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Characterization of Some Potentially Probiotic *Lactobacillus* Strains of Human Origin

Jelena Novaković Jovanović*, Biljana Nikolić[1], Svetlana Šeatović, Gordana Zavišić, Dragana Mitić-Ćulafić[1], Branka Vuković-Gačić[1], and Jelena Knežević-Vukčević[1]

Galenika ad, Batajnicki drum bb, 11000 Belgrade, Serbia
[1]*University of Belgrade, Faculty of Biology, Studentski trg 16, 11000 Belgrade, Serbia*

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*Corresponding Author
Tel/Fax: +381 11 307 12 05
E-mail: novakovic.jelena@gmail.com

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Abstract A novel preparation for human use was investigated for probiotic properties of new lactobacilli isolates from oral and fecal samples of children. Identified strains were *Lactobacillus plantarum* (Lac1, Lac2, Lac6, and Lac7), *Lactobacillus casei* (Lac3), and *Lactobacillus paracasei* (Lac4). Isolates were non-hemolytic, produced organic acids, were tolerant to wide ranges of temperature, NaCl, and pH, and were highly resistant to lysozyme, acidity, and bile salts. High survival rates in artificial gastric and intestinal fluids indicated abilities to survive passage through the gastrointestinal tract. Antimicrobial activities were restricted to bacteria, attributed to low pH values. Isolated strains possessed good aggregation abilities, high hydrophobicity values, and moderate abilities to adhere to HCT 116 cells. Substantial probiotic features were identified for all isolates. Lac2, Lac6, and Lac7 were identified as the most advantageous candidates for further study of other probiotic and technological properties.

Keywords: *Lactobacillus*, probiotic, adhesiveness, HCT116 cell line

Introduction

Preservation of a microbiological balance in the human gastrointestinal tract is of great importance. A disrupted equilibrium might result in the occurrence of different diseases (1,2). The pioneering work resulting in the Tessie, Moro, and Metchnikoff theory concerning longevity benefits of probiotics has prompted exploration of probiotics as a topic of great interest (3). An expert panel assembled by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as live microorganisms that confer health benefits on a host when administered in adequate amounts (4). Most species with probiotic properties belong to the lactic acid bacteria (LAB), especially to the genera *Lactobacillus* and *Bifidobacterium*, which are 'Generally Recognized as Safe' for human consumption (GRAS status) (4). *In vitro* testing is useful to gain preliminary information about probiotic properties of isolated strains. Several *in vitro* tests are recommended by FAO/WHO (4), including evaluation of gastrointestinal tract (GIT) colonization (5) where probiotics can survive, grow, and perform beneficial actions.

Lactobacilli have an ability to adhere and interact with the intestinal epithelium and mucosal layers (6), which is presumably necessary to confer health benefits (7,8). Potential probiotics should possess antimicrobial activities against certain pathogenic bacteria. Although probiotic strains can be isolated from different sources, a human source is recommended for human use (9). Autochthonous probiotic strains have evolved under the influence of environmental factors in the human gut, and the advantage for therapeutic use comes from the fact that these strains are isolated from the environment, similar to one they would colonize after ingestion (10). This study was conducted to isolate and characterize strains of lactobacilli of human origin, and to evaluate probiotic properties in order to develop a novel pharmaceutical preparation for human use.

Materials and Methods

Bacterial, fungal, and eukaryotic cell cultures *Staphylococcus aureus* ATCC 6538-P, *Micrococcus luteus* ATCC 93419, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Bacillus pumilus* NCTC 8241, *Lactobacillus rhamnosus* ATCC 7469, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830, *Clostridium sporogenes* ATCC 19404, *Listeria monocytogenes* ATCC 19111, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella abony* NTCC 6017, *Aspergillus brasiliensis* ATCC 16404, and *Candida albicans* ATCC 10231 were used in this study. Adhesive

properties of isolates were examined using colorectal carcinoma cells HCT 116 (ATCC CCL-247).

Media and growth conditions Lactobacilli were grown in de Man, Rogosa, and Sharpe broth and agar (MRS; Merck, Darmstadt, Germany) at 37°C under microaerophilic conditions in an anaerobic gas-jar (BioMerieux Co., Marcyl'Etoile, France). Bacterial strains were incubated (D115; Binder, Tuttlingen, Germany) at 37°C for 48 h on Tryptic Soy Agar (TSA) (Merck) and Tryptic Soy Broth (TSB) (Merck). Fungal strains were incubated (D115; Binder) at 25°C for 72 h on Sabouraud Dextrose Agar (SDA) (Merck). Lactose broth (Merck) containing inverted Durham tubes was used to determine gas production. Blood agar plates (Merck) containing 5% sheep blood were used to determine hemolytic activity. Artificial gastric fluid (AGF) (pH 2.0) containing 3 mg/mL of porcine gastric mucosa pepsin (Sigma-Aldrich, Saint Louis, MO, USA), 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, and artificial intestinal fluid (AIF) (pH 8.0) containing 0.1% (w/v) pancreatin USP (ICN Biomedicals, Santa Ana, CA, USA) and 0.15% (w/v) bile salts (Oxgall; Sigma-Aldrich), were prepared immediately before use and sterilized using filtration (0.22 μm cellulose acetate filter; Rotilabo Syringe Filters; Roth, Mannheim, Germany).

Human HCT 116 cells were grown (CB53; Binder) in DMEM (Sigma-Aldrich) with 4.5% D-glucose and 2 mM L-glutamine (PAA Laboratories GmbH, Pasching, Austria), supplemented with 10% fetal bovine serum (PAA Laboratories GmbH) and penicillin/streptomycin (PAA Laboratories GmbH) at 37°C under 5% CO_2 and 100% humidity. HCT116 cells were detached from the flask surface, after the treatment with 0.1% trypsin (PAA Laboratories GmbH) in order to obtain a single cell suspension. The cells were washed by centrifugation (Universal 30 RF; Hettich, Tuttlingen, Germany), and resuspended in Dulbecco's phosphate buffered saline (PBS) (PAA Laboratories GmbH).

Bacterial isolation Bacterial strains were isolated from oral and fecal samples aseptically taken from healthy Serbian infants. Sampling procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national), and with the Helsinki Declaration of 1975 as revised in 2008. Informed consent was obtained from parents of all infants for inclusion in the study. Oral and fecal samples were stored at 4°C (T0022; Euroengel, Brescia, Italy) during transfer, and used for isolation up to 2 h. Each oral and fecal sample was mixed with sterile physiological saline (Merck) and homogenized by vortexing (MS2; IKA Werke, Staufen, Germany). Serial dilutions of oral and fecal samples were plated on MRS agar (Merck) and incubated (D115; Binder) for 48 h at 37°C. After incubation, provisional identification of lactobacilli was carried out as described by Hammes and Hertel (11). Briefly, positive strains were characterized as Gram-positive non-spore-forming rods and negative for the catalase reaction and capable of growth in MRS broth. Purified isolates of lactobacilli were preserved at −20°C (MPR-414 F 500; Sanyo Electric Co., Osaka, Japan) in MRS broth (Merck) containing 15% (v/v) glycerol (Merck).

Phenotypic characterization of bacterial isolates Further identification was carried out for isolates that were positive in provisional identification. Gas production and growth at temperatures between 10 and 50°C for 5 days were monitored. Growth for 3 days at different pH values of MRS broth (Merck) ranging from 2.0 to 8.0 was also investigated, as well as salt tolerance in MRS broth (Merck) containing 2.0, 4.0, and 6.5% NaCl (Merck). Finally, carbohydrate fermentation profiles were developed and biochemical identification was carried out using a standard API 50 CHL kit (BioMerieux Co.).

Genotypic characterization of bacterial isolates Genotypic characterization of isolates was performed based on sequence analyses of the 16S rRNA gene. Total genomic DNA was extracted using a QIAGEN Dneasy® Blood&Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Amplification of the 16S rRNA gene was conducted using the universal bacterial primers 27F 5'-GAGAGTTTGATCCTGGCTCAG-3' (12) and 1523R 5'-AGGAGGT GATCCAGCCG-3' (13) for amplification of products approximately 1,500 bp in size. DNA amplification was performed in 20 μL of a total reaction volume containing 1 μL of extracted DNA, 2 mM $MgCl_2$, 0.3 mM each dNTPs, 0.75 μM of each primer, and 0.75 U of *Taq* polymerase (Fermentas, Vilnius, and Lithuania). After 5 min of initial denaturation at 95°C, 33 amplification cycles in a thermal cycler (Mastercyclerep Gradient S; Eppendorf, Hamburg, Germany) were performed following a thermal profile of 1 min at 95°C for denaturation, 1 min at 50°C for annealing, and 1 min at 72°C for primer extension. Final extension was carried out for 7 min at 72°C. PCR products were separated in 1% agarose gel (Sigma-Aldrich) in TBE buffer (90 mM Tris-Borate, 1 mM EDTA) (Sigma-Aldrich), stained with ethidium bromide (Biorad Laboratories, Berkeley, CA, USA), and visualized under a UV transilluminator (LKB 2011; Macrovue, Bromma, Sweden).

Obtained amplimers of the expected size were purified using a QIA quick PCR Purification Kit (Qiagen) following the manufacturer's instructions. Both strands were sequenced on automated equipment (BMR Service, Padova, Italy), and sequences were deposited in the National Centre for Biotechnology Information (NCBI) GenBank database under accession numbers JN315657, JN315658, JN315659, JN315660, JN315662, and JN315663. Sequence identity was determined based on comparison with bacterial sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank) using BLAST analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Hemolytic activity Isolates were grown in MRS broth (Merck) for 18-24 h at 37°C, streaked on Blood agar plates (Merck) containing 5% sheep blood, and incubated for 24 h at 37°C under both aerobic and anaerobic conditions for hemolytic activity testing. *Staphylococcus aureus* ATCC 6538-P, *Bacillus cereus* ATCC 11778, and *Clostridium*

sporogenes ATCC 19404 were used as positive controls (14).

Detection of antimicrobial activity Agar well diffusion assays (9) were performed to monitor the antimicrobial activities of lactobacilli isolates against S. *aureus* ATCC 6538-P, *M. luteus* ATCC 93419, *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. pumilus* NCTC 8241, *L. rhamnosus* ATCC 7469, *L. lactis* ATCC 7830, *E. coli* ATCC 8739, *K. pneumoniae* ATCC 10031, *P. aeruginosa* ATCC 9027, *S. abony* NTCC 6017, *A. brasiliensis* ATCC 16404, and *C. albicans* ATCC 10231. One hundered microlitres of overnight cultures of each indicator strain containing approximately 10^8 CFU/mL were mixed with 20 mL of melted MRS agar (Merck) for lactobacilli, TSA (Merck) for other bacteria, and SDA (Merck) for fungi, and poured into sterile Petri plates. Cell-free supernatants (CFS) of lactobacilli isolates were obtained after centrifugation (Universal 30 RF; Hettich) of overnight cultures at 3,500×*g* for 20 min at 4°C. CFS was split into 2 fractions. One fraction remained unmodified while the second was adjusted to pH 7.0 with 1 M KOH. Both fractions were filter-sterilized using a cellulose acetate filter with 0.22 μm pores (Rotilabo Syringe Filters; Roth). Wells of 6 mm diameter were cut into agar plates and aliquots of 100 μL of CFS were placed into the wells. Plates were first incubated at 4°C (MPR-414 F 500; Sanyo Electric Co.) for 2 h to allow diffusion of the CFS in the agar, then incubated (D115; Binder) at 37 and 25°C for bacterial and fungal strains, respectively, then inhibition zone sizes were measured and reported as the mean of inhibition zone sizes (n=3) measured from the edge of a well to the margin.

Aggregation and co-aggregation abilities Aggregation and co-aggregation abilities of lactobacilli strains were studied spectrophotometrically as described by Collado *et al.* (15). Overnight cultures were centrifuged twice (Universal 30 RF; Hettich) at 3,500×*g* for 20 min at 4°C, and resuspended in PBS (PAA Laboratories GmbH) with adjustment of the initial absorbance (A_0) value at 600 nm to approximately 0.6 for monitoring of aggregation properties. After incubation at 37°C for 24 h, the absorbance value of the mixture (A_x) was measured (UV/VIS 5625; Unicam, Loughborough, England) at time intervals of 2, 4, and 24 h. The aggregation percentage (Ag%) was calculated as:

$$Ag\% = (1\ A_x/A_0) \times 100\%$$

For assessment of co-aggregation abilities, equal volumes of overnight isolate cultures, and pathogens were mixed and incubated (D115; Binder) for 24 h at 37°C without agitation. Absorbance values of the mixture (A_{mix}), pure pathogen (A_{path}), and pure lactobacilli (A_{lac}) suspensions were measured (UV/VIS 5625; Unicam) at time intervals of 2, 4, and 24 h. The percentage of co-aggregation (Co-Ag%) was calculated for every time interval as:

$$Co\text{-}Ag\% = [1 - 2A_{mix}/(A_{path}+A_{lac})] \times 100\%$$

Bacterial adhesion to a hydrophobic solvent Hydrophobic characteristics of the cell surface of isolated lactobacilli strains were evaluated using a microbial adhesion to hexadecane (MATH) assay, previously described by Deepika *et al*. (16). Overnight lactobacilli cultures were centrifuged twice (Universal 30 RF; Hettich) at 3,500×*g* for 20 min at 4°C, and resuspended in 10 mM KH_2PO_4 (Sigma-Aldrich). pH values of bacterial suspensions were adjusted to 3 with 10 M HCl for minimization of electrostatic interactions between bacterial cells and hexadecane. Initial absorbance (A_0) values at 600 nm were adjusted to approximately 0.6. The same volumes of bacterial suspension and hexadecane were mixed by vortexing (MS2; IKA Werke), and left undisturbed for 20 min to allow complete phase separation, after which the lower aqueous phase was removed and the absorbance value was measured (A_1) (UV/VIS 5625; Unicam). The percentage adhesion to hexadecane (%A) was calculated as:

$$\%A = (1 - A_1/A_0) \times 100\%$$

Bacterial adhesion to HCT 116 cells An adhesion assay was performed following a modified protocol previously explained by Sanchez *et al.* (17). HCT 116 cells as a model of the colon epithelium were inoculated into 12-well tissue culture plates (Sarstedt Inc, Newton, NC, USA) at a density of 8×10^4 cells/well. The cell density was determined using a trypan-blue assay and a light microscope (BOE5000.900; Boeko, Hamburg, Germany). Cells were grown in a medium containing penicillin and streptomycin until 90% confluence and full cell differentiation, then were grown in a medium without antibiotics for 24 h.

Overnight cultures were centrifuged twice (Universal 30 RF; Hettich) at 3,500×*g* for 20 min at 4°C, and resuspended in DMEM (Sigma-Aldrich) without antibiotics for preparation of lactobacilli suspensions for an adhesion assay. The cell monolayer was centrifuged, resuspended with PBS (PAA Laboratories GmbH), and lactobacilli suspensions were added to each well at a ratio of HCT 116 cells/bacteria approximately 1/10 for monitoring of isolate adhesive properties. Cells were co-incubated (CB53; Binder) with bacteria for 60 min at 37°C under 5% CO_2. The medium was transferred to sterile tubes, and 1 mL of PBS (PAA Laboratories GmbH) was added to the monolayer, (also transferred to the tubes), in order to collect all non-adhered bacteria. The monolayer was treated with 0.1% trypsin (PAA Laboratories GmbH) for 3 min and appropriate dilutions of obtained cell suspensions were plated on MRS agar (Merck) and incubated (CB53; Binder) at 37°C for 24 h for enumeration of adherent bacterial cells (CFU_{adh}). The number of non-adhered bacterial cells ($CFU_{non\text{-}adh}$) in the collected medium was also determined. The adhesion value (%A) was calculated as:

$$\%\ A = [CFU_{adh}/(CFU_{adh}+CFU_{non\text{-}adh})] \times 100\%$$

Effect of trypsinization on bacterial survival The bactericidal effect of trypsinization was quantified based on preparation of lactobacilli suspensions in empty wells of a tissue culture plate (Sarstedt), followed by simulation of the trypsinization procedure used in bacterial adhesion assays. In order to determine CFUs before and after trypsinization, appropriate dilutions of starter and trypsin-

treated suspensions were plated in triplicate onto MRS agar (Merck) and incubated (D115; Binder) at 37°C for 24 h. Comparison of obtained values allowed determination of the fraction of bacterial cells that survived trypsinization.

Lysozyme tolerance The effect of lysozyme on lactobacilli growth was examined using an agar-well diffusion assay (11). Eighteen mL of melted MRS agar cooled to 45°C was inoculated with 2 mL of a suspension of selected isolate (McFarland 1.0) and poured into Petri plates. Wells of 6 mm diameter were cut into agar and 100 μL of lysozyme (Fluka, St. Gallen, Switzerland) solutions at concentrations of 0.2, 0.6, 1, and 10 mg/mL were placed in wells. Plates were incubated (D115; Binder) at 37°C for 24 h and inhibition zone sizes were measured. *Micrococcus luteus* ATCC 93419 was used as a positive control.

Acid tolerance The acid tolerance of lactobacilli was studied in MRS broth (Merck) with the pH adjusted with 10 M HCl to 2.0, 3.0, and 4.0. MRS broth (Merck) at pH 6.4 was used as a control. An amount of 200 μL of a bacterial suspension (approximately 10^9 cells/mL) was inoculated into 10 mL of MRS broth (Merck) for each pH solution and mixed by vortexing (MS2; IKA Werke). Aliquots were taken immediately after inoculation (0 h), and after 1, 2, and 4 h of incubation. Appropriate dilutions were plated in order to determine the number of cells (CFU/mL).

Bile tolerance Strains bile tolerance was studied as described by Mishra and Prasad (18). Bovine bile salts (Oxgall; Sigma-Aldrich) at final concentrations 0.5, 1.0, and 2% were prepared in MRS broth (Merck) while broth without bovine bile salts was used as a negative control. A 200 μL bacterial suspension containing approximately 10^9 cells/mL was inoculated into 10 mL of MRS broth (Merck) containing bile salt concentrations of 0.5, 1.0, and 2%, and incubated (D115; Binder) at 37°C for 24 h. Serial dilutions were prepared immediately after inoculation and after 2 and 4 h of incubation. Appropriate dilutions were plated in order to determine the number of cells (CFU/mL).

***In vitro* gastrointestinal digestion** Gastrointestinal digestion was examined as described by Zarate *et al.* (19). Briefly, bacterial cells were grown at 37°C for 24 h, centrifuged (Universal 30 RF; Hettich), and resuspended in PBS (PAA Laboratories GmbH), to obtain about 10^8-10^9cells/mL. Four hundred μL of a cell suspension was added to 20 mL of AGF, mixed by vortexing (MS2; IKA Werke) and incubated for 2 h under microaerophilic conditions at 37°C with agitation (ThermoLab; Adolf Kuhner, Birsfelden, Switzerland) (200 rpm) to simulate peristalsis. Bacterial suspensions were then centrifuged (Universal 30 RF; Hettich) at 3,500×*g*, resuspended in AIF, and incubated (ThermoLab; Adolf Kuhner) for an additional 2 h. Aliquots for enumeration of CFUs were taken at 1 h intervals and plated on MRS agar (Merck) and incubated (D115; Binder) for 48 h at 37°C.

Statistical analyses All tests were performed in triplicate and results were presented as mean values with standard deviations (SD). Student's *t*-test was used for statistical analyses at $p<0.05$.

Results and Discussion

Phenotypic characterization of bacterial isolates Bacterial species that are currently of commercial interest as probiotics mainly belong to the general *Lactobacillus* and *Bifidobacterium* (1,20,21). In a search for new probiotic lactobacilli, 126 new bacterial strains from oral and fecal samples taken from 43 healthy infants in Novi Beograd Health Centre, Belgrade, and Serbia were isolated in this study. Among these isolates, 14 were Gram-positive rods, 104 were Gram-positive cocci, and 8 were Gram-negative rods. After provisional identification, 7 isolates were determined to be lactobacilli and labeled as Lac1 to Lac7. Biochemical identification using a standard API 50 CHL kit confirmed that all isolates belonged to Genus *Lactobacillus*.

Isolates Lac1, Lac2, Lac3, Lac4, Lac6, and Lac7 were further investigated while Lac5 was excluded due to extremely poor growth on solid media. Phenotypic characterization included tolerance to temperature, pH, and NaCl concentrations. Lac1 and Lac2 isolates grew at temperatures ranging from 10 to 40°C, while all the others grew at temperatures ranging from 15 to 40°C. Tolerance to pH and NaCl ranged from 2.0 to 8.0 and from 2.0 to 6.5%, respectively. Further phenotypic characterization showed production of lactic and butyric acids from lactose, but no gas production (data not shown).

Genotypic characterization of bacterial isolates BLAST analyses of 16S rRNA gene sequences of bacterial isolates and comparison with GenBank deposited sequences allowed identification of isolates to the species level. Lac1 was closely related to *Lactobacillus plantarum* subsp. *plantarum* TO 1003, Lac2 to *Lactobacillus plantarum* G8, Lac3 to *Lactobacillus casei* MGB65-2, Lac 4 to *Lactobacillus paracasei* NRIC 1942, and Lac6 and Lac7 to *Lactobacillus plantarum* strain Lp-01. Similarity of all isolated strains with deposited sequences was 100%.

Hemolytic activity Monitoring of hemolytic activity indicated that no isolates caused lysis while positive controls caused complete β-hemolysis.

Antimicrobial activity An important feature of probiotics is antimicrobial activity against potential pathogenic microorganisms in GIT. Although tested isolates were completely ineffective against fungal indicator strains, some antibacterial effect against a majority of Gram negative and Gram positive bacteria, except *Lactobacillus* species, was obtained with non-neutralized CFSs of tested isolates (Table 1). However, after neutralization of CFSs, no inhibition zone was detected for any of the isolates (data not shown), indicating that the observed antibacterial activity could be attributed to production of lactic and butyric acids and low pH values of non-neutralized CFSs,

Table 1. The antimicrobial spectra of the non-neutralized CFSs of lactobacilli isolates

Indicator strains	Zone of inhibition (mm)[1)]					
	Lac1	Lac2	Lac3	Lac4	Lac6	Lac7
S. aureus ATCC 6538-P	0	0	3.7±0.2	0	3.5±0.1	3.5±04
M. luteus ATCC 93419	4.1±0.1	3.6±0.2	3.4±0.1	2.7±0.1	3.7±0.2	3.0±0.2
B. cereus ATCC 11778	3.2±0.3	3.3±0.3	3.2±0.3	3.4±0.1	3.5±0.1	3.0±0.2
B. pumilus NCTC 8241	3.5±0.3	3.7±0.3	3.4±0.2	3.6±0.1	3.6±0.2	4.0±0.2
B. subtilis ATCC 6633	0	4.0±0.2	3.8±0.3	3.7±0.1	3.9±0.2	3.0±0.3
L. rhamnosus ATCC 7469	0	0	0	0	0	0
L. lactis ATCC 7830	0	0	0	0	0	0
P. aeruginosa ATCC 9027	2.7±0.3	0	0	3.1±0.1	3.7±0.1	4.0±0.2
E. coli ATCC 8739	2.6±0.2	2.8±0.2	0	2.8±0.2	3.9±0.3	4.2±0.3
K. pneumoniae ATCC 10031	3.4±0.2	3.5±0.3	0	3.3±0.2	3.7±0.2	3.8±0.2
S. abony NTCC 6017	3.2±0.3	3.3±0.2	3.1±0.1	3.0±0.2	3.7±0.1	4.5±0.3
C. albicans ATCC 10231	0	0	0	0	0	0
A. brasiliensis ATCC 16404	0	0	0	0	0	0

[1)]Inhibition zone was measured from the edge of the well to the margin of the inhibition zone.

Table 2. Aggregation abilities of lactobacilli isolates during a period of 24 h at 37°C (unit: %)

Isolates	h	Auto-aggregation	Co-aggregation with *L. monocytogenes*	Co-aggregation with *E. coli*
Lac1	2	19.52±0.21[1)]	3.90±0.08	2.16±0.15
	4	31.67±0.22	24.57±0.16	9.37±0.29
	24	53.70±0.45	28.67±0.39	22.98±0.32
Lac2	2	18.06±0.32	13.56±0.18	16.30±0.01
	4	34.43±0.60	13.79±0.35	4.32±0.17
	24	44.99±0.48	35.11±0.32	44.37±0.13
Lac3	2	17.53±0.38	12.40±0.14	7.45±0.15
	4	35.29±0.67	25.69±0.04	9.68±0.18
	24	46.51±0.45	33.33±0.14	47.55±0.30
Lac4	2	13.07±0.36	3.52±0.22	5.09±0.15
	4	21.13±0.57	7.41±0.18	7.93±0.04
	24	52.60±0.40	16.48±0.03	27.86±0.15
Lac6	2	13.05±0.31	10.11±0.30	3.74±0.08
	4	29.53±0.32	10.44±0.23	7.90±0.04
	24	73.31±0.57	55.49±0.27	42.44±0.60
Lac7	2	23.38±0.12	11.62±0.23	1.33±0.14
	4	24.80±0.28	12.01±0.39	13.50±0.14
	24	70.26±0.59	22.65±0.17	45.41±0.43

[1)]There was no statistically significant difference between isolates (p>0.05).

different from many previously reports indicating bacteriocin production by some *L. plantarum* strains (22). However, production of organic acids could be important for antibacterial activities against pathogens, including *Salmonella* and *Clostridium* species, which is considered beneficial for maintenance of host GIT health, as well as of the female genital tract (23,24).

Auto-aggregation and co-aggregation abilities Aggregation abilities are influenced by factors of surface charge and cell surface molecules, including S-layer proteins, lipoteichoic acid, exopolysaccharides, and mucus-binding proteins. The ability to form auto-aggregates enables better survival under fluctuating GIT conditions, while co-aggregation with pathogens might interfere with an ability to infect a host and prevent GIT colonization with food borne pathogens (25). During incubation, aggregation values increased, with the highest values (>70%) observed for Lac7 and Lac6, but there were no statistically significant differences (p>0.05) between the isolates (Table 2). Co-aggregation abilities with the pathogens *L. monocytogenes* and *E. coli* were also monitored. All isolates co-aggregated with the pathogens, but the obtained values depended on both lactobacilli and pathogen strain used.

Bacterial adhesion to a hydrophobic solvent and cultured HCT 116 cells Some physicochemical properties of probiotic strains, such as cell hydrophobicity, are considered to be good indicators of positive adhesion to intestinal cells (25). Adhesion to hydrocarbons is widely used as a measure of cell surface hydrophobicity of lactobacilli. Hexadecane, a non-polar *n*-alkane solvent, was used to assay adhesion potentials of isolates. High hydrophobicity values, without statistically significant differences (p>0.05) between the isolates, were observed for all lactobacilli (Table 3).

The colorectal cancer cell line HCT 116 was used for monitoring adhesive properties to intestinal epithelium. Adhesion of bacteria to host epithelial cells is a strain specific character and, along with hydrophobicity and aggregation abilities, can influence interactions with the host gut (8). Adhesion is usually expressed as a percentage of adhered to total inoculated bacterial cells. However, a bactericidal effect obtained with trypsin treatment ranging from 37 to 51% was observed in preliminary experiments. Therefore, the percentage of adhered bacterial cells with respect to the sum of adhered and non-adhered cells was calculated. A moderate adhesion potential was determined with the highest values observed for Lac2, Lac6, and Lac7 (Table 3). There were statistically significant differences (p≤0.05) in adhesion ability among isolates.

Lysozyme, acid, and bile tolerance The main *in vitro* testing currently

Table 3. Adhesion of lactobacilli isolates to hexadecane and HCT 116 cells

Isolate	Adhered bacteria[1)]	Non-adhered bacteria[2)]	Adhesion to HCT 116 cells (%)	Adhesion to hexadecane (%)[3)]
Lac1	78.50±3.54	127.00±2.52	5.82	70.56±0.41
Lac2	100.00±2.83	100.33±2.52	9.06	77.63±0.33
Lac3	48.50±3.54	69.00±3.61	6.57	76.65±0.41
Lac4	55.50±4.95	123.00±3.00	4.32	80.51±0.14
Lac6	66.00±2.83	85.00±5.00	7.21	78.26±0.26
Lac7	81.00±4.24	99.00±2.00	7.56	66.51±0.59

[1)]CFU/plate obtained with dilution 10^{-4}; statistically significant difference between all isolates ($p \leq 0$ .05).
[2)]CFU/plate obtained with dilution 10^{-5}; statistically significant difference between all isolates ($p \leq 0.05$).
[3)]No statistically significant difference between isolates ($p > 0.05$).

used for study of probiotic functionalities includes tolerance to lysozyme, gastric acidity, and bile salts. Lysozyme is an enzyme found in saliva in concentrations of 20 to 80 μg/mL (26,27) and is effective in killing some Gram-positive bacteria. No inhibition zones in agar-well diffusion assays were observed, regardless of the lactobacilli isolate and the lysozyme concentration. However, growth inhibition of the positive control *M. luteus* ATCC 93419 was evident (data not shown), indicating high tolerance to lysozyme for all isolates.

Tolerance to gastric acidity and bile salts is also needed for bacterial cells to survive passage through the stomach and the proximal part of the small intestine (28). All isolates survived at pH values ranging from 2.0 to 4.0 (Table 4). Moreover, isolates were also resistant to concentrations of bile salts ranging from 0.5 to 2.0% (Table 5). The bile concentration in the duodenum depends on food ingestion and varies from 0.15 to 0.6% (29). Therefore, isolate tolerance to higher than physiological concentrations was observed. When testing acid and bile tolerance, statistically significant differences between isolates ($p \leq 0.05$) were observed, with some exceptions specified in Table 4 and 5.

Gastrointestinal digestion *in vitro* Survival of lactobacilli isolates was monitored in AGF and AIF during time intervals corresponding to actual digestion time in the stomach and duodenum. All isolates showed a high degree of survival under AGF and AIF conditions (Table 6), with statistically significant differences between isolates ($p \leq 0.05$), with some exceptions specified in Table 6. High isolate survival rates under gastric and duodenal conditions were comparable with previous reports other investigated probiotic strains (11,28-35).

In conclusion, screening of lactobacilli strains of human origin recovered 126 bacterial isolates and resulted in isolation and identification of 6 lactobacilli strains with probiotic features. Based on adhesion abilities, antimicrobial spectra, and degrees of survival under artificial GIT conditions, Lac2, Lac6, and Lac7 can be considered as candidates for further study, including *in vitro* assays of competitive inhibition of pathogens, adhesion to colon cells, and *in vivo* studies in animal models and human trials in accordance with FAO/WHO recommendations (4).

Table 4. Tolerance of lactobacilli isolates to different acidic conditions

Isolates	h	V[1)]	R[2)]	%S[3)]	V	R	%S	V	R	%S
		pH=2			pH=3			pH=4		
Lac1	0	8.153±0.022			8.161±0.037			8.162±0.018		
	2	6.982±0.018	1.171		7.491±0.013	0.671		7.961±0.011	0.201	
	4	5.551±0.015	2.602	68.1	6.111±0.042	2.050	74.9	7.447±0.025	0.715	91.2
Lac2	0	8.146±0.014[a4)]			8.145±0.022[a]			8.146±0.006[a]		
	2	7.563±0.010	0.583		7.677±0.007	0.468		7.996±0.004	0.150	
	4	5.002±0.006	3.144	61.4	7.352±0.026	0.793	90.3	7.990±0.002	0.156	98.1
Lac3	0	7.073±0.01			8.179±0.016[ab]			7.072±0.010		
	2	5.373±0.014	1.700		8.015±0.030	0.163		6.644±0.007	0.428	
	4	4.492±0.010	2.581	63.5	7.380±0.018[b]	0.709	90.2	6.361±0.034	0.710	89.9
Lac4	0	7.932±0.013			7.934±0.005			7.934±0.002		
	2	3.002±0.006	4.932		5.472±0.087[a]	2.457		5.318±0.017	2.616	
	4	3.000±0.000	4.934	37.8	4.001±0.007	3.933	50.4	4.544±0.014	3.390	57.3
Lac6	0	8.193±0.017[a]			8.193±0.017[ac]			8.192±0.031[a]		
	2	7.747±0.004	0.446		7.956±0.007	0.237		8.169±0.011	0.024	
	4	6.909±0.006	1.284	84.3	7.278±0.023	0.914	88.8	8.134±0.016	0.059	99.3
Lac7	0	8.179±0.010[ae]			7.070±0.017			8.672±0.011		
	2	7.540±0.014[b]	0.639		6.510±0.006	0.519		8.179±0.005[e]	0.493	
	4	6.000±0.013[d]	2.179	73.4	5.903±0.009	1.168	83.5	7.910±0.003	0.762	91.2

[1)]Viability of isolates, expressed as log (CFU/mL).
[2)]Reduction of viability, expressed as decrease of log (CFU/mL).
[3)]Survival of isolates, expressed in percentages of log (CFU/mL).
[4)]Lower case letters indicate Lac1-a, Lac2-b, Lac3-c, Lac4-d, Lac6-e; groups with the same letter are not significantly different from corresponding isolate ($p > 0.05$).

Table 5. The tolerance of lactobacilli isolates to different bile salt concentrations

Isolates	h	V[1)]	R[2)]	%S[3)]	V	R	%S	V	R	%S
		Bile salts 0.5%			Bile salts 1%			Bile salts 2%		
Lac1	0	7.826±0.013			7.826±0.017			7.826±0.006		
	2	7.998±0.048	-0.174		7.826±0.003	0.000		7.785±0.016	0.041	
	4	8.556±0.029	-0.730	109.3	7.342±0.014	0.484	93.8	7.002±0.013	0.824	89.5
Lac2	0	7.408±0.025			7.408±0.012			7.408±0.012		
	2	7.903±0.014	-0.495		7.517±0.005	-0.098		7.358±0.018	0.056	
	4	8.001±0.032	-0.593	108.0	7.524±0.009	-0.119	101.6	7.326±0.025	0.082	98.9
Lac3	0	7.312±0.042			7.314±0.011			7.313±0.013		
	2	7.842±0.063[b4)]	-0.531		7.591±0.020	-0.277		7.069±0.026	0.244	
	4	8.075±0.073[b]	-0.765	110.4	7.602±0.017	-0.299	103.9	7.001±0.014	0.312	95.7
Lac4	0	7.952±0.048[a]			7.954±0.008			7.954±0.012		
	2	7.792±0.014[c]	0.162		7.708±0.009	0.247		7.629±0.008	3.326	
	4	7.699±0.017	0.255	96.8	7.602±0.015[c]	0.353	95.6	7.295±0.088[b]	0.653	91.7
Lac6	0	7.651±0.009			7.651±0.010			7.651±0.010		
	2	7.778±0.000[cd]	-0.127		7.613±0.007	0.038		7.380±0.025[b]	0.270	
	4	7.812±0.033	-0.162	102.1	7.476±0.038[b]	0.174	97.7	6.052±0.045[c]	1.598	79.1
Lac7	0	7.536±0.010			7.537±0.008			7.536±0.010		
	2	8.053±0.046[a]	-0.518		7.477±0.013	0.060		6.663±0.023	0.873	
	4	8.321±0.035	-0.786	110.4	7.040±0.036	0.496	93.4	6.612±0.010	0.924	87.7

[1)]Viability of isolates, expressed as log (CFU/mL).
[2)]Reduction of viability, expressed as decrease of log (CFU/mL).
[3)]Survival of isolates, expressed in percentages of log (CFU/mL).
[4)]Lower case letters indicate Lac1-a, Lac2-b, Lac3-c, Lac4-d, Lac6-e; groups with the same letter are not significantly different from corresponding isolate (p>0.05).

Table 6. Survival of isolates in artificial gastrointestinal fluids

Isolates	h	V[1)]	R[2)]	%S[3)]	V	R	%S
		AGF			AIF		
Lac1	0	10.475±0.058			6.991±0.012		
	1	9.903±0.011	0.574		6.978±0.009	0.014	
	2	8.579±0.037	1.897	81.9	6.914±0.009	0.077	98.9
Lac2	0	10.405±0.111[a4)]			7.334±0.024		
	1	9.954±0.010	0.460		7.263±0.236	0.033	
	2	8.857±0.024	1.557	85.1	7.259±0.034	0.074	98.9
Lac3	0	10.633±0.026			6.038±0.066		
	1	9.952±0.048[ab]	0.679		5.996±0.085[b]	0.040	
	2	8.731±0.042	1.901	82.1	5.380±0.018	0.661	89.1
Lac4	0	10.300±0.027[bc]			6.246±0.115[bc]		
	1	9.903±0.014[ac]	0.398		6.253±0.048[b]	0.022	
	2	8.623±0.021[a]	1.678	83.7	6.144±0.056	1.132	98.4
Lac6	0	10.756±0.015			6.830±0.051		
	1	10.259±0.101	0.455		6.813±0.012	0.020	
	2	9.355±0.096	1.394	86.9	6.732±0.014	0.100	98.6
Lac7	0	10.792±0.025[e]			6.924±0.010[e]		
	1	10.251±0.069[e]	0.491		6.903±0.016	0.021	
	2	9.341±0.040[e]	1.450	86.6	6.857±0.016	0.067	99.0

[1)]Viability of isolates, expressed as log (CFU/mL).
[2)]Reduction of viability, expressed as decrease of log (CFU/mL).
[3)]Survival of isolates, in percentages of log (CFU/mL), in comparison with the corresponding 0 h value.
[4)]Lower case letters indicate Lac1-a, Lac2-b, Lac3-c, Lac4-d, Lac6-e; groups with the same letter are not significantly different from corresponding isolate (p>0.05).

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