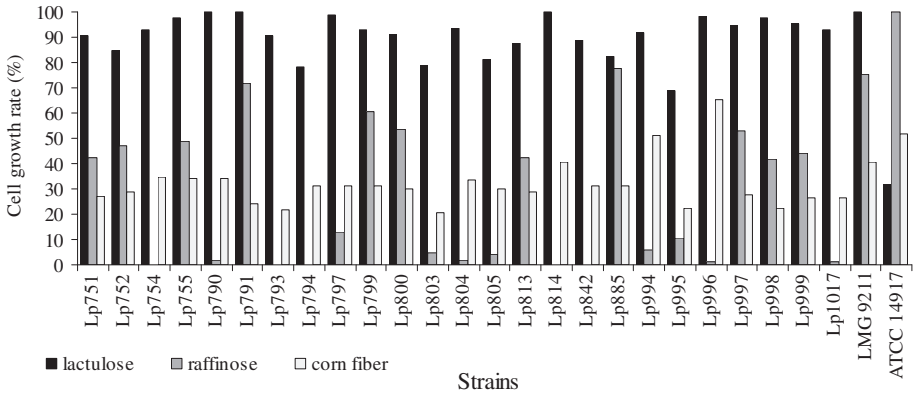

Fig. 2. Lactulose, raffinose, and corn fiber utilization by *L. plantarum* strains during growth. Values are expressed as growth cell percentage of the three prebiotics with respect to glucose (100% utilization), used as control, after 24 h incubation.

3.9. *In vivo trials*

For *in vivo* tests, strains Lp790, Lp813, and Lp998 were chosen on the basis of their high degree of agglutination, surface hydrophobicity and good tolerance to simulated gastric juice. In relation to the translocation assay, no bacterial colonies were detected on both MacConkey agar and LAPTg agar. Therefore, no microbial translocation was induced to extra-intestinal sites (liver and spleen) by the oral administration of the three strains at the chosen doses. The histological examination of the small intestine (haematoxilin–eosin assay) showed no lymphocyte infiltrates in the villi lamina propria, or the presence of edema or mucosal atrophy, compared to control mice. No significant morphological changes in the overall architecture of the small intestine tissue were observed when compared to histological slices of control mice (data not shown). The phagocytic activity of peritoneal macrophages was significantly enhanced in mice that received *L. plantarum* Lp790, Lp813 and Lp998 for 2, 5 and 7 consecutive days, respectively (Fig. 3). The number of IgA-producing cells was enhanced for all strains for all feeding periods assessed, but with different profiles of immune stimulation (Fig. 4).

4. Discussion

Several functional attributes have already been demonstrated for *L. plantarum* strains isolated from fermented foods (Adlerberth et al., 1996; Cebeci and Gürakan, 2003; Nguyen et al., 2007; Vizoso-Pinto et al., 2006; Fuentes et al., 2008; Georgieva et al., 2008; Mathara et al., 2008; Pisano et al., 2008; Belviso et al., 2009). In view of the role that *L. plantarum* plays in food systems, the aim of this work was to select potentially probiotic *L. plantarum* strains from a wide collection of dairy isolates.

A preliminary subtractive screening based on the presence of *msa* and *bsh* genes was applied to 98 genotypically different strains. The *msa* gene was selected to indicate the mannose-specific adhesion of *L. plantarum*, being one of the first genes to be identified that is associated with a definite probiotic effect (Pretzer et al., 2005). The *bsh* gene, coding for the BSH activity, was selected for its importance in bile salt hydrolysis which, ultimately, leads to lower blood serum cholesterol levels (Begley et al., 2006). Twenty-five strains carrying both amplicons were further subjected to a series of *in vitro* and *in vivo* analyses to assess their probiotic properties.

An important step towards the selection of potentially probiotic candidates is to investigate the strain behavior under conditions which mimic the GIT. This allows a selection of strains likely to survive such conditions, and which can be further investigated for their potential as probiotic cultures. Stresses to microorganisms begin in the mouth, with the lysozyme-containing saliva, and continues in the stomach, which has a pH between 1.5 and 3.0, and the upper intestine, which contains bile (Corzo and Gilliland, 1999). The selected strains showed a high resistance to 100 mg/l of lysozyme under conditions simulating the *in vivo* dilution by saliva. The same lysozyme levels did not affect the survival of *L. plantarum* strains in a simulated stomach passage (Vizoso-Pinto et al., 2006).

Strains also showed a good adaptation to simulated gastric juice at pH 2.5, and a moderate to low bile tolerance. The bile concentration to which the selected strains should be tolerant to, has been object of different experimental protocols. In more recent investigations, bile concentrations ranging from 0.15% to 0.5% were used (Vizoso-Pinto et al., 2006), while Mathara et al. (2008) established a limit of 0.3% bile to select strains considered to have good resistance (more than 50% growth when compared to the control without bile added to the medium). By applying the same bile level and selection criteria, the *L. plantarum* strains Lp790, Lp797, Lp800, Lp803, Lp805 and Lp999 were able to better survive simulated stomach duodenum passage (1.0 h incubation at pH 2.2 in the presence of 0.3% bile), confirming their interesting adaptation ability. A strain-dependent tolerance to conditions similar to those found in the GIT was also observed in *L. plantarum* strains isolated from Bulgarian cheeses (Georgieva et al., 2008) and from Fiore Sardo cheese (Pisano et al., 2008).

Another relevant property of candidate probiotics is BSH activity, which involves deconjugation of bile salts to primary bile

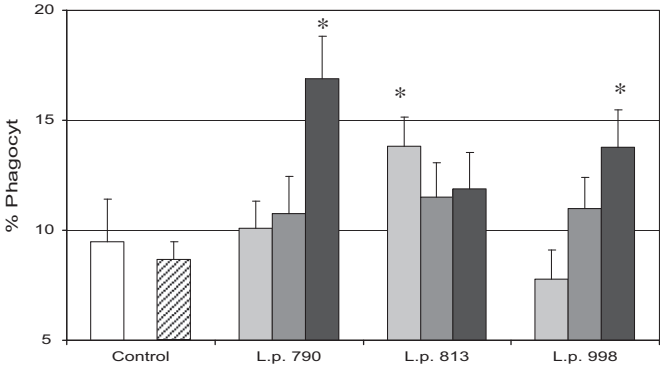

Fig. 3. Effect of the oral administration of *L. plantarum* Lp790, Lp813, and Lp998 for 2 (■), 5 (■) or 7 (■) consecutive days on the phagocytic activity of peritoneal macrophages, compared to control animals that received water (□) or 1% skim milk (striped bars). *Significantly different from controls ($P < 0.05$).

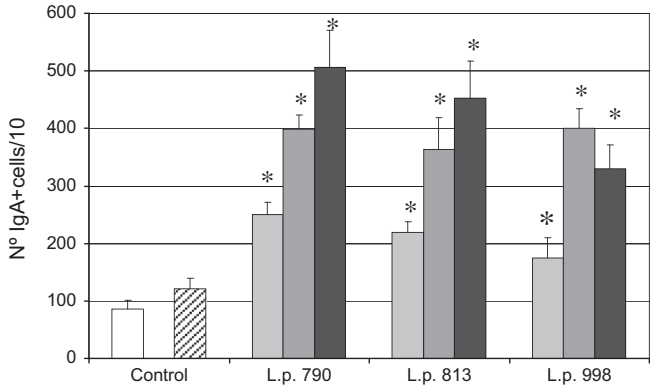

Fig. 4. Effect of the oral administration of *Lactobacillus plantarum* Lp790, Lp813, and Lp998 for 2 (■), 5 (■) or 7 (■) consecutive days on the number of IgA + cells on histological slices of the small intestine, compared to control animals that received water (□) or 1% skim milk (striped bars). *Significantly different from controls ($P < 0.05$).

salts, protects bacteria from the toxicity of conjugated bile salts, being considered a detoxification mechanism of vital importance to bacterial communities (such as lactobacilli), which are typically associated to the human GIT (De Smet et al., 1995). All 25 *L. plantarum* strains possessed the *bsh* gene and expressed BSH activity but did not show full resistance to bile acids. Schmidt et al. (2001) also showed that, at least in lactobacilli, bile salt resistance could not be related to the presence of BSH. BSH activity, however, is a widespread trait in *L. plantarum* (Vizoso-Pinto et al., 2006; Pisano et al., 2008).

The ability to adhere to mannose residues is a potentially interesting characteristic with regard to colonization of the intestinal surface and competitive exclusion of pathogens. The product of the *msa* gene, identified as a mannose-specific adhesion, was found to mediate adhesion to human colonic cell line HT-29 (Adlerberth et al., 1996), and to agglutinate *S. cerevisiae* cells (Pretzer et al., 2005). All our strains carried the *msa* gene, but the degree of agglutination of yeast cells was variable. The involvement of mannose in the mechanism of adhesion was confirmed by the loss of agglutination after addition of methyl-D-mannopyranoside, that interacting with the yeast inhibited the adhesion of *L. plantarum* strains. The cell surface hydrophobicity was also highly variable, and the comparison with the capacity to agglutinate, confirmed previously reported trends that hydrophobicity values do not correlate with adhesion properties (Vinderola and Reinheimer, 2003; Mathara et al., 2008).

Beta-galactosidase activity and metabolism of prebiotics are important features in strains envisaged as probiotics. Lactose intolerance is found in people lacking the enzyme β-galactosidase, since lactose is not broken down in the upper regions of the small intestine and is thus used by the indigenous microbiota (De Vrese et al., 2001). Very high β-galactosidase activity was expressed from a considerable number of the tested *L. plantarum* strains, which may therefore be used to alleviate lactose intolerance in the gut, but the large variation among the strains emphasized the importance of selecting appropriate strains for use as a dietary adjunct. Prebiotics are defined as non-digestible carbohydrates that selectively stimulate the growth and/or activity of populations of beneficial bacteria in the colon, thus improving the host health (Gibson and Roberfroid, 1995). Our data showed variable utilization ability between the tested *L. plantarum* strains, with notable preference for lactulose, corn fiber, and, to a lesser extent, raffinose. The prebiotic utilization appeared strain-specific, which could lead to the likely selection of useful combinations of prebiotics and putative *L. plantarum* probiotic strains to enhance the physiological benefits.

Probiotic strains must be safe for human consumption. A Qualified Presumption of Safety (QPS) status, similar to the GRAS system in the Unites States, has been granted to a number of *Lactobacillus* species. *L. plantarum* is included in the list of taxonomic units within the QPS status, provided that the lack of acquired antibiotic resistance is systematically demonstrated (European Food Safety Authority, 2007). Of the 25 selected strains tested for susceptibility to four antibiotics belonging to the clinically most relevant antibiotic classes, only two were highly resistant to tetracycline and carried the *tet*M gene. Vizoso-Pinto et al. (2006) did not detect any antibiotic resistant strains within *L. plantarum* isolated from traditional African fermented products. Conversely, a high frequency of tetracycline-resistant strains was observed in *L. plantarum* from Italian fermented dry sausages (Zonenschain et al., 2009).

Safety assessment must also include the lack of harmful activities, such as β-glucuronidase activity. Beta-glucuronidases liberate toxins and mutagens that have been glucuronated in the liver and excreted into the gut with the bile. This can lead to high local concentrations of carcinogenic compounds within the gut, thus increasing the risk of carcinogenesis (Gill and Rowland, 2002). β-glucuronidase activity was reported in a human intestinal isolate of *Lactobacillus gasseri* (Russell and Klaenhammer, 2001), but similar data were not available for *L. plantarum*. None of the tested strains showed β-glucuronidase activity, nor other *Lactobacillus* spp. strains isolated from the feces of healthy humans (Delgado et al., 2007).

The immune response towards the oral ingestion of three of the most interesting *L. plantarum* putative probiotic strains was studied. It was observed that, in the absence of intestinal inflammation, a strain-specific increase in the phagocytic activity of peritoneal macrophages and in the number of IgA-producing cells in the small intestine was observed, indicating that the strains studied were active in stimulating the immune response of the host. The oral administration of viable probiotic bacteria is a suitable way to stimulate the host's non-specific immunity by enhancing the systemic immune response or by modulating the functions of immunocompetent cells (Perdigón et al., 1986). Similarly, the main function of secretory IgA in the gut is to exert immune exclusion of pathogens and foreign proteins by intimate cooperation with the innate non-specific defence mechanisms (Brandtzaeg et al., 1985). Among the possible adverse effects of regular probiotic feeding, the risk of bacterial translocation (passage of viable indigenous bacteria from the gut to other sites beyond the intestine) should be evaluated with caution. Bacterial translocation is therefore a potential indicator of probiotic toxicity, as it is one of the early steps in the pathogenesis process for opportunistic strains (Steffen and Berg, 1983). In this work, the translocation assay was used as a parameter to determine the ability to modulate the intestinal immune response in an animal model without interferences that could arise from an inflammatory response. Data suggested that high doses of the three test probiotics, orally administered to mice, do not increase bacterial translocation to spleen or liver. Similarly, orally administered *L. plantarum* PH04 and Lp-115 did not translocate to extra-intestinal organs of mice (Nguyen et al., 2007) or a mouse colitis model (Daniel et al., 2006).

In conclusion, this study revealed a considerable heterogeneity in probiotic properties among the different strains of *L. plantarum*, and highlighted the possibility to design multiple cultures to cooperatively link strains showing the widest range of useful traits. More specifically, results showed that strains Lp790, Lp813, and Lp998 display most of the probiotics and safety characteristics and would be the best candidates for inclusion as starter cultures for the

manufacture of probiotic fermented foods Although the most important characteristics of probiotic bacteria are their beneficial effects in host health, the evaluation of technological traits such as growth and survival in food, e.g. milk-based media, and during product manufacturing and shelf life, is important for the selection of strains for food applications (Vinderola et al., 2008). Further studies are in progress in the author's laboratories to evaluate the ability of *L. plantarum* strains to multiply in milk, to withstand food technological stresses, and to resist to phage attack.

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