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Characterization of functional, safety, and gut survival related characteristics of *Lactobacillus* strains isolated from farmhouse goat's milk cheeses

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ABSTRACT

A set of 80 *Lactobacillus* strains (36 *Lactobacillus plantarum* and 44 *Lactobacillus paracasei*) isolated from Spanish farmhouse cheeses have been studied as to their functional and safety properties and their survival under gut-related conditions. None of these 80 *Lactobacillus* strains were able to hydrolyse starch. A high percentage of *L. plantarum* and *L. paracasei* strains were, however, capable of hydrolysing casein (86.1% and 68.2% respectively). For the other characteristics investigated, *L. plantarum* strains generally had more positive responses than *L. paracasei*. The latter strains tested negative for most of these characteristics, with the exception of stachyose hydrolysis, which was positive in six strains of *L. paracasei*. A high percentage (91.7%) of *L. plantarum* produced haemo-dependent catalase. Phytase was present in 10 *L. plantarum* and in 2 *L. paracasei*. Most *L. plantarum* (83.3%) but no *L. paracasei* hydrolysed bile salts. All strains were completely resistant to a challenge of pH 3, but many showed a loss of viability after a subsequent exposure to 0.3% oxgall; in fact, only one *L. paracasei* strain and 33 *L. plantarum* strains (91.67%) were tolerant to both stresses. *L. plantarum* Mb25 and *L. plantarum* Mb26 were the most adherent to Caco-2 cells (adherence percentages of 36 and 7% respectively). These two strains were also the most adherent to HeLa 229 cells, with 19.3 and 16.0% adhesion respectively. The Mb26 strain inhibited the adhesion of *Listeria monocytogenes* to Caco-2 cells when added simultaneously to *Listeria* and also when added 1 h before the pathogen (21.0% and 51.6% adhesion inhibition, respectively). Production of H_2O_2 was detected in 38.9% of *L. plantarum* strains and in 9.1% of *L. paracasei*. Twelve *L. plantarum* and eight *L. paracasei* strains produced bacteriocin-like inhibitors. PCR amplifications of several plantaricin genes suggest that all the bacteriocinogenic strains may produce plantaricin E/F and some may also manufacture the plantaricin J/K. The nine *L. plantarum* strains assayed for antibiotic resistance were resistant to ciprofloxacin (MIC > 2 μg/ml), vancomycin (MIC > 16 μg/ml), and teicoplanin (MIC > 16 μg/ml). Moreover, some strains showed intermediate resistance to penicillin, tetracycline, rifampicin, and levofloxacin. We conclude that farmhouse cheeses are good sources of biotechnologically relevant lactobacilli and that the *L. plantarum* species shows better biotechnological properties than *L. paracasei*. This can be deduced from the finding of a high percentage of strains of *L. plantarum* that exhibit remarkable functional and inhibitory properties and high abilities to survive in gut-related conditions, which can be further developed for biotechnological applications.

1. Introduction

It has been estimated the human intestinal tract contains 500–1000 different species of bacteria, with a total population between 10^{13} and 10^{14} microorganisms. The gastrointestinal tract includes beneficial and pathogenic bacteria in a complex symbiosis, so the maintenance of optimal gut balance is very important to prevent pathologies. The composition of the gastrointestinal tract changes over one's life, so modifications in diet, climate, or drugs can alter the balance (Narayan et al., 2010).

Lactobacilli are Gram-positive, catalase-negative bacilli belonging to the lactic-acid bacteria (LAB) group. Lactobacilli are found in the gastrointestinal tract soon after birth. In healthy humans, lactobacilli are normally present in the oral cavity (10^3–10^4 cfu/g), the ileum (10^3–10^7 cfu/g), and the colon (10^4–10^8 cfu/g), and they are the dominant microorganisms in the vagina (Bernardeau et al., 2008; Hill et al., 1984; Merk et al., 2005). Lactobacilli are ubiquitous and have frequently been reported as the main autochthonous microbiota in cheeses made from different milk types (Martín-Platero et al., 2008, 2009; Abriouel et al., 2008), with *Lactobacillus plantarum* and

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Lactobacillus paracasei species being dominant. Lactobacilli have also been investigated with regard to their probiotic potential. The term "probiotic" refers to "live microorganisms which when administered in adequate amounts confer a health benefit on the host". (Anukam et al., 2006; FAO/WHO, 2002; Kotzamanidis et al., 2010; Martín et al., 2008). However, although there is good evidence that specific strains of lactobacilli are safe for human use and are able to confer some health benefits on the host, these benefits cannot be extrapolated to other strains without experimentation (FAO/WHO, 2002; Kotzamanidis et al., 2010). Before assaying the probiotic properties of a strain *in vivo* it is necessary to verify the presence of several characteristics concerning safety, gut survival, and colonization ability as well as other properties related with probiosis. Guidelines from the FAO/WHO (2001, 2002) indicate that the resistance to gastric acidity and bile salts, the production of antimicrobial compounds, the adhesion to gut tissues, bile salt hydrolysis, and the ability to modulate immune responses are properties to be studied for probiotic assessment (Vizoso et al., 2006). In addition, although *Lactobacillus* spp. are classified as QPS (Quality Presumption of Safety) microorganisms, it is important to assess the safety of microorganisms intended for use as probiotics in more depth. Therefore, analysing certain virulence determinants such as transmissible antibiotic resistance is key since strains harbouring resistance genes may act as a source of these genes to pathogenic bacteria (Teuber et al., 1999) in the gut and other body sites. It is also imperative to analyse the production of biogenic amines, which are mainly generated by specific amino acid decarboxylating enzymes produced by microorganisms present in foods since the occurrence of biogenic amines in food (such as tyramine, the most commonly encountered biogenic amine in fermented products) has toxicological effects (Landete et al., 2007).

The objective of this work was to investigate the functional and safety properties, production of inhibitory compounds, and survival in certain gut-related conditions of 80 *Lactobacillus* strains (36 *L. plantarum* and 44 *L. paracasei*) isolated from Spanish farmhouse cheeses by *in-vitro* studies in order to establish their potential as probiotics and as starter cultures. In addition, we have investigated the ability of selected *L. plantarum* strains to interfere with the adhesion of the pathogen *Listeria monocytogenes* to Caco-2 cells.

2. Material and Methods

2.1. Bacterial Strains

The *Lactobacillus* strains used in this assay were previously isolated from Spanish farmhouse goat's milk cheeses and identified at the species level by 16S DNA gene sequencing and species-specific PCRs as *L. plantarum* (17bP29, 17bP30, 17bP31, 17bP48, Mb1, Mb2, Mb3, Mb25, Mb26, Mb40, Mb45, Mb46, Mb50, Mb57, Mb58, Mb61, Mb67, Mb75, Mb96, Mc14, Mc29, Mc45, O6, O28, O79, O83, O86, O95, O124, O150, P1, P16, P17, P19, P23, P130) and *L. paracasei* (C3-52, C3-54, C3-59, C3-60, C3-63, C3-66, C3-70, C3-72, C3-73, C3-75, C3-80, C3-83, C3-84, C3-89, C3-102b, C3-108b, C3-110b, Mb4, Mb12, Mb13, Mb18, Mb22, Mb23, Mb30, Mb39, Mb55, Mb68, Mb70, Mb71, Mb79, Mc4, Mc8, Mc12, Mc13, Mc32, Mc41, Mc47, O99, O109, O114, O121, O147, P2, P11) (Martín-Platero et al., 2009). These strains were selected as representative of the RAPD-PCR groups established in a broader collection of *L. plantarum* and *L. paracasei* strains isolated and identified by Martín-Platero et al. (2008, 2009). All lactobacilli were grown routinely in Man Rogosa Sharpe (MRS) broth (Scharlau, Barcelona, Spain) at 28 °C for 24–48 h, and stored in Elliker agar plates at 4 °C. Other bacteria used in production assays for antimicrobial substances (*L. monocytogenes* CECT 4032, *Enterococcus faecalis* S-47 (from our collection), *L. plantarum* CECT 748, and *L. paracasei* CECT 4022 t) were cultivated in Brain Heart Infusion broth (BHI, Scharlau) at 37 °C (the listeria and enterococci) or in MRS at 28 °C (the lactobacilli) and maintained in BHI or Elliker agar plates at 4 °C. *Gardnerella vaginalis* was cultivated in Gardnerella agar (Oxoid, Paris, France) at 37 °C in anaerobic conditions. The isolates were kept in 20% glycerol at −80 °C.

2.2. Cell Line Cultures

Enterocyte-like Caco-2 ECACC 86010202 (from colon adenocarcinoma) and HeLa 229 ECACC 86090201 (from vaginal cervix carcinoma) (both from the Scientific Instrument Services of the University of Granada, Spain) were used in simple adhesion assays. Caco-2 cells were used in listeria adhesion interference assays. The cells were cultured aerobically in 25 cm^2 Falcon flasks (Falcon, Becton and Dickinson Company, Franklin Lakes, NJ) at standard conditions (37 °C, 95% humidity) using RPMI 1640 medium (Biowest, Nuaillé, France) supplemented with 10% heat-inactivated foetal bovine serum (Biowest), 2 mM L-glutamine (Sigma-Aldrich, St Louis, USA), 1 mM sodium pyruvate (Sigma), 50 μM 2-mercaptoethanol (Sigma), 100 UI/ml penicillin (Sigma), 100 μg/ ml streptomycin (Sigma), 5 μg/ml amphotericin B (Sigma), and 25 mM HEPES buffer (Biowest). For adhesion assays, Caco-2 or HeLa 229 cells in the monolayers were separated from the bottom and suspended in RPMI 1640 with antibiotic and finally seeded in 24-well plates (2 cm^2/well; Falcon) to obtain semiconfluent monolayers in five days. Cell cultures were replenished with RPMI 1640 without antibiotics 24 h before the assay was performed.

2.3. Detection of Functional Enzymatic Activities

Proteolytic activity against casein was checked by spotting 5 μl of an overnight culture on skim milk agar plates (Smibert and Krieg, 1994). Amylase production was performed according to Giraud et al. (1993). Phytase activity was detected using the procedure described by Bae et al. (1999). Detection of haemo-dependent catalase activity was carried out according to the method described by Knauf et al. (1992). Degradation of raffinose and stacchyose was tested according to Gilliland and Speck (1977).

2.4. Properties Related with Safety

DNAse activity was determined by spotting 5 μl of a liquid culture onto DNAse agar plates (Scharlau); after incubation at 37 °C for 48 h, plates were overlain with HCl 1 N for 5 min. Hydrolysis of gastrointestinal mucine was assayed according to Zhou et al. (2001) in two identical media with added glucose or not. Gelatinase production was assayed by spotting 5 μl of an overnight culture of the strains on BHA supplemented with 0.04% gelatine.

Biogenic amine production was investigated using two approaches. On one hand, 50 μl of each strain was inoculated in 100 μl of decarboxylase liquid medium as described by Maijala (1993) with the addition of 2 g/L final concentration of each precursor amino acid lysine, ornithine, histidine, arginine, and tyrosine (Sigma) and also without amino acids (as control). After incubation at 37 °C for 48 h in aerobic conditions, biogenic amine production was qualitatively detected by a colour shift from yellow to purple in the medium. *Escherichia coli* U9 was used as positive control. In the other approach, the presence of amino acid decarboxylase genes [*hdc* (histidine decarboxylase), *odc* (ornithine decarboxylase), and *tdc* (tyrosine decarboxylase)] was screened using multiplex-PCR as described elsewhere (De la Rivas et al., 2005). The primers used are listed in Table 1. Each PCR was carried out using a 50 μl (total volume) mixture containing 50 ng of template DNA, 2.5 mM $MgCl_2$, 400 μM of each deoxynucleoside triphosphate (dNTP), and 1 U of Taq DNA polymerase (MBL, Córdoba, Spain). Amplification was performed with an iCycler 170–8720 (Bio-Rad, Hercules, CA) according to conditions in Table 1. The PCR products were analysed by electrophoresis in 1.5% agarose gels at 30 V for 16 h in 1× TAE buffer (40 mM Tris-acetate, 2.5 mM EDTA; pH 8) and were revealed by staining with ethidium bromide (0.5 μg/ml).

Table 1
Primer pairs and PCR conditions used in the study.

PCR primers	Target	PCR conditions	Reference
JV16HC; 5′-AGATGGTATTGTTTCTTATG-3′ JV17HC; 5′-AGACCATACACCATAACCTT-3′	*hdc*	(94 °C 0:30 min, 52 °C 0:30 min, 71 °C 2 min) × 30	De la Rivas et al. (2005)
P2-for; 5′-GAYATNATNGGNATNGGNYTNGAYCARG-3′ P1-rev; 5′-CCRTARTCNGGNATAGCRAARTCNGTRTG-3′	*tdc*		
ODC- 3; 5′-GTNTTYAAYGCNGAYAARACNTAYTTYGT-3′ ODC- 4; 5′-ATNGARTTNAGTTCRCAYTTYTCNGG-3′	*odc*		
PLNA-F; 5′-GTACAGTACTAATGGGAG-3′ PLNA-R; 5′-CTTACGCCAATCTATACG-3′	*plnA*	(94 °C 1 min, 53 °C 1 min, 72 °C 0:30 min) × 30	Ben Omar et al. (2006)
PLNB-F; 5′-TTCAGAGCAAGCCTAAATGAC-3′ PLNB-R; 5′-GCCACTGTAACACCATGAC-3′	*plnB*	(94 °C 1 min, 51.5 °C 1 min, 72 °C 0:30 min) × 30	
PLNC-F; 5′-AGCAGATGAAATTCGGCAG-3′ PLNC-R; 5′-ATAATCCAACGGTGCAATCC-3′	*plnC*	(94 °C 1 min, 49.5 °C 1 min, 72 °C 0:30 min) × 30	
PLND-F; 5′-TGAGGACAAACAGACTGGAC-3′ PLND-R; 5′-GCATCGGAAAAATTGCGGATAC-3′	*plnD*	(94 °C 1 min, 53 °C 1 min, 72 °C 0:30 min) × 30	
PLNEF-F; 5′-GGCATAGTTAAAATTCCCCCC-3′ PLNEF-R; 5′-CAGGTTGCCGCAAAAAAAG-3′	*plnEF*	(94 °C 1 min, 53.2 °C 1 min, 72 °C 0:30 min) × 30	
PLNI-F; 5′-CTCGACGGTGAAATTAGGTGTAAG-3′ PLNI-R; 5′-CGTTTATCCTATCCTCTAAGCATTGG-3′	*plnI*	(94 °C 1 min, 52.5 °C 1 min, 72 °C 0:30 min) × 30	
PLNJ-F; 5′-TAACGACGGATTGCTCTG-3′ PLNJ-R; 5′-AATCAAGGAATTATCACATTAGTC-3′	*plnJ*	(94 °C 1 min, 51 °C 1 min, 72 °C 0:30 min) × 30	
PLNK-F; 5′-CTGTAAGCATTGCTAACCAATC-3′ PLNK-R; 5′-ACTGCTGACGCTGAAAAG-3′	*plnK*	(94 °C 1 min, 52.9 °C 1 min, 72 °C 0:30 min) × 30	
PLNG-F; 5′-TGCGGTTATCAGTATGTCAAAG-3′ PLNG-R; 5′-CCTCGAAACAATTTCCCCC-3′	*plnG*	(94 °C 1 min, 52.8 °C 1 min, 72 °C 0:30 min) × 30	
PLNN-F; 5′-ATTGCCGGGTTAGGTATCG-3′ PLNN-R; 5′-CCTAAACCATGCCATGCAC-3′	*plnN*	(94 °C 1 min, 51.9 °C 1 min, 72 °C 0:30 min) × 30	
NC8-F; 5′-GGTCTGCGTATAAGCATCGC-3′ NC8-R; 5′-AAATTGAACATATGGGTGCTTTAAATTCC-3′	Plantaricin NC8 structural gene	(94 °C 1 min, 60 °C 1 min, 72 °C 0:30 min) × 30	
PLNS-F; 5′-GCCTTACCAGCGTAATGCCC-3′ PLNS-R; 5′-CTGGTGATGCAATCGTTAGTTT-3′	Plantaricin S structural gene		
PLNW-F; 5′-TCACACGAAATATTCCA-3′ PLNW-R; 5′-GGCAAGCGTAAGAAATAAATGAG-3′	Plantaricin W structural gene	(94 °C 1 min, 55 °C 1 min, 72 °C 0:30 min) × 30	

2.4.1. Antibiotic resistance

ATB ENTEROC 5 strips (bioMérieux, Marcy-l'Etoile, France) were used to determine the susceptibility of isolates to 14 antibiotics. The test was performed and evaluated following the manufacturer's instructions, with the results recorded after 24 h of incubation at 37 °C.

2.5. Properties Related with Survival and Implantation Under In-vitro gastrointestinal conditions

2.5.1. Resistance to HCl and oxgall

For testing survival under gastric and intestinal conditions, each *Lactobacillus* strain (aprox. 9 log cfu/ml) was exposed for 1 h to HCl at pH 3 and immediately for another hour to oxgall 0.3% (Difco-BBL, Franklin Lakes, NJ, USA) to simulate gastric and intestinal conditions in a two-step procedure based on Rodríguez et al. (2003). Lactobacilli surviving after each treatment were determined by diluting and plating on MRS agar. Each assay was repeated twice and the average data from duplicate trials ± standard deviations were determined with the Excel program (Microsoft Corp., USA).

2.5.2. Bile salt hydrolase (BSH) activity

BSH activity was tested by the plate-screening procedure described by Franz et al. (2001) using sodium taurocholate (Sigma) as bile salt.

2.5.3. Biofilm formation

The ability of lactobacilli to form biofilms was quantified essentially as described in Toledo-Arana et al. (2001) by measuring with a microplate reader (Multiskan sprectrum; Thermo Fisher Scientific, Vantaa, Finland) the crystal violet retained (OD_{620}) by the biofilm cells formed in 96-well polystyrene microtiter plates at 37 °C overnight. Each assay was performed in triplicate and repeated twice. *Escherichia coli* U-9 was used as positive control for biofilm formation.

2.6. Adhesion to Cellular Lines and Inhibition of L. monocytogenes CECT 4032 Adhesion

Adhesion assays were conducted as described by Moroni et al. (2006). For individual adhesion assays, cell monolayers in 24-well plates were separately inoculated with 250 μl (10^8 CFU/mL) of each bacterial strain. Plates were then incubated at 37 °C for 30 min, after which free bacteria were eliminated by washing the cell layers twice with phosphate-buffered saline (PBS, Sigma). Cells with adherent bacteria were harvested with EDTA-trypsin, centrifuged at 10,000 ×*g* for 5 min, suspended in PBS, serially diluted, and finally inoculated on TSA plates. *L. monocytogenes* CECT 4032 and *Bacillus subtilis* CECT 498 were used as positive and negative controls respectively in the simple adhesion assays to Caco-2 cells. *G. vaginalis* HC3 and *B. subtilis* CECT 498 were used as positive and negative controls, respectively, in simple adhesion assays to HeLa 229 cells.

To test listeria adhesion inhibition by the selected *Lactobacillus* strains, adhesion of *L. monocytogenes* CECT 4032 and *Lactobacillus* strains to epithelial cells were evaluated separately and in competition assays. Adherent *L. monocytogenes* cells were enumerated on *Listeria* selective medium (PALCAM with added *Listeria* selective supplement, Merck, Darmstadt, Germany) after incubation for 24 to 48 h at 37 °C. Adherent lactobacilli were enumerated on MRS agar with 0.04% sodium azide after incubation at 37 °C for 24 to 48 h. The individual adhesion capacity was expressed as the number of adherent bacteria (CFU/well) divided by the total number of bacteria added, multiplied by 100. For competition assays, cell monolayers in 24-well plates were inoculated with a combination of *Listeria* and lactobacilli (250 μl) either simultaneously or 1 h before or after one another. The inhibition of adhesion of *L. monocytogenes* in the competition assays

was expressed as a percentage using the following formula: Inhibition of adhesion = 100(1 − *T*1/*T*2), where *T*1 and *T*2 are the numbers of adherent *Listeria* cells (CFU/well) in the presence and absence of each *Lactobacillus* strain, respectively. Each assay was repeated twice and the average data from duplicate trials ± standard deviations were determined with the Excel program (Microsoft Corp., USA).

2.7. Production of Antimicrobial Substances

2.7.1. Production of Hydrogen Peroxide

Detection of H_2O_2 production was performed according to Rabe and Hillier (2003) in MRS agar containing 0.25 g/l 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) and 10 ml/L horseradish peroxidase (1.0 mg/ml, Sigma). Bacterial isolates were spotted on agar and incubated at 37 °C for 72 h in an anaerobic environment. After incubation, the plates were exposed to air for 30 min to allow colour production. Colonies that were positive for hydrogen peroxide production turned blue.

2.7.2. Production of bacteriocins

Production of bacteriocin-like inhibitory substances was tested by the spot-on-lawn method. Five microlitres of an overnight culture of each strain was spotted onto plates containing Elliker agar dissolved in PBS. After incubating for 16–18 h, the plates were overlain with 6 mL of soft Elliker agar (Elliker broth with 0.8% agar) previously inoculated with one of the following indicator strains: *L. monocytogenes* CECT 4032, *L. plantarum* CECT 748, *L. paracasei* CECT 4022 t or *E. faecalis* S-47. They were then incubated for 16–18 h at the appropriate temperature to allow the growth of the indicator strains. The appearance of inhibition zones around the strain spots indicated the production of a type of inhibitor substance against the specific indicator strain. To test the proteinaceous nature of the inhibitory substances, 10 μl of a trypsin (Sigma) solution (10 mg/ml in distilled water) was deposited on each bacterial spot. The plates were incubated at 37 °C for 3 h before they were overlain with the indicator strain as above. The absence of inhibition zones around the trypsin-treated spots revealed the protease sensitivity of the inhibitors and hence their peptide nature.

2.8. Detection of Plantaricin Genes in the Bacteriocinogenic Strains

The presence of the main plantaricin genes was screened by PCR amplification following the method described by Ben Omar et al. (2006) using the 13 primer pairs shown in Table 1. The genomic DNA of strains was extracted as described in Martín-Platero et al. (2007). The PCRs were performed in a reaction mixture containing 50 ng of template DNA, 2.5 mM of $MgCl_2$, 400 μM of each deoxynucleoside triphosphate (dNTP), and 1 U of Taq polymerase. The primer concentration was 10 pmol. The reactions were performed in an iCycler 170–8720 (Bio-Rad, Hercules, CA) according to the conditions in Table 1. PCR products were analysed by electrophoresis in 1.5% agarose gels as above.

2.9. Statistical Analyses

The average data from duplicate trials ± standard deviations were determined with the Excel program (Microsoft Corp., USA). Statistical analyses were performed using the SPSS 19.0 software (SPSS, Chicago, Ill. USA). Data on microbiological counts in adhesion and in the decrease of adhesion experiments were subjected to the Shapiro–Wilk test and the Levene test for checking data normality and homogeneity. Finally,the one-way ANOVA using Tukey's test was used to determine the significance of differences between strains, where a P value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Functional Properties

None of the 80 *Lactobacillus* strains studied were able to hydrolyse starch. Although not a common metabolic trait in this microbial group, α-amylase activity has been reported in some strains of *L. plantarum* and *L. manihotivorans* and in the species of *L. amylovorus* (Giraud and Cuny, 1997; Talamond et al., 2002).

In contrast to amylase, the presence of exoenzymes hydrolysing casein is a very common characteristic in LAB and other microorganisms in dairy products. These proteinases catalyse the initial steps in the hydrolysis of milk proteins, providing the cell with the amino acids essential for LAB growth and enabling more efficient growth in protein-rich media (Kunji et al., 1996). The proteinases also play an important role in cheese maturation because they contribute to milk protein breakdown and therefore have an impact on cheese texture (Olson, 1990). In this study, 31 strains of *L. plantarum* (86.1%) and 30 strains of *L. paracasei* (68.2%) were able to hydrolyse casein (Tables 4A, 4B).

Raffinose and stachyose are α-galactosides that are indigestible in the human gut due to the absence of α-galactosidase secretion by intestinal mucosa. Consequently, intact oligosaccharides pass directly into the lower intestine, where they are metabolized by bacteria that possess this enzyme, resulting in the production of flatulence (Donkor et al., 2007). In our study, raffinose was fermented by 16.7% of *L. plantarum* isolates and stachyose by 15.9% of *L. paracasei* isolates, but none of the strains degraded both sugars (Tables 4A, 4B).

Phytase activity was present in 10 *L. plantarum* strains (27.8%) and in two *L. paracasei* strains (4.5%) (Tables 4A, 4B). This is a desirable activity in probiotic bacteria since phytic acid (*myo*-Inositol hexaphosphate), the main source of phosphorus in cereal grains, is considered to be an anti-nutritional factor for humans and animals as it acts as an excellent chelator of essential dietary minerals, thereby decreasing the dietary bioavailability of these nutrients (De Angelis et al., 2003). In addition, it is also reported that lower inositol phosphate derivatives can have health benefits consisting of protection against colon cancer (Shamsuddin, 2002), arteriosclerosis, neural tissue degeneration, and coronary heart disease. Phytase activity has been reported in many microorganisms, including lactic acid bacteria (Reale et al., 2004). It has been shown that lactic acid bacteria used in milk and milk-based products such as strains of *L. acidophilus*, *L. casei*, *Lactococcus lactis*, *L. delbrueckii*, and *Streptococcus* sp. are very poor producers of phytase, whereas *L. amylovorus* and *L. plantarum*, present in a variety of microbial systems of plant origin, result in the maximum enzyme yield (De Angelis et al., 2003).

The accumulation of hydrogen peroxide in food fermentations has undesired effects since it may cause colour defects and rancidity (Hertel et al., 1998). Lactobacilli are commonly regarded as devoid of catalase activity, but there are several reports of strains of lactobacilli that exhibit catalase activity if exogenous heme (naturally present in meat products) is provided (Whittenbury, 1964; Wolf and Hammes, 1988). None of the *L. paracasei* strains assayed were able to decompose hydrogen peroxide when exogenous heme was provided. In contrast, all *L. plantarum*, with the exceptions of strains Mb1, Mb57, and Mb58, showed this activity (Tables 4A, 4B).

3.2. Properties Related with Survival and Implantation Under In-vitro Gastrointestinal Conditions

Properties related with survival in the transit through the gastrointestinal tract and gut colonization include resistance to acid pH, bile tolerance, bile salt hydrolysis, biofilm formation, and adhesion to different human cellular lines.

Prior to reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach, where the secretion of gastric acid constitutes a primary defence mechanism against the majority

of ingested micro-organisms. The pH in the human stomach ranges from 1, during fasting, to 4.5, after a meal, and food digestion can take up to 3 h (Maragkoudakis et al., 2006). In order to test survival under gastrointestinal conditions, we decided to examine the viability of lactobacilli under pH 3 and after a subsequent exposure to bile acids. All *Lactobacillus* strains were completely resistant after a challenge of pH 3 for 1 h. Nevertheless, many lactobacilli displayed a loss of viability when, after low pH exposure, they suffered a second stress of 0.3% oxgall for one more hour. In fact, only one *L. paracasei* strain (2.7%) and 33 *L. plantarum* strains (91.67%) were tolerant to both pH 3 and bile acid stresses (loss of viability > 3 log cycles) (Tables 4A, 4B). These results are in concordance with those obtained for BSH activity since *L. plantarum* strains in general presented this ability and, in contrast, *L. paracasei* isolates did not hydrolyse bile salts.

Resistance to bile salts is considered an important property in strains envisaged as probiotics (FAO/WHO, 2001, 2002). However, there is no consensus about the precise concentration to which the selected strain should be tolerant (Begley et al., 2005; De Smet et al., 1995; Vizoso et al., 2006). Since the physiological concentrations of bile acids in the small intestine range between 5000 and 20,000 μM (Vizoso et al., 2006), in this study we have used a concentration of 0.5% bile salts, equivalent to 12,255 μM. The inference of BSH activity in gut colonization and in the probiosis of *Lactobacillus* is controversial. Although some researchers maintain that deconjugation of bile salts might be a detoxification mechanism of vital importance to the lactobacilli (Begley et al., 2005; De Smet et al., 1995), others infer that the higher toxicity of deconjugated salts might negatively affect their viability (Grill et al., 2000). With respect to probiosis, the results obtained by Vizoso et al. (2006) on the *in-vitro* probiotic properties of several strains of *Lactobacillus johnsonii* and *L. plantarum* showed that BSH activity could be a valuable trait for these bacteria. In fact, this functional property has also been suggested to be important in lowering serum cholesterol levels through the increased demand of this compound to *de novo* bile salt synthesis in liver. However, other authors (Marteau et al., 1995) have demonstrated that lactic acid bacteria can cause the 7α-hydroxylation of primary bile salts, thus converting them to secondary bile salts, which are pro-carcinogenic. In our study, 33 *L. plantarum* strains (83.3%) and no *L. paracasei* were able to hydrolyse bile salts (Tables 4A, 4B).

The formation of biofilms that mask the epithelial cell receptors, thereby preventing the implantation of undesirable pathogenic microorganisms, may be considered as a positive trait in probiotics (Martín et al., 2008). However, none of the strains under study were able to produce biofilms. Nonetheless, the fact that a bacterium is not able to form biofilms by itself does not mean that it may not be included in a biofilm initiated by other pioneer bacteria.

Adhesion to mammalian epithelial cells is a key process for bacteria to survive and to colonize the gastrointestinal tract. For pathogenic bacteria, adhesion to epithelia is a critical step since it allows the release of enzymes and toxins initiating necrotic processes directly into the target cell, thereby facilitating the invasion (Jankowska et al., 2008). Several studies indicate that lactic acid bacteria (LAB) could prevent the attachment of pathogens, thereby reducing colonization and preventing infection (Zárate and Nader-Macias, 2006). In order to complete the study of *Lactobacillus* strains, we performed *in-vitro* experiments on the adhesion capability to human epithelial cells derived from colon adenocarcinoma (Caco-2 line) and vaginal cervix carcinoma (HeLa 229 line) of the strains most resistant to low pH-oxgall and positive for several of the functional properties assayed (caseinase, phytase, and catalase) and, specially, for bacteriocin production: *L. plantarum* 17bP29, 17bP30, 17bP31, 17bP48, Mb25, Mb26, Mb40, Mc14, and Mc45. Specifically, the bacteriocinogenic strains *L. plantarum* Mb40, Mc14, and Mc45 were positive for several genes codifying the structural genes and the transport system operon of plantaricin E/F and for several production and immunity genes of bacteriocin J/K, suggesting that these strains produce both bacteriocins (see below in Table 2). Fig. 1 shows the adhesion efficiency of *L. plantarum* strains to Caco-2 and HeLa cells and also the adhesion of *L. monocytogenes* CECT 4032 and *B. subtilis* CECT 498, used as positive and negative controls, respectively. *L. monocytogenes*, *L. plantarum* Mb25, and *L. plantarum* Mb26 were the most adherent to Caco-2 cells, with adherence percentages of 46.2, 36.0, and 7.0% respectively, but only *Listeria* and *L. plantarum* Mb25 showed a significant adherence ($P < 0.05$) as compared with the negative control *B. subtilis* CECT 498 (0.65% adhesion). Both *Lactobacillus* strains also showed significant adherence ($P < 0.05$) to HeLa 229 cells (as compared with the 0.40% exhibited by the negative control *B. subtilis* CECT 498) with 19.27% and 16% adhesion, respectively, which is moderate in relation to adhesion of the vaginal pathogen *G. vaginalis* H3 (35.88%) (Fig. 1B). In any case, this value was higher than that reported for the bifidobacterial species (less than or equal to 7.68%) (Moroni et al., 2006).

Although adhesion ability to epithelial cells is a criterion for defining a new probiotic strain (Saarela et al., 2000; Salminen et al., 1996), current research also focuses (rather than merely on the simple adhesion of putative probiotics) on their interference with pathogen adhesion. Because of their adherence ability to cell lines, the strains *L. plantarum* Mb25 and Mb26 were selected to carry out competition assays in Caco-2 cells. When the Mb26 strain was added 1 hour before *Listeria*, a significant decrease of 51.58% ($P < 0.05$) in adhesion was observed (Fig. 2). A lower non-significant (P □ 0.05) inhibition of adhesion (21%) was observed when lactobacilli were added simultaneously to *Listeria* (Fig. 2). In contrast, when *L. plantarum* Mb26 was added 1 h

Table 2
PCR amplification of plantaricin genes in the bacteriocinogenic *L. plantarum* strains isolated from Spanish farmhouse cheeses.

Plantaricin genes	Strains											
	17bP29	17bP30	17bP31	17bP48	Mb1	Mb25	Mb26	Mb40	Mb57	Mb58	Mc14	Mc45
plnA	+	+	+	+	+	+	+	+	+	+	+	+
plnB	+	+	+	+	+	+	+	+	+	+	+	+
plnC	+	+	+	+	+	+	+	+	+	+	+	+
plnD	+	+	+	+	+	+	+	+	+	+	+	+
plnEF	+	+	+	+	+	+	+	+	+	+	+	+
plnI	+	+	+	+	+	+	+	+	+	+	+	+
plnJ	−	−	−	−	−	−	−	+	−	−	+	+
plnK	−	−	−	−	−	−	−	+	−	−	+	+
plnG	+	+	+	+	+	+	+	+	+	+	+	+
plnN	−	−	−	−	−	−	−	+	−	−	+	+
P-NC8[a]	−	−	−	−	−	−	−	−	−	−	−	−
P-S[b]	−	−	−	−	−	−	−	−	−	−	−	−
P-W[c]	−	−	−	−	−	−	−	−	−	−	−	−

[a] Plantaricin NC8 structural gene.
[b] Plantaricin S structural gene.
[c] PLANTARICIN W structural gene.

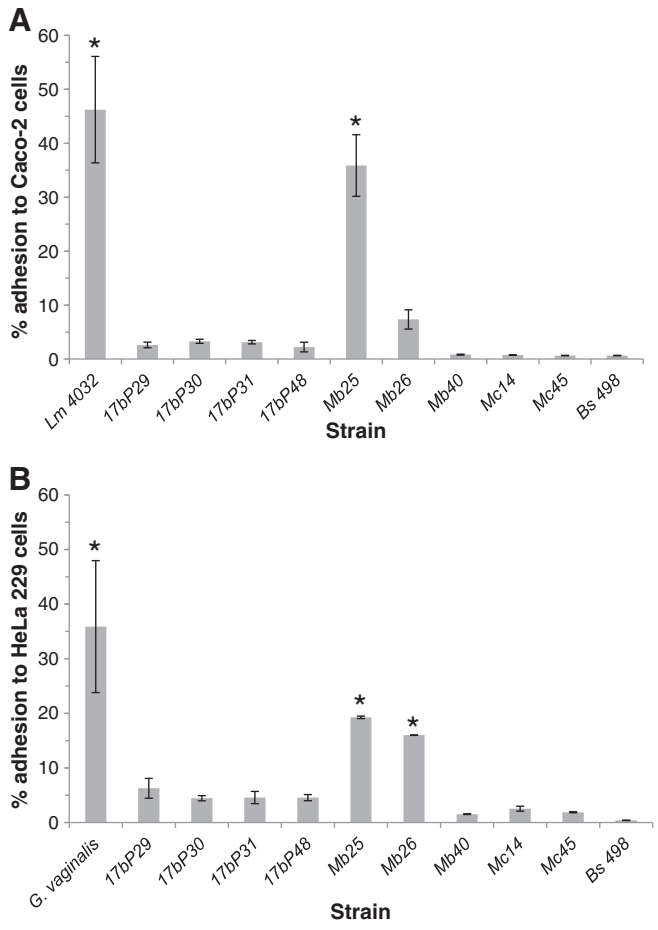

Fig. 1. Adhesion of *Lactobacillus plantarum* isolates to Caco-2 and HeLa 229 cell lines. A) Adhesion to Caco-2 cells; B) adhesion to HeLa 229 cells. (*) $P < 0.05$, indicating statistically significant differences.

after *Listeria*, no adhesion inhibition of this pathogen could be detected (Fig. 2). *L. plantarum* Mb25 was not capable of inhibiting *Listeria* adhesion in any experimental condition (results not shown). This behaviour is quite rare since both strains have a very similar response to the traits investigated. In the study by Moroni et al. (2006), when *Listeria* and bifidobacteria were added simultaneously, no inhibition of listeria adhesion was observed. Similar results were obtained when *L. monocytogenes* was added 1 h before bifidobacteria. In contrast, when bifidobacteria were added at a concentration of 10^8 CFU/ml 1 h before *Listeria*, decreases in invasion ranged from 38% to 90% for the different *Bifidobacterium* strains used.

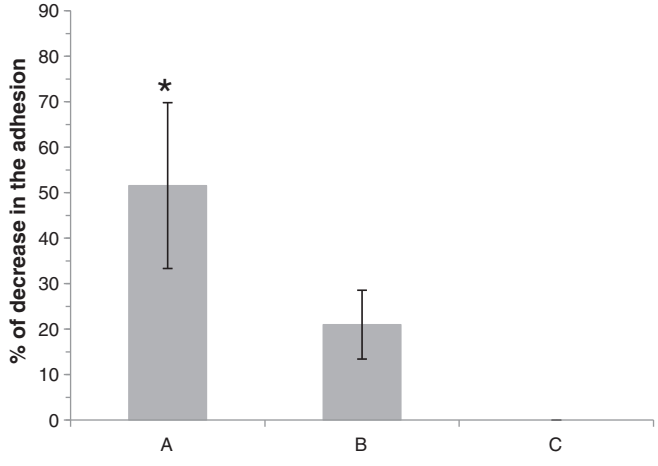

Fig. 2. Inhibition of *Listeria monocytogenes* CECT 4032 adhesion by *Lactobacillus plantarum* Mb26. The Mb26 10 strain was inoculated 1 h before (A), simultaneously (B), or 1 h after (C) *L. monocytogenes*. (*) $P < 0.05$, indicating statistically significant differences.

3.3. Production of Antimicrobial Substances

The production of H_2O_2 was detected in 14 *L. plantarum* strains (38.88%) and in 4 *L. paracasei* strains (9.1%) (Tables 4A, 4B). Certain data suggest that H_2O_2 production by lactobacilli may be more relevant than lactic acid production in dominating some ecosystems, such as the vagina (Rabe and Hillier, 2003). In an earlier study carried out by Martín et al. (2008), for instance, 71% of *Lactobacillus* spp. isolated from the human vagina generated H_2O_2, although the single *L. plantarum* isolated did not produce this antimicrobial.

Bacteriocin production is often proposed as a beneficial characteristic of probiotics that may contribute to host protection against gastrointestinal pathogens (Avonts et al., 2004). The role of bacteriocins in the protection against *Listeria* infection has been conclusively proven *in vivo* for the bacteriocin Abp118 produced by *L. salivarius* UCC118 (Corr et al., 2007). In addition, bacteriocin production may facilitate the establishment of a strain in the competitive environment of the gut. We have screened for the production of inhibitory substances using as indicator strains *L. plantarum* CECT748, *L. paracasei* CECT4022, *L. monocytogenes* CECT4032, and *E. faecalis* S-47 with the deferred antagonism technique. Twelve strains of *L. plantarum* (33.3%) were capable of inhibiting all indicator strains used with the exception of *L. monocytogenes*, and eight *L. paracasei* strains (18.2%) inhibited only *L. plantarum* CECT748 and *L. paracasei* CECT4022 (excepting *L. paracasei* C3-83, which inhibited only *L. paracasei*). All inhibitory activities were sensitive to trypsin, indicating the proteinaceous nature of the substances. *L. plantarum* strains were more active against *L. plantarum* CECT748 and *E. faecalis* S-47, and *L. paracasei* strains were more active against *L. plantarum* CECT748 and *L. paracasei* CECT4022 (results not shown).

The production of several bacteriocins (plantaricins) has been described in *L. plantarum*, with the best known being that produced by the C11 strain, isolated from cucumber fermentation (Daeschel et al., 1990). The *pln* locus responsible for bacteriocin biosynthesis in *L. plantarum* C11 is organized into five operons. The regulatory

Table 3
Frequency of antibiotic resistances in the selected *Lactobacillus plantarum* strains.

Antimicrobial	Concentration (μg/ ml)	Frequency of antimicrobial resistance (%)
Ampicillin	8	0.00
Penicillin	8	33.33
Erythromycin	0.5	0.00
	4	0.00
Tetracycline	4	33.33
	8	0.00
Chloramphenicol	8	0.00
	16	0.00
Rifampicin	1	55.56
	2	0.00
Ciprofloxacin	1	100.00
	2	100.00
Levofloxacin	2	22.22
	4	0.00
Vancomycin	4	100.00
	16	100.00
Teicoplanin	8	100.00
	16	100.00
Nitrofurantoin	32	0.00
	64	0.00
Gentamycin	500	0.00
Streptomycin	1000	0.00
Quinupristin/ Dalfopristin	1	0.00
	2	0.00

operon *plnABCD* encodes an inducing peptide (PlnA), a histidine protein kinase (PlnB), and two response regulators (PlnC and PlnD). The *plnEFI* and *plnJKLR* operons encode two-peptide bacteriocins (PlnEF and PlnJK) and their corresponding immunity proteins. The *plnMNOP* codes for four putative proteins, of which PlnN appears to be a putative prebacteriocin with an N-terminal double glycine leader consensus. The last *plnGHSTUVW* operon encodes for an ABC transport system and an accessory protein that secretes and processes the bacteriocin precursors. PlnA peptide induces the transcription of these five operons (revised in Diep et al., 2009). In addition to the C11 strain, four other strains of *L. plantarum* (WCFS1, NC8, J51, and J23) have each been found to harbour a similar bacteriocin locus in their genomes, part being highly conserved and the other part being a mosaic. Together these strains produce four different class IIb two-peptide bacteriocins, the plantaricins EF, JK, NC8 and J51, and a pheromone peptide plantaricin A with antimicrobial activity (Diep et al., 2009). Many studies support the wide diffusion of plantaricin genes between *L. plantarum* strains of different sources. As an example, in a study on plantaricin production carried out on 33 *L. plantarum* isolates obtained from grape musts and wines during fermentation, Sáenz et al. (2009) found that 74% of the strains contained *plnABCD*, the two-peptide bacteriocin plnEF genes, and the genes *plnGHSTUVW*. Ben Omar et al. (2006) screened plantaricin genes in six *L. plantarum* strains isolated from ben saalga (a traditional fermented gruel from Burkina Faso), but only the *plnG* gene was positive in five of the strains. In addition, one strain tested positive for *plnA*, *plnC*, *plnD*, *plnEF*, *plnI*, *plnJ*, *plnK*, and *plnN*. Another work that Ben Omar et al. (2008) carried out on 31 lactobacilli isolated from poto poto (a Congolese fermented maize product) revealed the complete *plnEFI* operon in thirteen isolates. Genes encoding for the plantaricin J/K were detected in six isolates, whereas others only tested positive for *plnK*. Finally, the *plnG* gene was also found in 16 isolates.

In our study, all the bacteriocinogenic strains were positive for the *plnABCD* transport system operon, the *plnEFI* operon that codes for bacteriocins and immunity and the *plnG* gen (part of the large operon involved in plantaricin export), suggesting that all the strains may produce plantaricin E/F (Table 2). In addition, the strains *L. plantarum* Mb40, Mc14, and Mc45 were positive for *plnJK* operon, which also codes for J/K bacteriocin and immunity, and for *plnN*, suggesting that the three strains may produce the plantaricin J/K as well. None of the strains were positive for plantaricins NC8, S, or W structural genes (Table 2).

Table 4A
Summary of results of properties studied in *Lactobacillus paracasei* strains. Properties for which all strains tested negative are not presented. Susceptibility to pH 3 is expressed as log UFC/ml T_0 − Log UFC/ml $T_{1h\ pH3}$. Susceptibility to pH and oxgall is expressed as log UFC/ml T_0 − Log UFC/ml $T_{1h\ pH3\ +\ 1h\ oxgall}$.

Strain	Functional properties					Inhibitory properties			Gut survival properties	
	Raffinase	Stachyose	Caseinase	Phytase	Catalase	H_2O_2	Bacteriocins	BSH	Susceptibility to pH 3	Susceptibility to pH 3 + Oxgall
L. paracasei O114	−	+	+	−	−	−	+	−	0.00 ± 0.00	5.59 ± 0.11
L. paracasei O121	−	+	+	−	−	−	+	−	0.02 ± 0.03	6.02 ± 0.45
L. paracasei O99	−	+	−	−	−	−	+	−	0.10 ± 0.13	5.68 ± 0.22
L. paracasei O109	−	+	−	−	−	−	+	−	0.31 ± 0.10	6.08 ± 0.38
L. paracasei C3-60	−	−	+	−	−	−	+	−	0.11 ± 0.04	5.17 ± 0.71
L. paracasei C3-70	−	−	+	+	−	−	−	−	0.84 ± 0.01	5.80 ± 0.49
L. paracasei C3-89	−	−	+	+	−	−	−	−	0.43 ± 0.60	5.75 ± 0.58
L. paracasei Mb4	−	+	+	−	−	−	−	−	0.09 ± 0.02	6.15 ± 0.02
L. paracasei Mb22	−	+	+	−	−	−	−	−	0.21 ± 0.24	6.46 ± 0.04
L. paracasei Mb55	−	−	+	−	−	+	−	−	0.00 ± 0.00	4.83 ± 2.05
L. paracasei Mb68	−	−	+	−	−	+	−	−	0.07 ± 0.01	6.12 ± 0.19
L. paracasei Mb79	−	−	+	−	−	+	−	−	0.06 ± 0.10	5.91 ± 0.22
L. paracasei Mc4	−	−	+	−	−	+	−	−	0.24 ± 0.23	5.26 ± 0.65
L. paracasei C3 − 83	−	−	−	−	−	−	+	−	0.06 ± 0.03	6.01 ± 0.19
L. paracasei C3 − 110b	−	−	−	−	−	−	+	−	0.01 ± 0.01	5.97 ± 0.71
L. paracasei Mb71	−	−	−	−	−	−	+	−	0.15 ± 0.12	6.35 ± 0.28
L. paracasei O147	−	+	−	−	−	−	−	−	0.13 ± 0.18	4.33 ± 0.79
L. paracasei C3 − 52	−	−	+	−	−	−	−	−	0.09 ± 0.13	6.30 ± 0.23
L. paracasei C3-59	−	−	+	−	−	−	−	−	0.00 ± 0.00	6.28 ± 0.22
L. paracasei C3-63	−	−	+	−	−	−	−	−	0.07 ± 0.10	6.24 ± 0.23
L. paracasei C3-66	−	−	+	−	−	−	−	−	0.05 ± 0.02	6.22 ± 0.24
L. paracasei C3-72	−	−	+	−	−	−	−	−	0.03 ± 0.03	6.04 ± 0.35
L. paracasei C3-73	−	−	+	−	−	−	−	−	0.00 ± 0.00	6.00 ± 0.19
L. paracasei C3-80	−	−	+	−	−	−	−	−	0.05 ± 0.06	6.10 ± 0.33
L. paracasei C3-84	−	−	+	−	−	−	−	−	0.10 ± 0.13	5.51 ± 0.05
L. paracasei C3-102b	−	−	+	−	−	−	−	−	0.00 ± 0.00	5.61 ± 0.33
L. paracasei C3-108b	−	−	+	−	−	−	−	−	0.01 ± 0.01	4.50 ± 0.24
L. paracasei Mb12	−	−	+	−	−	−	−	−	0.09 ± 0.05	2.98 ± 0.67
L. paracasei Mb18	−	−	+	−	−	−	−	−	0.17 ± 0.19	6.09 ± 0.01
L. paracasei Mb70	−	−	+	−	−	−	−	−	0.13 ± 0.04	6.23 ± 0.10
L. paracasei Mc8	−	−	+	−	−	−	−	−	0.12 ± 0.15	6.02 ± 0.10
L. paracasei Mc12	−	−	+	−	−	−	−	−	0.01 ± 0.01	6.34 ± 0.23
L. paracasei Mc41	−	−	+	−	−	−	−	−	0.04 ± 0.05	5.22 ± 1.18
L. paracasei Mc47	−	−	+	−	−	−	−	−	0.21 ± 0.30	5.00 ± 0.18
L. paracasei P2	−	−	+	−	−	−	−	−	0.10 ± 0.14	4.87 ± 0.45
L. paracasei P11	−	−	+	−	−	−	−	−	0.16 ± 0.01	5.00 ± 0.87
L. paracasei C3-54	−	−	−	−	−	−	−	−	0.09 ± 0.13	6.11 ± 0.10
L. paracasei C3-75	−	−	−	−	−	−	−	−	0.02 ± 0.03	6.10 ± 0.04
L. paracasei Mb13	−	−	−	−	−	−	−	−	0.05 ± 0.06	6.48 ± 0.04
L. paracasei Mb23	−	−	−	−	−	−	−	−	0.34 ± 0.47	6.33 ± 0.12
L. paracasei Mb30	−	−	−	−	−	−	−	−	0.10 ± 0.03	5.41 ± 0.59
L. paracasei Mb39	−	−	−	−	−	−	−	−	0.06 ± 0.09	5.01 ± 1.2
L. paracasei Mc13	−	−	−	−	−	−	−	−	0.14 ± 0.19	4.87 ± 1.53
L. paracasei Mc32	−	−	−	−	−	−	−	−	0.00 ± 0.00	4.80 ± 1.25

Table 4B
Summary of results of properties studied in *Lactobacillus plantarum* strains. Properties for which all strains tested negative are not presented. Susceptibility to pH 3 is expressed as log UFC/ml T_0 − Log UFC/ml $T_{1h\ pH3}$. Susceptibility to pH and oxgall is expressed as log UFC/ml T_0 − Log UFC/ml $T_{1h\ pH3\ +\ 1h\ oxgall}$.

Strain	Functional properties					Inhibitory properties		Safety[a]	Gut survival properties		
	Raffinase	Stachyase	Caseinase	Phytase	Catalase	H_2O_2	Bacteriocins	Antibiotic resistance	BSH	Susceptibility to pH 3	Susceptibility to pH 3 + Oxgall
L. plantarum 17bP30	−	−	+	+	+	−	+	Tet-c, Rfa-c, Cip, Lvx-c, Van, Tec	+	0.06 ± 0.00	0.09 ± 0.12
L. plantarum 17bP31	−	−	+	+	+	−	+	Pee, Cip, Van, Tec	+	0.09 ± 0.10	0.05 ± 0.07
L. plantarum 17bP48	−	−	+	+	+	−	+	Pee, Cip, Van, Tec	+	0.10 ± 0.14	0.04 ± 0.02
L. plantarum Mb25	−	−	+	+	+	−	+	Cip, Van, Tec	+	0.46 ± 0.06	0.44 ± 0.17
L. plantarum Mb26	−	−	+	+	+	−	+	Pee, Rfa-c, Cip, Van, Tec	+	0.33 ± 0.17	0.31 ± 0.13
L. plantarum Mb40	+	−	−	−	+	+	+	Tet-c, Rfa-c, Cip, Van, Tec	+	0.05 ± 0.06	0.26 ± 0.00
L. plantarum Mc45	+	−	+	−	+	−	+	Rfa-c, Cip, Lvx-c, Van, Tec	+	0.01 ± 0.01	0.46 ± 0.25
L. plantarum 17bP29	−	−	−	+	+	−	+	Cip, Van, Tec	+	0.06 ± 0.09	0.00 ± 0.00
L. plantarum Mc14	+	−	+	−	+	−	+	Tet-c, Rfa-c, Cip, Van, Tec	+	0.04 ± 0.06	3.63 ± 0.03
L. plantarum O83	−	−	+	−	+	+	−	N. A.	+	0.05 ± 0.02	0.61 ± 0.18
L. plantarum O86	−	−	+	−	+	+	−	N. A.	+	0.03 ± 0.04	0.53 ± 0.14
L. plantarum O95	−	−	+	−	+	+	−	N. A.	+	0.16 ± 0.02	1.21 ± 0.01
L. plantarum O124	−	−	+	−	+	+	−	N. A.	+	0.28 ± 0.40	1.72 ± 0.39
L. plantarum Mb46	−	−	+	+	+	−	−	N. A.	+	0.48 ± 0.58	2.44 ± 1.46
L. plantarum Mb50	−	−	+	+	+	−	−	N. A.	+	0.16 ± 0.08	0.47 ± 0.28
L. plantarum Mb61	−	−	+	+	+	−	−	N. A.	+	0.34 ± 0.44	1.70 ± 0.77
L. plantarum Mb67	−	−	+	+	+	−	−	N. A.	+	0.18 ± 0.26	1.56 ± 0.24
L. plantarum P1	−	−	+	−	+	+	−	N. A.	+	0.19 ± 0.27	2.34 ± 1.18
L. plantarum P16	−	−	+	−	+	+	−	N. A.	+	0.30 ± 0.23	2.73 ± 0.41
L. plantarum P19	−	−	+	−	+	+	−	N. A.	+	0.34 ± 0.35	1.58 ± 1.7
L. plantarum P23	−	−	+	−	+	+	−	N. A.	+	0.11 ± 0.15	2.15 ± 1.47
L. plantarum P130	−	−	+	−	+	+	−	N. A.	+	0.21 ± 0.13	2.43 ± 1.86
L. plantarum O150	−	−	−	−	+	+	−	N. A.	+	0.05 ± 0.07	0.72 ± 0.18
L. plantarum Mb2	−	−	+	−	+	−	−	N. A.	+	0.06 ± 0.08	0.28 ± 0.10
L. plantarum O6	−	−	+	−	+	−	−	N. A.	+	0.03 ± 0.04	0.08 ± 0.04
L. plantarum O28	−	−	+	−	+	−	−	N. A.	+	0.47 ± 0.66	0.07 ± 0.10
L. plantarum P17	−	−	+	−	+	−	−	N. A.	+	0.16 ± 0.07	1.62 ± 0.04
L. plantarum Mb186	−	−	+	−	+	−	−	N. A.	+	0.22 ± 0.12	2.40 ± 1.20
L. plantarum O79	−	−	+	−	+	−	−	N. A.	+	0.96 ± 0.22	3.00 ± 1.06
L. plantarum Mb45	−	−	−	−	+	−	−	N. A.	+	0.17 ± 0.06	0.26 ± 0.19
L. plantarum Mb3	−	−	+	−	+	−	−	N. A.	−	0.01 ± 0.02	0.22 ± 0.12
L. plantarum Mc29	−	−	+	−	+	−	−	N. A.	−	0.06 ± 0.08	0.11 ± 0.03
L. plantarum Mb57	+	−	+	−	−	+	+	N. A.	−	0.47 ± 0.58	5.87 ± 0.50
L. plantarum Mb58	+	−	+	−	−	+	+	N. A.	−	0.02 ± 0.03	5.34 ± 1.25
L. plantarum Mb1	+	−	−	−	−	+	+	N. A.	−	0.05 ± 0.02	5.51 ± 0.77
L. plantarum Mb75	−	−	+	−	+	−	−	N. A.	−	0.11 ± 0.16	3.97 ± 0.48

[a] N. A.: not available; Pee: penicillin entero 8 μg/ml; Tet: tetracycline 8 μg/ml, Tet-c: tetracycline 4 μg/ml; Rfa: rifampicin 2 μg/ml, Rfa-c: rifampicin 1 μg/ml;Cip: ciprofloxacin 2 μg/ml, Cip-c: ciprofloxacin 1 μg/ml; Lvx: levofloxacin 4 μg/ml, Lvx-c: levofloxacin 2 μg/ml; Van: vancomycin 16 μg/ml, Van-c: vancomycin 4 μg/ml; Tec: teicoplanin 16 μg/ml, Tec-c: teicoplanin 8 μg/ml.

3.4. Safety Aspects

With the purpose of determining the safety status of *Lactobacillus* isolates, we have investigated the presence of certain enzymatic activities related with virulence, antibiotic resistance, and the production of biogenic amines.

All the lactobacilli isolated showed neither gelatinase, DNase, nor mucinase activity. Lack of mucin degradation has been reported as an important marker for the safety assessment of potential probiotic strains (Salminen et al., 1998) since these glycosylated proteins form the mucus layer, an important component of the physical gut barrier. In addition, none of the strains was able to decarboxylate the precursor aminoacids and form histamine, tyramine, putrescine, cadaverine, or agmatine when this property was investigated by production in a liquid medium. In addition, the presence of genes for the production of histidine decarboxylase (*hdc*, 367pb), ornithine decarboxylase (*odc*, 1446 pb), and tyrosine decarboxylase (*tdc*, 924 pb) was screened by multiplex PCR. No amplification occurred for either of the aminoacid decarboxylase genes (data not shown).

ATB ENTEROC 5 strips were used to determine the susceptibility of *Lactobacillus* strains to antimicrobials, and were evaluated in accordance with the manufacturer's instructions. The antibiotic concentrations on the ATB ENTEROC 5 strip comply with Clinical and Laboratory Standards Institute (CLSI) (2007) committee recommendations. Only the nine *L. plantarum* strains that showed the most promising traits with respect to functional and gut implantation related properties as well as the production of antimicrobial substances were analysed for antimicrobial resistance. The percentage of *L. plantarum* isolates able to grow in liquid medium at a given antimicrobial concentration provided by the strip and therefore resistant to it is shown in Table 3. All lactobacilli were sensitive to ampicillin (MIC ≤ 8 μg/ml), but 33.33% of the strains showed resistance to penicillin (MIC > 8 μg/ml). This is an unusual trait in lactobacilli, which are generally sensitive to this antibiotic (Danielsen and Wind, 2003). Erythromycin and chloramphenicol were highly active against all isolates. However, 33.33% of strains showed intermediate resistance to tetracycline (MIC > 4 μg/ml, but all were susceptible to 8 μg/ml). All strains assayed were resistant to ciprofloxacin (MIC > 2 μg/ml), vancomycin (MIC > 16 μg/ml), and teicoplanin (MIC > 16 μg/ml). Many species of *Lactobacillus* carry intrinsic resistance to vancomycin, which is not considered a risk factor in lactobacilli, as many strains have a long history of safe use as probiotics and there is no indication that they can transfer resistance to other species (Mattila-Sandholm et al., 1999). Resistance to ciprofloxacin also appears to be a natural trait in many species of *Lactobacillus* (Elisha and Courvalin, 1995). Previous reports also indicate poor ciprofloxacin activity against many *Lactobacillus* isolates (Hamilton-Miller and Shah, 1994; Herra et al., 1995; Zarazaga et al., 1999). All isolates were sensitive to gentamicin, streptomycin, and quinupristin/dalfopristin. Our results are, on the

whole, in agreement with those reported by Pérez-Pulido et al. (2005) for a collection of 43 *L. plantarum* isolated from caper fermentations. Nevertheless, lower resistance was detected in our isolates for penicillin, tetracycline, rifampicin, and levofloxacin and an absence of resistance to nitrofurantoin.

Finally, for a general view of the results for the properties investigated and to highlight the more interesting strains with the aim of selecting a strain or a few strains to be assayed *in vivo*, a summary of results is presented in Tables 4A and 4B.

4. Conclusions

This study on 36 *L. plantarum* strains and 44 *L. paracasei* strains isolated from farmhouse cheeses reveals that these foods are good sources of biotechnologically relevant *Lactobacillus* strains. Of special interest are *L. plantarum* strains, many of which tested positive for a great number of functional and gut survival related characteristics and also for the production of antimicrobial substances, mainly bacteriocins. Especially remarkable are the high proportions of strains with caseinase, haemo-dependent catalase, and BSH activities. Other relevant traits in *L. plantarum* isolates were a high resistance to acid pH and oxgall stresses and the presence of plantaricin genes in twelve of the strains. *L. plantarum* Mb26 particularly stands out due to its adhesion to Caco-2 and HeLa 229 cells and, especially, to the inhibition of *L. monocytogenes* adhesion to Caco-2 cells. In addition, this strain presents caseinase, phytase, haemo-dependent catalase, and BSH activities and probably produces the plantaricin E/F. Mb26 strain also resistant to the acid pH-oxgall challenge. All of these features make *L. plantarum* Mb26 a worthy candidate to carry out in-vivo assays on its probiotic potential.

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