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ORIGINAL ARTICLE

In vitro assessment of safety and probiotic potential characteristics of *Lactobacillus* strains isolated from water buffalo mozzarella cheese

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Abstract The aim of this study was to evaluate the safety and probiotic potential characteristics of ten *Lactobacillus* spp. strains (*Lactobacillus fermentum* SJRP30, *Lactobacillus casei* SJRP37, SJRP66, SJRP141, SJRP145, SJRP146, and SJRP169, and *Lactobacillus delbrueckii* subsp. *bulgaricus* SJRP50, SJRP76, and SJRP149) that had previously been isolated from water buffalo mozzarella cheese. The safety of the strains was analyzed based on mucin degradation, hemolytic activity, resistance to antibiotics and the presence of genes encoding virulence factors. The in vitro tests concerning probiotic potential included survival under simulated gastrointestinal (GI) tract conditions, intestinal epithelial cell adhesion, the presence of genes encoding adhesion, aggregation and colonization factors, antimicrobial activity, and the production of the β-galactosidase enzyme. Although all strains presented resistance to several antibiotics, the resistance was limited to antibiotics to which the strains had intrinsic resistance. Furthermore, the strains presented a limited spread of genes encoding virulence factors and resistance to antibiotics, and none of the strains presented hemolytic or mucin degradation activity. The *L. delbrueckii* subsp. *bulgaricus* strains showed the lowest survival rate after exposure to simulated GI tract conditions, whereas all of the *L. casei* and *L. fermentum* strains showed good survivability. None of the tested lactobacilli strains presented bile salt hydrolase (BSH) activity, and only *L. casei* SJRP145 did not produce the β-galactosidase enzyme. The strains showed varied levels of adhesion to Caco-2 cells. None of the cell-free supernatants inhibited the growth of pathogenic target microorganisms. Overall, *L. fermentum* SJRP30 and *L. casei* SJRP145 and SJRP146 were revealed to be safe and to possess similar or superior probiotic characteristics compared to the reference strain *L. rhamnosus* GG (ATCC 53103).

Keywords Lactic acid bacteria · Dairy · Safety · Antibiotic resistance · Beneficial properties · Gastrointestinal tract survival

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Introduction

Lactobacillus spp. belong to the group of lactic acid bacteria (LAB) and have a long history of use in the production of dairy products due to their ability to convert lactose into lactic acid (Tulumoğlu et al. 2014). In addition to their use as technological agents in the food industry, some *Lactobacillus* species can confer health benefits to the host when they are administered adequately as probiotics. Probiotics are currently defined "as live microorganisms that, when administered in adequate amounts, confer health benefit on the host" (Hill et al. 2014). Although probiotics have been extensively

studied and commercialized, and are the subject of national and international regulations, there is no agreement concerning the amount of probiotic bacteria necessary to produce their beneficial effects. Generally, probiotic food products must contain 10^6 CFU/mL or CFU/g (Shah 2000). Nevertheless, some authors state that beneficial effects can be achieved even when bacteria lose their viability (Adams 2010).

Some of the health effects attributed to probiotic consumption include the regulation of gastrointestinal (GI) functions, relief of lactose intolerance, prevention of different types of diarrhea besides urogenital infections, reduction in cholesterol levels, reduction in atopic and food allergies, and modulation of the immune system. Furthermore, in vitro studies have shown that probiotic bacteria reduce the number of pathogens and their metabolic activities in the human intestine and compete with these microorganisms for attachment sites to intestinal epithelial cells and nutrients (Guarner and Malagelada 2003; Mishra et al. 2015).

Although a large number of probiotic strains are available for commercial use worldwide, the isolation and characterization of new strains from different species is desirable; thus, many studies in this field have been published in recent years (Jeronymo-Ceneviva et al. 2014; Peres et al. 2014; de Paula et al. 2015; Oh and Jung 2015). Probiotics targeted for human consumption are usually isolated from humans or animals because strains from these origins can better adapt to the conditions encountered in the human/animal GI tract, which enables more successful gut colonization (Argyri et al. 2013). However, certain food-associated *Lactobacillus* strains have probiotic characteristics even though they do not belong to the gut microbiota (Solieri et al. 2014; Tulumoğlu et al. 2014).

According to the FAO/WHO (2002), a bacterial strain should fulfill a number of requirements to be considered probiotic; these requirements must be verified by in vitro and in vivo tests. In vitro tests are useful for the selection of strains that have greater probiotic potential; these tests increase knowledge regarding the strain as well as the mechanisms underlying the beneficial effects. Although LAB, particularly *Lactobacillus*, are generally recognized as safe (GRAS), additional tests should be performed to check the safety of these strains because some cases recently associated systemic infection with the consumption of probiotics (Liong 2008; Sharma and Devi 2014). Thus, evaluating their safety, assessing their resistance to antibiotics, investigating the presence of virulence genes, and determining hemolytic activity are important (Jeronymo-Ceneviva et al. 2014; Vijayakumar et al. 2015).

Given these points, the aim of this study was to characterize the safety features and probiotic potential attributes of autochthonous *Lactobacillus* spp. isolated from water buffalo mozzarella cheese using in vitro tests. Candidates that met the established criteria may be used in the production of fermented products to promote their probiotic characteristics.

Materials and methods

Bacterial strains

Ten *Lactobacillus* strains previously isolated and identified through 16S rRNA gene sequencing by our group (Silva et al. 2015; Silva 2015) as *Lactobacillus fermentum* (SJRP30), *Lactobacillus casei* (SJRP37, SJRP66, SJRP141, SJRP145, SJRP146, and SJRP169), and *Lactobacillus delbrueckii* subsp. *bulgaricus* (SJRP50, SJRP76 and SJRP149) were screened for their safety and probiotic potential. *Lactobacillus rhamnosus* GG (ATCC 53103) was used as a probiotic reference strain. The strains were maintained at −80 °C in MRS broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 25% (v/v) glycerol (Vetec, Duque de Caxias, RJ, Brazil). Each culture was sub-cultured at least twice in MRS broth before use in the assays.

Assessment of safety characteristics

Hemolytic activity

Fresh lactobacilli broth cultures (8.0–9.0 log CFU/mL) were streaked in triplicate on Columbia agar plates containing 5% (w/v) sheep blood (NewProv, Pinhais, PR, Brazil). After 48 h of incubation at 37 °C, the plates were examined for hemolytic reactions. The *Lactobacillus rhamnosus* GG (ATCC 53103) and *Staphylococcus aureus* ATCC 6538 strains were used as the negative and positive controls, respectively (Pieniz et al. 2014). The assay was repeated on three independent occasions in triplicate.

Mucin degradation

Mucin degradation was determined according to Zhou et al. (2001). *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *Lactobacillus rhamnosus* GG (ATCC 53103) were used as the positive and negative controls, respectively. The assay was repeated on three independent occasions in triplicate.

Presence of genes encoding virulence factors, antibiotic resistance and biogenic amines

The *Lactobacillus* strains were tested for the presence of virulence, antibiotic resistance and amino acid decarboxylase genes (Table 1). DNA was extracted using the QIAgen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), followed by DNA concentration estimation using the NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA). PCRs were performed according to the references listed in Table 1, and the amplified products were separated by electrophoresis in 0.8 to 2.0% (w/v) agarose gels in

Table 1 Presence of genes implicated in virulence factors, antibiotic resistance and biogenic amine production in *Lactobacillus* spp. strains

Gene	Encoded factor	*L. fermentum*	*L. casei*						*L. delbrueckii* subsp. *bulgaricus*			Reference
		SJRP30	SJRP37	SJRP66	SJRP141	SJRP145	SJRP146	SJRP169	SJRP50	SJRP76	SJRP149	
Virulence												
*gel*E	Gelatinase	–[a]	–	+	–	–	–	–	–	+	+	Vankerckhoven et al. (2004)
hyl	Hyaluronidase	–	–	–	–	–	–	–	+	+	–	Vankerckhoven et al. (2004)
*asa*1	Aggregation substance	–	–	–	–	–	–	–	+	–	–	Vankerckhoven et al. (2004)
esp	Enterococcal surface protein	–	+	+	+	–	–	+	–	–	–	Vankerckhoven et al. (2004)
*cyl*A	Cytolysin	–	–	–	–	–	–	+	–	–	–	Vankerckhoven et al. (2004)
*efa*A	Endocarditis antigen	–	–	–	–	–	–	–	–	–	–	Martín-Platero et al. (2009)
ace	Adhesion of collagen	–	–	–	–	–	–	–	–	–	–	Martín-Platero et al. (2009)
fsrA	Gelatinase	–	+	+	+	+	–	+	–	–	–	Lopes et al. (2006)
fsrB	Gelatinase	–	+	+	+	–	–	+	–	–	–	Lopes et al. (2006)
fsrC	Gelatinase	–	–	–	–	–	–	–	+	+	–	Lopes et al. (2006)
sprE	Serine protease	–	–	–	–	+	–	–	–	–	+	Lopes et al. (2006)
ccf	Sex pheromones, chemotactic for human leukocytes; facilitate conjugation	–	+	–	+	+	+	–	+	–	–	Eaton and Gasson (2001)
cob	Sex pheromones, chemotactic for human leukocytes; facilitate conjugation	–	–	–	–	–	–	–	+	–	+	Eaton and Gasson (2001)
cpd	Sex pheromones, chemotactic for human leukocytes; facilitate conjugation	–	+	–	+	–	+	+	+	–	–	Eaton and Gasson (2001)
Biogenic amine												
hdc1	Histidine decarboxylase	–	+	–	–	–	–	+	–	–	+	de Las Rivas et al. (2005)
hdc2	Histidine decarboxylase	–	–	–	–	–	–	–	–	–	–	de Las Rivas et al. (2005)
tdc	Tyrosine decarboxylase	–	–	–	–	–	–	–	+	+	–	de Las Rivas et al. (2005)
odc	Ornithine decarboxylase	–	–	–	–	–	–	–	–	–	–	de Las Rivas et al. (2005)
Antibiotic resitance												
van A	Vancomycin resistance	–	+	+	+	–	–	+	–	–	–	Martín-Platero et al. (2009)
van B	Vancomycin resistance	–	+	+	+	–	+	+	–	–	–	Martín-Platero et al. (2009)
vanC1	Vancomycin resistance	–	–	–	+	–	–	–	+	+	+	Dutka-Malen et al. (1995)
vanC-2,vanC-3	Vancomycin resistance	+	–	–	–	–	–	+	–	–	–	Dutka-Malen et al. (1995)
vanC1	Vancomycin resistance	+	–	–	–	–	–	+	–	–	–	Miele et al. (1995)
tet(M)	Tetracycline resistance	–	–	–	–	–	–	–	–	+	–	Aarestrup et al. (2000b)
tet(L)	Tetracycline resistance	–	–	–	+	–	–	–	+	+	–	Aarestrup et al. (2000b)

Table 1 (continued)

Gene	Encoded factor	*L. fermentum*	*L. casei*						*L. delbrueckii* subsp. *bulgaricus*			Reference
		SJRP30	SJRP37	SJRP66	SJRP141	SJRP145	SJRP146	SJRP169	SJRP50	SJRP76	SJRP149	
tet(K)	Tetracycline resistance	–	+	–	–	–	–	–	+	–	+	Aarestrup et al. (2000b)
tet(O)	Tetracycline resistance	–	+	+	+	+	+	+	–	–	–	Aarestrup et al. (2000b)
tet(S)	Tetracycline	–	–	–	–	–	–	–	–	–	–	Aarestrup et al. (2000a)
bcr(B)	Bacitracin resistance	–	–	+	–	+	+	+	–	–	–	Manson et al. (2004)
bcr(D)	Bacitracin resistance	–	–	–	–	–	–	–	–	–	–	Manson et al. (2004)
bcr(R)	Bacitracin resistance	–	–	–	–	–	+	–	–	–	–	Manson et al. (2004)
erm(A)	Erythromycin resistance	–	–	–	–	–	–	–	–	–	–	Sutcliffe et al. (1996)
erm(B)	Erythromycin resistance	–	–	–	–	–	–	–	–	–	–	Sutcliffe et al. (1996)
erm(C)	Erythromycin resistance	–	+	–	+	+	+	+	–	+	–	Sutcliffe et al. (1996)
erm(B)	Erythromycin resistance	–	–	–	–	–	–	–	–	–	–	Gevers et al. (2003)
ant(4')-Ia	Aminoglycoside resistance	+	+	+	+	+	–	+	–	–	–	Fortina et al. (2008)
aph(3')-III-a	Aminoglycoside resistance	–	+	–	–	–	–	+	+	+	+	Fortina et al. (2008)
aph(2")-Ib	Aminoglycoside resistance	+	+	–	+	+	+	–	–	–	–	Fortina et al. (2008)
aph(2")-Ic	Aminoglycoside resistance	–	+	+	+	+	–	+	–	–	–	Fortina et al. (2008)
aph(2")-Id	Aminoglycoside resistance	–	–	–	+	+	–	–	+	+	+	Fortina et al. (2008)
aac(6')-Ie-aph(2")-Ia	Aminoglycoside resistance	+	+	+	–	+	–	–	+	–	+	Fortina et al. (2008)
aac(6')-Ii	Aminoglycoside resistance	+	+	+	+	+	–	+	+	+	–	Costa et al. (1993)
catA(PIP501)	Chloramphenicol resistance	–	+	+	–	–	–	–	–	–	–	Aarestrup et al. (2000a)
int-Tn	Tetracycline resistance	–	–	+	–	–	–	–	–	–	+	Fortina et al. (2008)
int	Transposon integrase gene	+	+	+	+	–	+	–	+	+	+	Gevers et al. (2003)

[a] + Indicates the presence and – absence of genes

0.5× TAE buffer. The gels were stained in 0.5× TAE buffer containing 0.5 μg/mL of ethidium bromide (Sigma-Aldrich, St. Louis, MO).

Antibiotic susceptibility

The disc diffusion assay was applied to determine the antibiotic susceptibility of the strains. Diluted culture (100 μL; 6.0 log CFU/mL) was spread onto MRS agar media (Difco), and antibiotic discs (Oxoid, Basingstoke, UK) containing (per disc) ampicillin (10 μg), vancomycin (30 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (300 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), and clindamycin (2 μg) were placed manually on the surface of the inoculated plates using sterile forceps. These antibiotics were chosen according to the list proposed by the European Food Safety Authority (EFSA 2012). The plates were incubated at 37 °C under anaerobic conditions, and the diameters of the inhibition zones were evaluated 24 h after incubation. The susceptibility of the isolates was scored as resistant, moderately susceptible, or susceptible according to the cut-off values proposed by Charteris et al. (1998). The assay was repeated on three independent occasions in triplicate.

Assessment of probiotic potential characteristics

Tolerance to simulated GI tract conditions

The tolerance to simulated GI tract conditions test was performed by successively exposing the strains to gastric and enteric simulated juices as described by Botta et al. (2014). The lactobacilli strains were grown for 18 h at 37 °C in MRS broth, and 1 mL of each culture (8.0–9.0 CFU/mL) was distributed into four sterile flasks (two for the gastric phase and two for the enteric phase). The solutions simulating the gastric and enteric juices were prepared according to the method of Bautista-Gallego et al. (2013). The pH values used in the gastric and enteric phases were 2.5 and 8.0, respectively. All enzyme solutions were prepared and filter-sterilized using a 0.22-μm membrane filter (Merck Millipore, Cork, Ireland) on the day of analysis.

The cells were counted at the beginning (T_0) and the end of the gastric phase (T_{120}) and after the enteric phase (T_{360}). The cell count was performed by serial dilution and plating in MRS agar (Difco). The plates were incubated at 37 °C for 48 h under anaerobic conditions (Anaerobac, Probac, São Paulo, Brazil). The commercial probiotic *L. rhamnosus* GG (ATCC 53103) was used as a reference strain. The assay was repeated on three independent occasions in duplicate.

Bile salt hydrolase activity

Fresh bacterial cultures of the studied lactobacilli (8.0–9.0 log CFU/mL) were screened for bile salt hydrolase (BSH) activity as previously described by de Paula et al. (2014) using MRS plates supplemented with taurodeoxycholic acid sodium salt (TDCA) or taurocholic acid sodium salt hydrate (TC); MRS plates without TDCA and TC were used as negative controls, whereas *L. mesenteroides* SJRP 55 was used as a positive control. The plates were incubated anaerobically at 37 °C for 48 h. The presence of precipitated bile acid around the spots was considered a positive result (Rodríguez et al. 2012). The assay was repeated on three independent occasions in triplicate.

Adhesion to Caco-2 cells

The Caco-2 cell line BCRJ 0059 (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) was cultured (passages 29–31) in Dulbecco's modified Eagle's minimum (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Cultilab, Campinas, Brazil), a mixture of penicillin (100 UI/mL) and streptomycin (100 μg/mL) (Sigma-Aldrich), and 1% non-essential amino acid solution (Sigma-Aldrich) at 37 °C in a 5% CO_2 atmosphere.

The adhesion assay was performed as described by Argyri et al. (2013). All bacterial cultures were grown for 18 h in MRS at 37 °C before the assays, harvested by centrifugation (7000 *g*, 7 min, 5 ° C), washed twice with phosphate-buffered saline (PBS) and re-suspended in DMEM without any serum or antibiotics. The commercial probiotic *L. rhamnosus* GG (ATCC 53103) was used as a reference strain. Subsequently, 1 mL containing approximately 8.0–9.0 log CFU bacterial cells was added to each well, and each strain was evaluated for adherence in duplicate wells in each experiment. After incubation for 2 h at 37 °C, the cells were washed three times with sterile PBS to remove non-adherent bacteria, and then detached from each well by the addition of 1 mL Triton X-100 (0.5% v/v) (Sigma-Aldrich). Following incubation for 5 min at 37 °C, the cell lysates were serially diluted and plated on MRS agar. Bacterial adhesion (%) was calculated by the ratio of adhered bacteria to the total number of added bacteria. The experiment was performed on three independent occasions.

Presence of genes encoding adhesion, aggregation and colonization factors

The investigated *Lactobacillus* strains were tested for the presence of adhesion, aggregation and colonization genes (Table 2) as described in the section "Presence of genes encoding virulence factors, antibiotic resistance and biogenic amines".

Table 2 Presence of genes implicated in adhesion, aggregation and colonization in *Lactobacillus* spp. strains

Gene	Encoded factor	*L. fermentum*	*L. casei*						*L. delbrueckii* subsp. *bulgaricus*			Reference
		SJRP30	SJRP37	SJRP66	SJRP141	SJRP145	SJRP146	SJRP169	SJRP50	SJRP76	SJRP149	
Mub	Adhesion proteins	–[a]	–	–	–	–	–	–	+	–	+	Ramiah et al. (2007)
mapA	Adhesion proteins	–	–	–	–	+	–	–	+	–	+	Ramiah et al. (2007)
EF-Tu	Elongation factor	–	+	+	+	+	+	–	+	+	+	Ramiah et al. (2007)
EF2662-cbp	Choline binding protein	–	–	–	–	–	–	–	–	–	+	Fortina et al. (2008)
EF1249-fbp	Fibrinogen binding protein		–	–	–	–	+	+	–	–	+	Fortina et al. (2008)
EF2380-maz	Membrane-associated zinc metalloprotease	–	–	+	+	–	+	–	+	–	–	Fortina et al. (2008)
prgB	Surface protein	–	–	–	–	–	+	–	–	–	–	Fortina et al. (2008)

[a] + Indicates the presence and – absence of genes

Antimicrobial activity

All lactobacilli strains were tested for antimicrobial activity against *Escherichia coli* ATCC 25922, *E. coli* ATCC 8739, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 15313, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 10031, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *Shigella sonnei* ATCC 25931 according to the method described by de Paula et al. (2014). The antibiotic ciprofloxacin (5 μg) was used as a positive control, whereas MRS broth adjusted to pH 6.5 and filtered was used as a negative control. The assay was repeated on three independent occasions in triplicate.

β-Galactosidase activity

The β-galactosidase activity of the *Lactobacillus* spp. strains was assessed by employing sterile filter paper discs impregnated with *o*-nitrophenyl-β-D-galactopyranose (ONPG Discs, Fluka, Buchs, Switzerland) according to the manufacturer's instructions. The test was performed in three independent experiments in duplicate. *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *E. coli* ATCC 25922 were used as the negative and positive controls, respectively.

Statistical analysis

The statistical analysis was performed using the Statistica 7.0 software (StatSoft, Inc., 2004, Tulsa, OK). One-way ANOVA followed by Tukey's test was applied to detect significant differences ($P \leq 0.05$) in the data regarding tolerance to simulated GI tract conditions and in vitro adhesion to Caco-2 cells.

Results

Hemolytic activity

None of the examined strains revealed β-hemolytic (i.e., red blood cell lysis) activity when grown in Columbia sheep blood agar. Most of the strains (*L. fermentum* SJRP30 and *L. casei* strains SJRP37, SJRP66, SJRP145, SJRP146, and SJRP169) were γ-hemolytic (i.e., no hemolysis), whereas four strains showed partial hemolysis (*L. delbrueckii* subsp. *bulgaricus* SJRP50, SJRP76, and SJRP149 and *L. casei* SJRP141). *Staphylococcus aureus* ATCC 6538 (positive control) showed hemolytic activity.

Mucin degradation

Neither the *Lactobacillus* spp. nor the reference strain (negative control) showed mucinolytic activity in either type of

tested medium (with or without glucose). Conversely, *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (positive control) was able to degrade mucin in vitro in a medium in which mucin was the only energy source.

Presence of genes encoding virulence factors, antibiotic resistance and biogenic amines

None of the tested isolates presented a positive result for the *efaA*, *ace*, *hdc2*, *odc*, *tet(S)*, *erm(A)*, and *erm(B)* genes, which were related to endocarditis antigen, collagen adhesion, tyrosine decarboxylase, ornithine decarboxylase, tetracycline resistance and erythromycin resistance, respectively (Table 1). *L. casei* SJRP169 had the highest frequency of genes encoding virulence factors, antibiotic resistance and biogenic amines (42.55%). Conversely, the *L. fermentum* SJRP30 strain showed the lowest frequency (14.89%) of positive results.

Antibiotic susceptibility

All of the strains were sensitive to ampicillin, tetracycline, chloramphenicol, erythromycin, and clindamycin, which are frequently used to treat bacterial infections (Table 3). All of the *L. delbrueckii* subsp. *bulgaricus* strains were sensitive to vancomycin and gentamicin, whereas the other strains were resistant. Most strains were susceptible to streptomycin, with the exception of *L. fermentum* SJRP30, which was classified as moderately susceptible. All of the tested strains were classified as resistant to kanamycin.

Tolerance to simulated GI tract conditions

There was a significant decrease ($P \leq 0.05$) in the populations of all strains evaluated after consecutive exposure to the gastric and small intestine conditions (Fig. 1). The *L. delbrueckii* subsp. *bulgaricus* strains showed the lowest population at the end of the in vitro assay, with a cell count reduction of 3.38 log CFU/mL on average. In contrast, *L. casei* and *L. fermentum* showed good viability during the simulated GI digestion, with reductions of 0.85–2.48 log units (Fig. 1). Two groups of tolerance were outlined after exposure to simulated gastric juice at pH 2.5; *L. casei* SJRP37, SJRP66, SJRP141, SJRP145, SJRP146 and SJRP169, *L. delbrueckii* subsp. *bulgaricus* SJRP76 and *L. rhamnosus* GG maintained the same populations, whereas *L. fermentum* SJRP30 and *L. delbrueckii* subsp. *bulgaricus* SJRP50 and SJRP149 showed a significant ($P \leq 0.05$) reduction in their populations. Tolerance to the enteric condition was variable among the strains. The *L. fermentum* SJRP30 and *L. casei* SJRP146 strains suffered a reduction of less than 1 log unit after exposure to simulated enteric juice. The other *L. casei* strains and *L. delbrueckii* subsp. *bulgaricus* SJRP50 suffered a reduction between 1 and 2 log units, whereas *L. delbrueckii* subsp. *bulgaricus* SJRP76 and SJRP149 revealed a reduction of 3.07 and 3.59 log CFU/mL, respectively.

BSH activity

All *L. casei* and *L. fermentum* strains were able to grow in MRS agar plates containing 0.5% (w/v) TDCA sodium salts, whereas the growth of *L. delbrueckii* subsp.

Table 3 Antibiotic susceptibility[a] of *Lactobacillus* spp. strains. *AMP* Ampicillin, *VA* vancomycin, *CN* gentamicin, *K* kanamycin, *S* streptomycin, *TE* tetracycline, *C* chloramphenicol, *E* erythromycin, *DA* clindamycin

Species	Strains	AMP (10 μg)	VA (30 μg)	CN (10 μg)	K (30 μg)	S (300 μg)	TE (30 μg)	C (30 μg)	E (15 μg)	DA (2 μg)
L. fermentum	SJRP30	29 S	0 R	9 R	0 R	14 MS	26 S	27 S	27 S	27 S
L. casei	SJRP37	29 S	0 R	9 R	0 R	19 S	30 S	29 S	30 S	27 S
	SJRP66	32 S	0 R	13 S	0 R	23 S	35 S	31 S	37 S	34 S
	SJRP141	33 S	0 R	12 R	0 R	26 S	36 S	29 S	37 S	33 S
	SJRP145	28 S	0 R	10 R	0 R	20 S	31 S	27 S	31 S	27 S
	SJRP146	33 S	0 R	13 S	0 R	22 S	38 S	32 S	37 S	32 S
	SJRP169	35 S	0 R	14 S	0 R	25 S	36 S	34 S	36 S	33 S
L. delbrueckii subsp. *bulgaricus*	SJRP50	28 S	21 S	10 R	11 R	24 S	31 S	29 S	32 S	30 S
	SJRP76	27 S	21 S	10 R	7 R	23 S	31 S	30 S	32 S	29 S
	SJRP149	38 S	23 S	10 R	0 R	25 S	31 S	30 S	33 S	31 S
L. rhamnosus GG	ATCC 53103	30 S	0 R	10 R	0 R	22 S	33 S	31 S	32 S	28 S

[a] Inhibition zones were measured in millimeters, and the susceptibility of the isolates was scored as resistant (R), moderately susceptible (MS) and susceptible (S) according to the cut-off values proposed by Charteris et al. (1998)

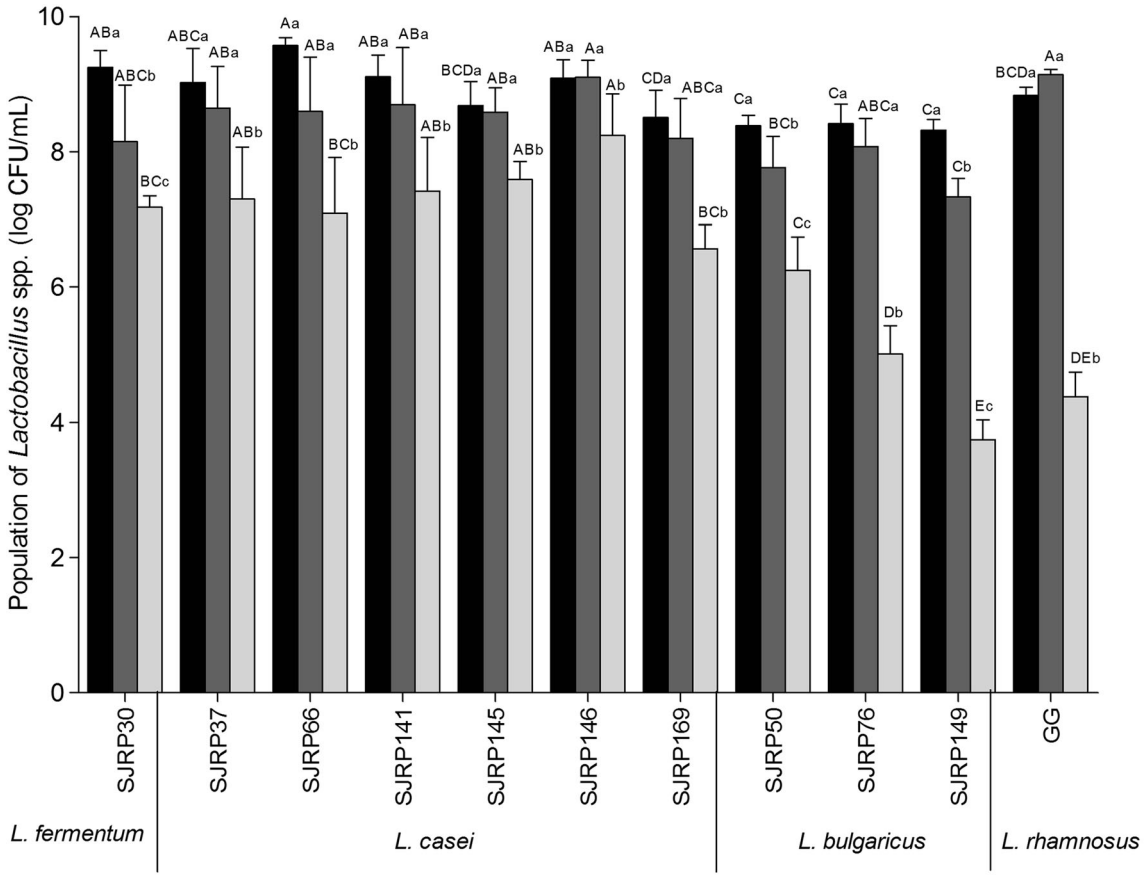

Fig. 1 Survival of *Lactobacillus* spp. strains before (■) and during exposure to in vitro simulated gastric conditions for 120 min (■, pH 2.5) and enteric conditions for 360 min (■, pH 8). Different capital letters denote significant differences ($P \leq 0.05$) among strains during the same sampling period of the in vitro assay. Different lower case letters denote significant differences ($P \leq 0.05$) among sampling periods for the same strain in the in vitro assay. The results are expressed as the mean ± SD. $n = 3$

bulgaricus was completely inhibited. Conversely, all strains grew in MRS agar plates with 0.5% (w/v) TC. Nevertheless, none of the tested lactobacilli strains showed BSH activity.

Adhesion to Caco-2 cells

All of the tested strains could adhere to Caco-2 cells, but they did so to different degrees. The adhesion rates ranged from 2.59% to 18.58%. *L. fermentum* SJRP30, *L. casei* strains SJRP37, SJRP145, and SJRP146 and *L. delbrueckii* subsp. *bulgaricus* SJRP76 showed similar adherence to the reference strain after 2 h of incubation ($P \leq 0.05$). *L. casei* SJRP141 was the most adhesive strain and presented adherent bacteria counts higher than the positive control *L. rhamnosus* GG. *L. casei* SJRP66 and *L. delbrueckii* subsp. *bulgaricus* SJRP149 had the lowest adhesion capacities. The results indicate that adhesion properties are strain-specific because the strains do not show similar adhesion values even though they are from the same species or genus (Fig. 2).

Presence of genes encoding adhesion, aggregation and colonization factors

L. delbrueckii subsp. *bulgaricus* SJRP149 showed positive results for all tested genes encoding adhesion, aggregation and colonization factors with the exception of the *prgB* gene. In contrast, strain *L. fermentum* SJRP30 did not harbor any of these genes. The other studied strains possessed at least one of the genes (Table 2).

Antimicrobial activity

The growth of pathogenic target microorganisms was not inhibited by any of the cell-free supernatants (CFS) (adjusted to pH 6.5) obtained from the tested LAB strains (data not shown).

β-galactosidase activity

Strains *L. casei* SJRP146 and *L. delbrueckii* subsp. *bulgaricus* SJRP50 and SJRP76 displayed an intense yellow color in their

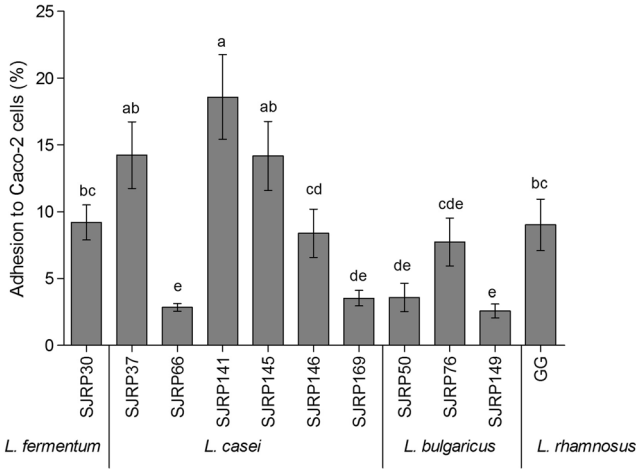

Fig. 2 Adhesion capacity of *Lactobacillus* spp. strains to Caco-2 cells. The adhesion capacity is calculated using the ratio of the number of bacterial cells that remained attached to the total number of bacterial cells added initially to each well. Different lower case letters denote significant differences ($P \leq 0.05$) among the adhesion capacities of the strains. The results are expressed as the mean ± SD. $n = 3$

tests. The other strains, except for *L. casei* SJRP145, also showed positive results; however, these strains produced a less intense yellow color. The reference strain *L. rhamnosus* GG and *L. casei* SJRP145 did not present β-galactosidase activity.

Discussion

In this study, we performed an in vitro analysis to determine the safety and probiotic potential characteristics of ten *Lactobacillus* strains. The assays were chosen based on international guidelines for evaluation of probiotic potential (FAO/WHO 2002). Although a large number of studies has been published in this field in the past, the identification of new strains with probiotic potential is always desirable, mainly because each strain shows different methods of action and several benefits to health. Nevertheless, before being used as a probiotic, the safety of the strains needs to be assessed to ensure that they will not represent a risk to consumer health. Recently, some cases relating infections to probiotic consumption have been reported (Kochan et al. 2011; Zbinden et al. 2015). Therefore, determining whether a strain is safe is of great concern among researchers.

A lack of hemolytic activity is considered a safety requirement when selecting a probiotic strain (FAO/WHO 2002) because such bacteria are not virulent, and the lack of hemolysin ensures that opportunistic virulence will not appear among strains (Peres et al. 2014). Previous reports also revealed that different LAB species did not show hemolysis (Argyri et al. 2013; Bautista-Gallego et al. 2013; Ryu and Chang 2013; Ilavenil et al. 2015). The production of enzymes capable of degrading mucin was proposed as a determinant factor of virulence for some enteropathogens. Therefore, this property is not considered a desirable feature for probiotic strains because it contributes to changes in the intestinal mucosal barrier in addition to favoring mucosal invasion by pathogens and other toxic agents (Monteagudo-Mera et al. 2012; Peres et al. 2014). Our results suggest that the evaluated probiotic candidates may not be able to invade the intestinal mucosa. These findings were in agreement with previous studies that investigated mucin degradation by several LAB species (Fernández et al. 2005; Abe et al. 2010; Rodríguez et al. 2012).

The main concern regarding probiotic safety is the resistance to antibiotics because these strains may transfer antibiotic resistance genes to pathogenic bacteria in the intestinal habitat, which can represent a serious risk for the treatment of infected patients. Antibiotic resistance is considered a negative characteristic for probiotics (Lee et al. 2014). The strains were found to be resistant to vancomycin, gentamicin, streptomycin, and kanamycin; however, this resistance pattern is considered an intrinsic feature of LAB because it is chromosomally encoded and, thus, the corresponding genes will not be transferred to pathogens (Tulini et al. 2013; Botta et al. 2014; Sharma et al. 2015). Taking these reports into consideration, the resistance to vancomycin, kanamycin, streptomycin, and gentamicin found in the strains and in the reference strain *L. rhamnosus* GG can be considered acceptable.

During the course of testing the strains for the presence of virulence genes, at least one of the genes responsible for gelatinase production (*gelE*, *fsrA*, *fsrB* and *fsrC*) was detected in all strains except *L. fermentum* SJPR30. The *gelE* gene is responsible for the production of gelatinase, which is an enzyme that hydrolyzes gelatin and collagen. Moreover, *gelE* expression is thought to be regulated in a cell density-dependent manner by the products of *fsrA*, *fsrB* and *fsrC*. However, the presence of *gelE* does not seem to be sufficient for gelatinase activity, and a complete *fsr* operon may be mandatory for *gelE* expression (Lopes et al. 2006). In our study, none of the strains contained the complete *fsr* operon.

Only *L. casei* SJRP169 presented the *cylA* gene; however, four isolates (*L. casei* SJRP141 and *L. delbrueckii* subsp. *bulgaricus* SJRP50, SJRP76 and SJRP149) showed partial hemolysis in the phenotypic test, whereas SJRP169 did not. Other lytic genes most likely cause this hemolytic reaction in the phenotypic tests when the *cylA* gene is not expressed (Perin et al. 2014). Four *L. casei* strains contained the *esp* gene, which may be a result of horizontal transference by the *Enterococcus* genus. Conversely, the adhesion properties conferred by the *esp* gene can be a significant characteristic for potential probiotic bacteria (de Paula et al. 2014). Sex pheromone genes (*ccf*, *cob* and *cpd*) were present in some of the evaluated strains. These genes are also considered virulence factors because they might induce an inflammatory response. Moreover, these genes have shown

in vitro chemotactic activity for human and rat polymorphonuclear leukocytes, and elicited superoxide production and the secretion of lysosomal enzymes (Eaton and Gasson 2001).

Genes encoding antibiotic resistance were also tested. The antibiotic resistance genes *vanA* and *vanB* were frequently present among the tested strains. Almost all of the *L. casei* strains showed positive results for both genes, except *L. casei* SJRP145 and *L. casei* SJRP146 in which the *vanA* gene was absent. The *vanC* genes were harbored by *L. fermentum* SJRP30, *L. casei* SJRP141 and SJRP169 and all of the *L. delbrueckii* subsp. *bulgaricus* strains. The *vanA* phenotype is characterized by a higher resistance level to vancomycin than the *vanB* phenotype and cross-resistance to teicoplanin. The gene cluster for both *vanA* and *vanB* resistance is usually located on a plasmid that is transferable, and thus represents a major concern for safety due to the spread of antibiotic resistance via horizontal gene transfer (Klein et al. 2000; Perin et al. 2014). Conversely, *vanC* is located in the bacterial chromosome (Martín-Platero et al. 2009). Therefore, despite the observation of resistance during the disc diffusion test, the resistance towards vancomycin in *L. fermentum* SJRP30 and *L. casei* SJRP145 recorded in our study was considered intrinsic, chromosomally encoded, and not inducible or transferable (Tynkkynen et al. 1998). The lack of genes encoding vancomycin resistance has been reported (Casado Muñoz et al. 2014). However, other studies have demonstrated the presence of *vanA* and *vanB* genes in a variety of LAB (Jeronymo-Ceneviva et al. 2014; Perin et al. 2014).

In this study, all strains contained at least one gene encoding aminoglycoside resistance, which could be associated with the intrinsic resistance towards this antibiotic class among *Lactobacillus* spp. The most common tetracycline resistance gene among *Lactobacillus* spp. strains was *tet(O)*, which was detected in all of the *L. casei* strains. However, *tet(S)* was not recorded in any of the strains. Tetracycline resistance genes have been found in other *Lactobacillus* spp. strains isolated from fermented dry sausages, cheese and yogurt (Zonenschain et al. 2009; Zhou et al. 2012). The *int* gene was detected in eight strains, indicating that these strains might harbor the transposon responsible for *tet* gene dissemination. Although the *int* gene has not been found in *Lactobacillus* strains to date, it has been identified in *Enterococcus* and *Lactococcus* strains with food origins (Bulajić et al. 2015; Morandi et al. 2015; Jaimee and Halami 2016). Regarding erythromycin resistance, only the *erm(C)* gene was found among the *Lactobacillus* sp. strains. This gene was previously detected in a variety of *Lactobacillus* species (Kastner et al. 2006; Klare et al. 2007; Egervärn et al. 2009).

The *hdc1* and *tdc* genes, which are related to biogenic amine production, were present in five strains. The *hdc1, hdc2, tdc* and *odc* genes express enzymes that degrade histamine, tyramine and ornithine into biogenic amines, respectively. Low levels of biogenic amines in food are not considered a serious risk to the consumer; however, they can be toxic when present in high concentrations (50–100 mg) (Jeronymo-Ceneviva et al. 2014). The presence of these genes has been reported for other LAB (Coton et al. 2010; Jeronymo-Ceneviva et al. 2014).

Concerning the probiotic potential characteristics, the evaluated strains showed good resistance to simulated gastric juice. In response to stress caused by acid, LAB use various mechanisms to overcome the damage, including maintaining the intracellular pH and cell membrane functionality and inducing stress-response proteins (Wu et al. 2014). These mechanisms vary both within species and according to exogenous conditions, including the growth media and incubation conditions (Madureira et al. 2011). The negative effect of bile on the viability of most lactobacilli was more accentuated than the effect of low pH. The bile concentration in the human body usually ranges from 0.3% to 0.5% (García-Ruiz et al. 2014); therefore, probiotic bacteria must to be able to withstand these bile concentrations. Bile is a toxic component that damages the membrane by modifying its integrity and permeability. Additionally, bile disturbs the stability of macromolecules, including RNA, DNA and proteins, and may cause oxidative stress (Begley et al. 2006).

Generally, *L. casei* strains showed higher viability after successive exposures to gastric and intestinal conditions than *L. delbrueckii* subsp. *bulgaricus*. The survival of the selected strains, with the exception of *L. delbrueckii* subsp. *bulgaricus* SJRP149, was greater than the survival observed for the commercial strain used as a reference; similar results were obtained by Argyri et al. (2013). The good tolerance of *Lactobacillus* strains to gastric juice and bile is in accordance with the results reported in a previous study (Jensen et al. 2012). Nevertheless, no recovery in cell viability was observed for any of the strains in the subsequent treatment with enteric juice, which was in contrast to reports by other authors (Corsetti et al. 2008; Bautista-Gallego et al. 2013). This discrepancy most likely occurred because the strains did not have the ability to metabolize conjugated bile salts. The ability to hydrolyze bile salts is usually included as one of the criteria for the selection of strains with probiotic potential (Rodríguez et al. 2012). Nonetheless, BSH activity is rare among bacteria that have been isolated from environments with an absence of bile, such as the strains used in the present study. This finding is in agreement with other studies (Bautista-Gallego et al. 2013; Solieri et al. 2014).

Adhesion to intestinal epithelial cells is commonly included as an in vitro test to select probiotic strains. Although in vitro adhesion assays are useful for providing information on the differences among the strains being assessed, the results obtained from adhesion tests are different from the reality in vivo. The human GI tract has defense systems, resident

flora, and bowel movements that may change the strain adhesion ability (Jensen et al. 2012). Moreover, different values for lactobacillus adhesion to Caco-2 cells have been reported, ranging from less than 1% to more than 70% (Jensen et al. 2012; Nikolic et al. 2012; Ramos et al. 2013; Tulumoğlu et al. 2014), possibly due to variation in the conditions used during the assay, such as the type of cell line, incubation time and number of probiotic cells added.

The presence of genes encoding adhesion, aggregation and colonization factors is desirable in LAB and can also indicate that the bacteria are able to adhere to the mucus layer (Jeronymo-Ceneviva et al. 2014). However, we did not find a correlation between the in vitro adhesion assay and the presence of adhesion genes because *L. delbrueckii* subsp. *bulgaricus* SJRP149 had the lowest ability to adhere to the Caco-2 cell model, despite presenting genes encoding adhesion, aggregation and colonization factors (except the *prgB* gene). Although in vitro tests are considered useful indicators of strain adhesion, they do not always reflect the ability of the bacteria to adhere to the mucus covering intestinal cells (Ramiah et al. 2007).

Antimicrobial activity was not detected in any of the neutralized CFSs, leading to the conclusion that no antimicrobial peptides or bacteriocin-like compounds were produced by these strains. This result is consistent with findings for other LAB strains, including *Leuconostoc mesenteroides*, *Ln. pseudomesenteroides*, *L. plantarum*, *L. pentosus*, *L. paraplantarum*, and *L. paracasei* subsp. *paracasei* (Argyri et al. 2013; Briggiler Marcó et al. 2014). Additionally, Ren et al. (2014) reported that most of the neutralized supernatants (pH 6.5) from lactobacilli and washed lactobacilli cells resuspended in fresh MRS broth lost their inhibitory activities against *E. coli*, *B. cereus*, and *S. aureus* when compared with fresh overnight lactobacilli strain cultures.

The production of β-galactosidase was investigated because the ability to produce this enzyme is an advantageous feature for probiotic strains. β-Galactosidase hydrolyzes lactose and is important for both consumers of dairy products who have lactose intolerance and for the production of dairy products.

Taking all of the results into account, *L. fermentum* SJRP30 and *L. casei* SJRP145 and SJRP146 are considered safe for future application as probiotics in co-culture with starter strains according to the tests suggested by FAO/WHO (ATCC 53103). Additionally, the selected strains possessed similar or superior probiotic potential characteristics compared to the reference strain *L. rhamnosus* GG. The promising results found for these strains suggest that additional in vitro or in vivo tests should be performed to verify the possible beneficial effects toward human health, including cholesterol-reducing ability, immunomodulatory effects and lowering the risk of GI diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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