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Screening of Probiotic Activities of Lactobacilli Strains Isolated from Traditional Tibetan Qula, A Raw Yak Milk Cheese

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ABSTRACT: In this study, 69 lactobacilli isolated from Tibetan Qula, a raw yak milk cheese, were screened for their potential use as probiotics. The isolates were tested in terms of: Their ability to survive at pH 2.0, pH 3.0, and in the presence of 0.3% bile salts; tolerance of simulated gastric and intestinal juices; antimicrobial activity; sensitivity against 11 specific antibiotics; and their cell surface hydrophobicity. The results show that out of the 69 strains, 29 strains (42%) had survival rates above 90% after 2 h of incubation at pH values of 2.0 or 3.0. Of these 29 strains, 21 strains showed a tolerance for 0.3% bile salt. Incubation of these 21 isolates in simulated gastrointestinal fluid for 3 h revealed survival rates above 90%; the survival rate for 20 of these isolates remained above 90% after 4 h of incubation in simulated intestinal fluid. The viable counts of bacteria after incubation in simulated gastric fluid for 3 h and simulated intestinal fluid for 4 h were both significantly different compared with the counts at 0 h ($p<0.001$). Further screening performed on the above 20 isolates indicated that all 20 lactobacilli strains exhibited inhibitory activity against *Micrococcus luteus* ATCC 4698, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 19115, and *Salmonella enterica* ATCC 43971. Moreover, all of the strains were resistant to vancomycin and streptomycin. Of the 20 strains, three were resistant to all 11 elected antibiotics (ciprofloxacin, erythromycin, tetracycline, penicillin G, ampicillin, streptomycin, polymyxin B, vancomycin, chloramphenicol, rifampicin, and gentamicin) in this study, and five were sensitive to more than half of the antibiotics. Additionally, the cell surface hydrophobicity of seven of the 20 lactobacilli strains was above 70%, including strains *Lactobacillus casei* 1,133 (92%), *Lactobacillus plantarum* 1086-1 (82%), *Lactobacillus casei* 1089 (81%), *Lactobacillus casei* 1138 (79%), *Lactobacillus buchneri* 1059 (78%), *Lactobacillus plantarum* 1141 (75%), and *Lactobacillus plantarum* 1197 (71%). Together, these results suggest that these seven strains are good probiotic candidates, and that tolerance against bile acid, simulated gastric and intestinal juices, antimicrobial activity, antibiotic resistance, and cell surface hydrophobicity could be adopted for preliminary screening of potentially probiotic lactobacilli. (**Key Words:** Probiotic Activities, Screening, Lactobacilli, Qula Cheese)

INTRODUCTION

Probiotics are defined as living microorganisms that have beneficial effects on the host, and can adjust the host body's microecological balance, improve intestinal function, and stimulate digestion and immune function. *Lactobacillus* was the earliest discovered probiotic of the three types of probiotics, which also include *Bifidobacterium* and Gram-positive cocci (Tulumoglu et al., 2013).

Lactobacillus is the largest genus of lactic acid bacteria (LAB), with 183 species, and are a group of rod-shaped or rod-like-shaped Gram-positive bacteria that can ferment glucose and produce lactic acid. *Lactobacillus* is the dominant bacteria in animal and human gastrointestinal and urinary systems, and plays an important role in the maintenance and recovery of health. Many species of *Lactobacillus* are recognized as safe for consumption, and thus, are often used in food production (de Almeida Júnior et al., 2015). Some species of *Lactobacillus*, including *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus*

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johnsonii, *Lactobacillus reuteri*, and *Lactobacillus rhamnosus*, have been used as probiotics (de Vos, 2011). In recent years, scientific research has confirmed the presence of large amounts of probiotic LAB in fermented dairy products and has shown their positive impact on human health. Qula is a white or yellow dried, hard, grainy cheese handcrafted from yak's milk in Tibet (Tan et al., 2010). Traditional Qula is fermented by microorganisms in the natural environment, and contains a large number of unique probiotic microorganisms, and to our knowledge, the isolation and screening of probiotic *Lactobacillus* from Qula has not been reported in the literature.

Increased attention has been paid to the probiotic abilities of *Lactobacillus*. Many factors need to be considered during the screening of potential probiotic LAB strains *in vitro*, including acid–bile salt tolerance; survival in gastric and intestinal fluids; their capability to adhere to intestinal surfaces; inhibition of pathogenic bacteria; and sensitivity to antibiotics.

The purpose of this study was to identify potential probiotic lactobacilli isolated from traditional handmade Qula cheese in the Qinghai province of China, and provide data for the development and utilization of probiotics.

MATERIALS AND METHODS

Phenotypic and genotypic identification

A total of 192 strains were isolated from traditional Tibetan Qula cheese based on colony morphology on de Man Rogosa Sharpe (MRS) agar (Tan et al., 2010). The isolates were initially identified based on their Gram reactions, catalase activity, gas production in the presence of glucose, and carbohydrate fermentation. The species were further identified based on 16SrRNA gene sequence analysis. Genomic DNA from each strain was first extracted using the Genomic DNA Mini Preparation Kit (Beyotime, Hangzhou, China), and amplification of the 16SrRNA gene was carried out in a thermal cycler using prokaryotic 16S ribosomal DNA universal primers: 27F (5′-AGAGTTTG ATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTA CGACTT-3′) (Tan et al., 2010). All sequences were then compared to those in the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (NCBI), resulting in the identification of 69 *Lactobacillus* isolates for the tests described below.

Acid tolerance

Acid tolerance was determined in accordance with the method by Chung et al. (1999), with slight modifications. In brief, 10 μL of overnight bacterial culture in MRS broth was inoculated respectively into 1 mL of pH 2.0, 3.0, and 6.4 (control) MRS broth. The number of LAB was quantified using the plate count method on MRS agar after incubation at 37°C for 2 h. The survival rate was calculated as log values of colony-forming units per milliliter (colony-forming unit [CFU]/mL).

Bile salt tolerance

Bile salt tolerance was determined according to the method by Gilliland et al. (1984). One percent overnight cultures in MRS broth were inoculated respectively into MRS broth with added 0.3% (w/v) bile (test) and without bile (control). All samples were incubated in a 37°C water bath. Growth in the control (no bile) and test cultures (0.3% bile) was determined by measuring the absorbance at 600 nm until the absorbance was increased by 0.3 units (0.3 U). The difference (d) in the length of time between the two samples was considered to be the delay in growth due to inhibition by the bile salts.

Simulation of tolerance to the gastrointestinal tract

For the pancreatic fluid tolerance test, 0.35 g of pepsin and 100 mL of a 0.2% sterile NaCl solution were used at pH 2.5, as suggested by Charteris et al. (1998). To test tolerance to intestinal juice, in accordance with the method by Bao et al. (2010), 0.1 g of trypsin and 1.8 g of bile salts were added to a sterile solution of 1.1 g of $NaHCO_3$ and 0.2 g of NaCl in 100 mL distilled water. The pH of the solution was adjusted to 8.0 with 0.5 M NaOH. The simulated gastrointestinal fluid was sterilized by filtering through a 0.22 μm membrane.

The lactobacilli for each test was incubated in MRS broth at 37°C for 24 h. The cultures were then centrifuged for 5 min at 10,000 *g* and washed three times with pH 7.0 phosphate-buffered saline (PBS) (10^8 to 10^9 CFU/mL). A 10% solution of each sample was transferred into the simulated gastric and intestinal juices, respectively. Viability in the simulated gastric juice was counted at 0 and 3 h on MRS agar, and at 0 and 4 h in the simulated intestinal juice. The survival rate was calculated in the same manner as for the determination of the acid resistance.

Antibacterial activity

The inhibition of pathogenic bacteria was determined by the agar diffusion assay method (Ennahar et al., 2000) with *Micrococcus luteus* ATCC 4698, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 11775, *Listeria monocytogenes* ATCC 19115, *Staphylococcus aureus* ATCC 29213, *Salmonella enterica* ATCC 43971, and *Pseudomonas aeruginosa* ATCC 27853 as the indicator strains. After being activated, each pathogen was suspended in sterile water and standardized to an absorbance of 1 at 600 nm. The overnight lactobacilli cultures in MRS broth were centrifuged for 20 min at 10,000 *g*, and the supernatants were filtered through a 0.22 μm filter to remove residual cells. Twenty milliliters of MRS agar at 50°C were mixed

with 200 mL overnight culture of the indicator strains and poured into a sterile plate. Wells (7.80 mm in diameter) were made in the agar layer, and 300 μL of cell-free supernatant was placed in each well. After incubation for 24 h at 37°C, the diameters of the inhibition zones were measured.

Sensitivity to antibiotics

The sensitivity of the bacteria to antibiotics was determined by the agar overlay disc diffusion test (Charteris et al., 1998), using ciprofloxacin (CPFX; 5 μg), erythromycin (15 μg), tetracycline (30 μg), penicillin G (10 μg), ampicillin (10 μg), streptomycin (10 μg), polymyxin B (300 μg), vancomycin (30 μg), chloramphenicol (30 μg), rifampicin (5 μg), and gentamicin (10 μg) antibiotic discs (Oxoid, Basingstoke, Hampshire, UK). Add 10 mL of MRS fluid nutrient medium to the sterile plate, and wait for it to solidify at room temperature. Add 5 mL MRS agar culture-medium (50°C) into 250 μL of overnight cultured *Lactobacillus* bacterial suspension (10^8 CFU/mL), mix them rapidly, and then pour the mixture into the plate with a base layer. Wait for the mixture to solidify, and then put drug-susceptible paper pasters on the medium with spaces of above 24 mm. The inhibition zone diameters were measured after 24 h of incubation at 37°C. The results were compared with breakpoint values designated by the Clinical and Laboratory Standards Institute (CLSI, 2012).

Cell surface hydrophobicity

Cell surface hydrophobicity was determined by the bacterial adhesion to hydrocarbons assay according to Rosenberg et al. (1980). The overnight bacterial culture in MRS broth was centrifuged at 10,000 *g* for 5 min, and then equal volumes of the supernatant and PBS were mixed by vortexing for 30 s. The absorbance of the mixture was measured at 600 nm (A_0). The PBS mixture was vortexed with dimethylbenzene for 60 s and incubated at 37°C for phase separation. The aqueous phase was gently removed, and its absorbance was measured at 600 nm (A_t). The surface hydrophobicity (H%) was calculated as follows:

$$H\% = (A_t - A_0)/A_0 \times 100\%$$

Statistical analysis

Each assay was repeated on three independent occasions with triplicate determinations. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) with statistical significance determined at $p<0.01$ or 0.05. Results are expressed as the mean and standard error of the mean of three independent experiments. One-way analysis of variance followed by Least significant difference test was used to determine significant differences of viability of the tested strains in simulated gastrointestinal fluid and also with respect to the cell surface hydrophobicity.

RESULTS

Phenotypic and genotypic identification

A total of 69 Gram-positive, catalase-negative, rod-shaped isolates were acquired. The 69 strains used in this research were identified by a molecular method, and the isolates were characterized as *Lactobacillus plantarum* (34 strains), *Lactobacillus casei* (28 strains), *Lactobacillus buchneri* (3 strains), *Lactobacillus diolivorans* (1 strain), *Lactobacillus sakei* (1 strain), *Lactobacillus curvatus* (1 strain), and *Lactobacillus kefiri* (1 strain).

Acid tolerance

Sixty-nine *Lactobacillus* strains were examined for acid tolerance in this research. The acid tolerance of 29 isolates with good resistance to low pH are shown in Table 1. Additionally, Table 1 shows that the survival rates of 17 strains (*Lactobacillus* 70, 75, 1087, 1150, 1193-2, 1095, 1138, 1158, 1197, 1086-1, 1059, 32-2, 1156, 1033-1, 1089, 21, and 1133) were ≥90% at pH 3.0, that of 21 strains (*Lactobacillus* 1110, 1141, 49-1, 33, 1193-2, 1033-1, 30, 1134, 1150, 1138, 1158, 1035, 60, 1156, 1089, 1067, 1115, 1140, 70, and 1197) were ≥90% at pH 2.0; nine of the 69 *Lactobacillus* strains (*Lactobacillus* 70, 1089, 1197, 1138, 1150, 1156, 1158, 1033-1, and 1093-2) showed good tolerance at both pH 2.0 and 3.0. Strain 1133 was the most acid tolerant at pH 3.0, with a survival rate of 97%, and four strains (*Lactobacillus* 1067, 1115, 1140, and 70) were the most tolerant at pH 2.0, with survival rates of 93%. The viable counts of these 29 isolates in Table 1 all decreased at both pH 2.0 and 3.0 after 2 h compared with the control. The survival rates of 15 strains (*Lactobacillus* 30, 33, 60, 70, 1035, 1059, 1067, 1110, 1115, 1134, 1140, 1141, 1150, 49-1, and 1195-1) at pH 2.0 were higher than at pH 3.0. Further screening was performed on these 29 strains as shown in Table 1.

Bile tolerance

Bile salts at a concentration of 0.3% had different degrees of inhibition on the 29 tested strains in this study. The results were analyzed using the standards suggested by Gilliland et al. (1984): resistant strains ($d \leq 15$ min), tolerant strains ($15 < d \leq 40$ min), weakly tolerant strains ($40 < d < 60$ min), and sensitive strains ($d \geq 60$ min). Twenty-one (72%) of the tested strains resisted 0.3% bile; their tolerances to bile are showed in Table 2. Two strains (*Lactobacillus* 75 and 1089) were considered to be resistant strains; six strains (*Lactobacillus* 21, 1067, 1138, 1141, 1193-2, and 1195-1) were tolerant strains; and 13 strains (*Lactobacillus* 1035, 1059, 1087, 1110, 1115, 1133, 1140, 1150, 1158, 1197, 32-2,

Table 1. Viability (log CFU/mL) and survival percentage of lactobacilli strains incubated at different pH values

Strains	pH 6.2[1] Viable count (log CFU/mL)	pH 3.0 Viable count (log CFU/mL)	Percentage survival (%)	pH 2.0 Viable count (log CFU/mL)	Percentage survival (%)[2]
*L.buchneri*1158	8.91±0.28	8.07±0.17	91	8.08±0.30	91
*L.buchneri*1059	9.08±0.01	8.36±0.14	92	8.11±0.07	89
*L.casei*1067	8.90±0.16	7.76±0.12	87	8.29±0.09	93
*L.casei*1133	7.90±0.29	7.67±0.06	97	6.98±0.15	88
*L.casei*1138	9.09±0.06	8.26±0.19	91	8.26±0.03	91
*L.casei*1156	8.92±0.10	8.28±0.21	93	8.19±0.04	92
*L.casei*32-2	8.39±0.07	7.74±0.14	92	7.29±0.09	87
*L.casei*1095	7.81±0.01	7.11±0.02	91	6.36±0.21	81
*L.casei*1035	8.75±0.02	7.65±0.10	87	8.08±0.04	92
*L.casei*1089	7.48±0.07	7.09±0.23	95	6.90±0.19	92
*L.casei*21	8.61±0.12	8.29±0.17	96	7.53±0.11	87
*L.casei*30	8.67±0.07	7.48±0.06	86	7.90±0.02	91
*L.casei*70	8.97±0.02	8.04±0.01	90	8.31±0.06	93
*L.plantarum*1033-1	9.03±0.04	8.45±0.02	94	8.15±0.10	90
*L.plantarum*1086-1	8.73±0.07	7.98±0.13	91	7.67±0.10	88
*L.plantarum*1087	8.96±0.08	8.06±0.13	90	7.75±0.02	86
*L.plantarum*1110	8.96±0.03	7.76±0.16	87	8.04±0.26	90
*L.plantarum*1115	8.78±0.11	7.77±0.02	88	8.16±0.06	93
*L.plantarum*1134	8.28±0.41	7.26±0.13	88	7.51±0.17	91
*L.plantarum*1140	9.06±0.03	7.98±0.06	88	8.45±0.05	93
*L.plantarum*1141	8.94±0.03	7.90±0.05	88	8.06±0.08	90
*L.plantarum*1150	8.96±0.26	8.07±0.04	90	8.16±0.05	91
*L.plantarum*1193-2	8.77±0.21	7.88±0.16	90	7.90±0.09	90
*L.plantarum*1195-1	8.85±0.14	7.88±0.03	89	8.13±0.08	92
*L.plantarum*1197	9.00±0.11	8.15±0.16	91	8.18±0.14	91
*L.plantarum*33	8.59±0.33	7.65±0.10	89	7.74±0.08	90
*L.plantarum*49-1	8.45±0.18	7.41±0.20	88	7.57±0.13	90
*L.plantarum*60	8.55±0.09	7.65±0.08	89	7.90±0.17	92
*L.plantarum*75	8.75±0.23	7.90±0.05	90	7.73±0.19	88

CFU, colony-forming unit.
[1] Control. [2] Percentage survival = final (log CFU/mL)/control (log CFU/mL)×100%.

1033-1, and 1086-1) were weakly tolerant strains.

Tolerance to simulated gastric and intestinal juices

The viability of 21 strains with good bile acid tolerance after exposure to simulated gastric and intestinal juices is shown in Figure 1 and 2. Compared with the conditions at 0 h, the viable counts of bacteria after incubation in gastric fluid for 3 h and in intestinal fluid for 4 h were both significantly different ($p<0.001$). As shown in Figure 1, similar tolerances to simulated gastric juice were observed among the 21 strains tested; the viable counts for 3 h were all 1.00 log CFU/mL less than those for 0 h, with survival rates all ≥90%. Strain 1133 had the highest survival rate (95%) in gastric juice; its viable counts after 3 h were only 0.40 log CFU/mL less than that for 0 h. All strains (except strains 1035 and 1133) had better viability in simulated intestinal fluid for 4 h than in simulated gastric fluid for 3 h. Strains 1089 and 1138 had the highest survival rates (98%) in intestinal juice.

Antibacterial activity

The assay of antimicrobial ability against seven pathogens was performed on the 20 lactobacilli strains that passed the biological barriers screening, and the results are shown in Table 3. All 20 strains exhibited inhibitory activity against *M. luteus* ATCC 4698 (with an inhibition zone 12.50 to 25.42 mm in diameter), *B. subtilis* ATCC 6633 (8.92 to 18.00 mm), *L. monocytogenes* ATCC 19115 (11.00 to 22.14 mm), and *S. enterica* ATCC 43971 (10.60 to 21.28 mm). In addition, *Lactobacillus* strains 21, 75, 1067, 1087, 1089, 1110, 1115, 1138, 1140, 1141, 1150, 1033-1, and 1193-2 exhibited antibacterial activity against all the pathogens used in this study; *Lactobacillus* strains 1133, 1158, 1197, 32-2, and 1195-1 inhibited all pathogens other than *E. coli*

Table 2. Ability of lactobacilli strains to tolerate 0.3% (w/v) bile (min)

Strains	I (d≤15 min)	II (15<d ≤40 min)	III (40<d <60 min)	IV (d≥60 min)
*L.casei*21		+		
*L.plantarum*75	+			
*L.casei*1035			+	
*L.buchneri*1059			+	
*L.casei*1067		+		
*L.plantarum*1087			+	
*L.casei*1089	+			
*L.plantarum*1110			+	
*L.plantarum*1115			+	
*L.casei*1133			+	
*L.casei*1138		+		
*L.plantarum*1140			+	
*L.plantarum*1141		+		
*L.plantarum*1150			+	
*L.buchneri*1158			+	
*L.plantarum*1197			+	
*L.casei*32-2			+	
*L.plantarum*1033-1			+	
*L.plantarum*1086-1			+	
*L.plantarum*1193-2		+		
*L.plantarum*1195-1		+		

I, group of bile-resistant strains; II, group of bile-tolerant strains; III, group of bile weakly tolerant strains; IV, group of bile-sensitive strains; +, member of the group (according to Gilliland et al., 1984).

(8.18 to 11.52 mm); *Lactobacillus* 1086-1 inhibited all pathogens other than *S. aureus* (11.24 to 21.70 mm); and *Lactobacillus* 1059 inhibited all pathogens other than *P. aeruginosa* (8.54 to 17.04 mm). Further, *Lactobacillus* 1144 had the strongest inhibitory activity against *M. luteus* and *L. monocytogenes*; and *Lactobacillus* 1086-1, 1087, 1115, 1197, and 1138 had the strongest inhibition against *B. subtilis*, *E. coli*, *S. aureus*, *S. enterica*, and *P. aeruginosa*, respectively. Most strains had weaker inhibition against *E. coli* than against the other pathogens.

Antibiotic resistance

The resistance of the 20 lactobacilli strains was tested against 11 antibiotics; the results are shown in Table 4. All the lactobacilli exhibited resistance to vancomycin and streptomycin. Fourteen lactobacilli (70%) were sensitive to chloramphenicol, 11 lactobacilli (55%) were sensitive to rifampin, 13 lactobacilli (65%) were sensitive to ampicillin, 14 lactobacilli (70%) were sensitive to tetracycline, 14 lactobacilli (70%) were sensitive to erythromycin, 19 lactobacilli (95%) were resistant to CPFX, 17 lactobacilli (85%) were resistant to penicillin, 15 lactobacilli (75%) were resistant to gentamicin, and 18 lactobacilli (90%) were resistant to polymyxin B. Strains 32-2 and 1195-1 showed resistance against the 11 antibiotics used in the assay, and strains 21, 1089, 1110, and 1138 were sensitive to more than half of the antibiotics.

Cell surface hydrophobicity

For further analysis of the cell surface characteristics, the cell surface hydrophobicity of the 20 lactobacilli were measured. As shown in Figure 3, the hydrophobicity of the different strains were significantly different ($p<0.05$; $p<0.01$), and ranged from 15% to 92%. The hydrophobicities of seven strains were more than 70%: 1133 (92%), 1086-1 (82%), 1089 (81%), 1138 (79%), 1059 (78%), 1141 (75%), and 1197 (71%).

DISCUSSION

In order to have probiotic effects in the intestinal tract, LAB must be capable of surviving passage through the gastrointestinal tract (GIT). Therefore, for probiotic LAB, resistance to the gastric acid environment is a prerequisite for survival and function in the intestinal tract. Depending on the specific individual's diet, the pH of the human gastric environment varies from 1.5 to 3.0 (Solieri et al., 2014), and is usually around 3. With the intake of foods such as dairy products, the gastric pH value rises to 3.0 or

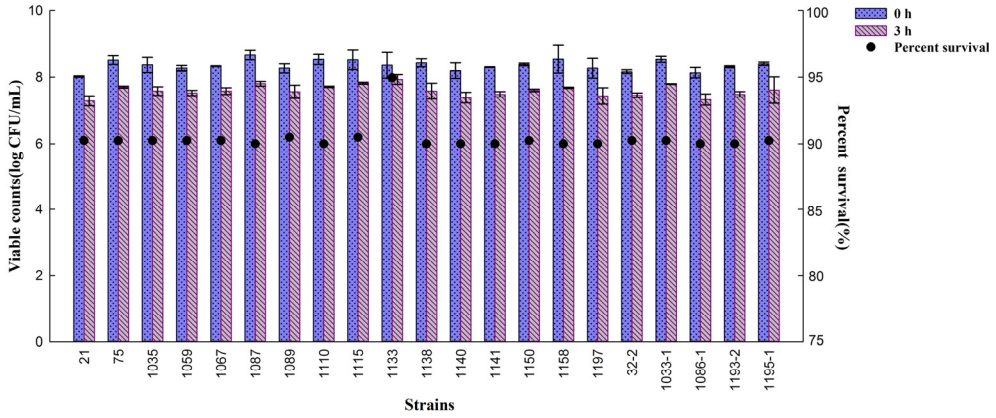

Figure 1. The viable counts (log CFU/mL) and survival rates of lactobacilli strains after 3 h in the simulated gastric juice.

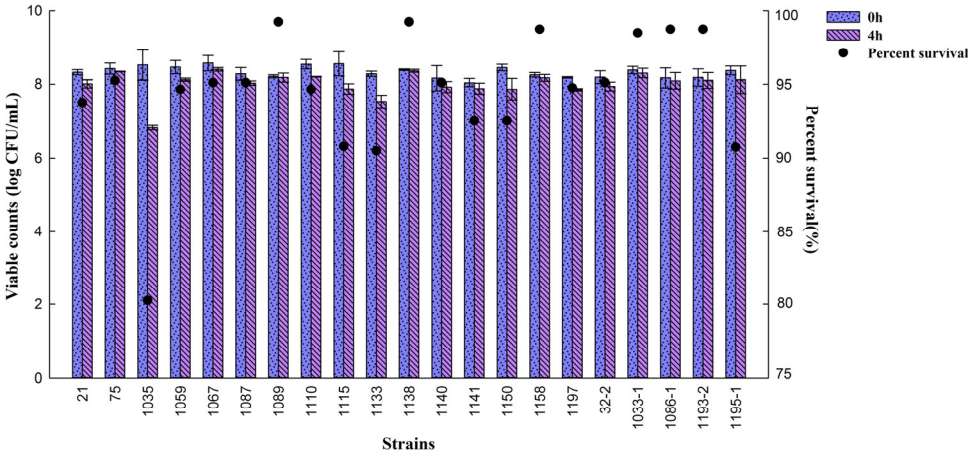

Figure 2. The viable counts (log CFU/mL) and survival rates of lactobacilli strains after 4 h in the simulated intestinal juice.

even higher. In most studies, MRS broth with a pH value of 2.0 to 3.0 has been used to determine the acid resistance of *Lactobacillus* (Jacobsen et al., 1999; Tulumoglu et al., 2013; Solieri et al., 2014). Acid conditions have a large effect on the growth of *Lactobacillus*. In the present study, only 29 of the 69 isolates had survival rates ≥90% at conditions of pH 2.0 or 3.0. At pH 3.0, the percentage of tested strains with survival rates ≥90% was 25%, which is lower than the percentage of 45% observed in a study by Tulumoglu et al. (2013). In the present study, the percentage of strains with a favorable anti-acid performance at pH 2.0 was 30%. This percentage is better than that seen in a study by Mathara et al. (2008), who isolated *Lactobacillus* from traditional fermented dairy products made by the Maasai people in Kenya; strains with a favorable resistance at a pH of 2.0 accounted for 22.2% of the overall strains. In the Tulumoglu et al. (2013) study, the percentage of strains with a favorable resistance at pH 2.0 was 25%. Moreover, Solieri

Table 3. Antimicrobial activity of lactobacilli strains

Strains (a = 20)	Antimicrobial activity						
	Micrococcus luteus ATCC 4698	*Bacillus subtilis* ATCC6633	*Escherichia coli* ATCC11775	*Listeria monocytogenes ATCC 19115*	*Staphylococcus aureus* ATCC29213	*Salmonella enterica* ATCC 43971	*Pseudomonas aeruginosa* ATCC27853
21 (n = 7)	+++	++	+	+++	+++	++	+
75 (n = 7)	+++	++	+	+++	+++	++++	+
1059 (n = 6)	++	+	+	+++	++	++	–
1067 (n = 7)	++++	++	+	+++	+++	+++	+
1087 (n = 7)	++++	+++	+	++++	+	++++	++
1089 (n = 7)	+++	+	+	+++	++++	+	++
1110 (n = 7)	++++	+++	+	++++	+++	+++	+
1115 (n = 7)	++++	+++	+	+++	++++	+++	+
1133 (n = 6)	++	+	–	+++	+	++	++
1138 (n = 7)	++++	++	+	++++	+++	+++	+++
1140 (n = 7)	++++	+++	+	++++	+++	++++	++
1141 (n = 7)	++++	+++	+	++++	++++	++++	++
1150 (n = 7)	++++	+++	+	++++	+++	+++	++
1158 (n = 6)	++	+	–	++	++	++	++
1197 (n = 6)	+++	++	–	++++	+++	++++	++
32-2 (n = 6)	++	+	–	+	++	++	+
1033-1 (n = 7)	++++	++	+	++++	+++	+++	++
1193-2 (n = 7)	++++	+++	+	++++	+++	+++	++
1195-1 (n = 6)	++	++	–	+++	+++	++++	++
1086-1 (n = 6)	++++	+++	+	++++	–	+++	++

+, Diameter of inhibition zone: 8.00 to 12.00 mm; ++, 12.00 to 16.00 mm; +++, 16.00 to 20.00 mm; ++++, more than 20.00 mm; –, not detected; the diameter of inhibition zone including that of Oxford cup (7.80 mm).
a, total number of lactobacilli strains. n, inhibition to number of pathogens.

Table 4. Antibiotic susceptibility test of lactobacilli strains

Strains	CHL	VA	GM	PB	RA	STR	AM	ERY	CIP	PG	TE
21	S	R	R	R	S	R	S	S	M	S	S
75	S	R	M	R	S	R	S	S	R	R	S
1089	S	R	S	S	S	R	S	S	R	S	S
1067	S	R	R	R	S	R	S	S	R	R	S
1087	S	R	R	R	M	R	S	M	R	R	S
1059	S	R	R	R	M	R	R	S	R	R	S
32-2	R	R	R	R	R	R	R	R	R	R	R
1086-1	S	R	R	R	M	R	S	M	R	R	S
1110	S	R	M	R	S	R	S	S	R	S	S
1115	S	R	R	R	S	R	S	S	R	R	S
1133	R	R	R	R	R	R	R	S	R	R	R
1033-1	R	R	R	R	R	R	R	S	R	R	R
1140	R	R	R	R	R	R	R	S	R	R	R
1141	S	R	S	R	S	R	R	S	R	R	S
1150	R	R	R	R	S	R	S	R	S	R	R
1158	S	R	R	R	S	R	S	S	R	R	S
1197	S	R	R	R	S	R	S	S	R	R	S
1138	S	R	S	S	S	R	S	S	R	R	S
1193-2	S	R	R	R	M	R	S	M	R	R	S
1195-1	R	R	R	R	R	R	R	R	R	R	R

CH, chloramphenicol; VA, vancomycin; GM, gentamicin; PB, polymyxin B; RA, rifampin; STR, streptomycin; AM, ampicillin; ERY, erythromycin; CIP, ciprofloxacin; PG, penicillin; TE, tetracycline; R, resistant; M, moderate resistance; S, susceptible.

et al. (2014) found that almost none of the 47 *Lactobacillus* strains isolated from ripened Parmigiano-Reggiano cheese could survive conditions of pH 2.0. Furthermore, in a study by de Almeida Júnior et al. (2015), strains with favorable resistance at pH 2.0 only accounted for 72% of the 50 *Lactobacillus* strains isolated from ewe's milk. In the present study, the survival rates of strains with the best anti-acid performances were 97% and 93% at pH values of 3.0 and 2.0, respectively. Additionally, the visual CFU of some strains was lower at pH 3.0 than at pH 2.0, and some exhibited certain acidophilic properties, which may be due to the acidification process during Qula production. The results are similar to those from a study by Zhang (2011), who assessed the anti-acid performance of strains isolated from homemade traditional fermented yak's milk in the Gansu pasturing area, although these results were different from those of Tulumoglu et al. (2013). In the study by Tulumoglu et al. (2013), the visual CFU of all strains at high pH values was higher than those at low pH values.

Cholate damages the structure of cell membranes, leading to leakage of substances inside the cell, and making it difficult for thallus to survive. Therefore, a strain's tolerance to cholate is also of vital importance when assessing probiotic ability. The concentration of cholate inside healthy intestinal tracts varies from 0.03% to 0.30%, and generally does not surpass 0.3% (w/v) (Gilliland et al., 1984), which is considered to be the critical concentration when screening for bile-tolerant strains (Gilliland et al., 1984; Jacobsen et al., 1999). Therefore, 0.3% bile was used in this study, and all strains tested showed growth delays in the 0.3% bile. Conversely, Jacobsen et al. (1999) found no growth delay in 0.3% bile for three strains isolated from Ghanaian fermented maize. In the present study, eight strains exhibited high levels of tolerance to bile (with delayed growth ≤40 min). This result is superior to that of the *Lactobacillus* strains isolated from cow excrement in a study by Hyronimus et al. (2000), in which the growth delay for all studied strains were >40 min. The strains with the best tolerance in this study had growth delays of <15 min. This performance is superior to that from the study by Gilliland et al. (1984), in which the *L. acidophilus* strains

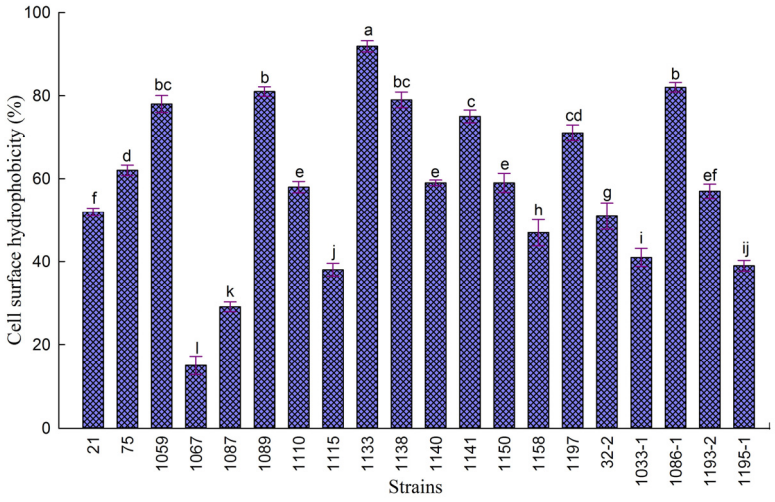

Figure 3. The surface hydrophobicity of selected lactobacilli strains as measured by their bacterial adherence to dimethylbenzene. [a,b,c,d,e,f,g,h,i,j,k,l] Superscripts of the same letters indicate no significant inter-group differences, superscripts of different letters indicate significant inter-group differences ($p<0.05$), and non-continuous letters indicate extremely significant inter-group difference ($p<0.01$).

with the best tolerance, which were isolated from the fecal or intestinal contents of 2- to 5-week-old calves, showed growth delays of 20 min.

The low pH of gastric juices and the gastric protease in gastric juices inhibit the growth of thallus. The small intestine is the major site of probiotic action, and various enzymes, bile acids, and other substances in small intestinal juice also inhibit probiotic growth. Therefore, GIT tolerance is an important criterion for the selection of potential probiotics. In the present study, during the GIT tolerance tests, almost all the strains exhibited better tolerance for simulated intestinal juice than simulated gastric juice. Further, Bao et al. (2010) reported that pancreatic fluid did not significantly affect LAB survival. In the present study, except for strain 1035, all the studied strains had survival rates >90% in the simulated gastrointestinal fluid, with <1.00 log CFU/mL decreases in the viable counts. This result is superior to that of de Almeida Júnior et al. (2015). In a study by Prasad et al. (1998), significantly inferior results were found compared to those in the present study in terms of the simulated GIT tolerance of two commercial fermented strains, with decreases in the viable counts of 7.60 log CFU/mL. The results of the present study are similar to those of studies by Charteris et al. (1998) and Musikasang et al. (2009).

Probiotics can protect organisms via various mechanisms, including bacteriostasis, which plays the most important role in the determination of the dominant bacterial communities within intestinal ecological systems (Tulumoglu et al., 2013). In this study, 20 *Lactobacillus* strains showed different levels of inhibition against *M. luteus*, *B. subtilis*, *S. aureus*, *L. monocytogenes*, *E. coli*, *S. enterica*, and *P. aeruginosa*. The inhibition of *Lactobacillus* against these pathogenic bacteria had been reported in previous studies (Ammor et al., 2006; Tulumoglu et al., 2013; Asurmendi et al., 2015). Both Gram-positive and -negative bacteria were tested in the present study. Aymerich et al. (2000) reported that Gram-positive bacteria are more sensitive to *Lactobacillus*. Generally, although the 20 tested strains in this study could inhibit both Gram-positive and -negative bacteria, showing a wide antimicrobial spectrum, they had the poorest inhibiting effect on *E. coli*.

Sensitivity to antibiotics is the most important factor in the safety evaluation of probiotics. Antibiotic resistance is a potential risk of probiotic application, as horizontal transfer of the antibiotic resistance gene has been demonstrated between lactobacilli and *Enterococcus faecalis* both *in vivo* (Ouoba et al., 2008) and *in vitro* (Jacobsen et al., 2007). Whether LAB can transfer tolerance to the pathogenic bacteria inside the intestinal tract is an important issue in the application of LAB. In the present study, 70% of the strains showed sensitivity to chloramphenicol, and LAB are usually sensitive to antibiotics such as chloramphenicol according to Klare et al. (2007). LAB isolated from wine by Rojo-Bezares et al. (2006) were all sensitive to chloramphenicol. In a study by Mathara et al. (2008), all 12 *Lactobacillus* strains isolated from kimchi were sensitive to chloramphenicol. Further, de Almeida Júnior et al. (2015) found that 96% of the studied strains were sensitive to chloramphenicol. Vancomycin was the first glycopeptide antibiotic applied clinically, and all the *Lactobacillus* strains in the present study showed tolerance for vancomycin, which is consistent with the results of Tulumoglu et al. (2013). According to Tulini et al. (2013), *Lactobacillus* has natural resistance against glycopeptide antibiotics such as vancomycin. In the study by de Almeida Júnior et al. (2015), 84% of the strains were sensitive to vancomycin, while Dasen et al. (2003) and Zhang (2011) found that all the *Lactobacillus* isolates tested were sensitive to vancomycin. These results do not support the statement of natural resistance against vancomycin in all *Lactobacillus*. Streptomycin and gentamicin both belong to the aminoglycoside antibiotic class, which strongly inhibits aerobic Gram-negative bacilli. In the present study, all the *Lactobacillus* strains showed resistance to streptomycin, while 75% of the strains were resistant to gentamicin; Tulumoglu et al. (2013) found that 90% of the *Lactobacillus* strains tested were resistant to gentamicin. These studies indicate weak inhibition of *Lactobacillus* by aminoglycoside antibiotics, which is consistent with the aforementioned statements. Only two strains in the present study showed sensitivity to polymyxin B; conversely, Zhang (2011) found that most of their tested strains were sensitive to polymyxin B. More than half of the *Lactobacillus* strains in the present study were sensitive to rifampicin, a rifamycin semisynthetic broad-spectrum antibiotic. This result was consistent with that of Zhang (2011). However, Essid et al. (2009) found that most of the 17 *L. plantarum* strains isolated from a Tunisian traditional salted meat showed resistance to rifampicin, which is not consistent with the results in the present study. In the present study, less than 50% of the strains showed tolerance for ampicillin, which is consistent with the results of Zhang (2011). However, Tulumoglu et al. (2013) found that all the studied strains were sensitive to ampicillin; further, Essid et al. (2009) observed that most strains showed tolerance for ampicillin, which is not consistent with the results in the present study. Additionally, most of the *Lactobacillus* strains in the present study were sensitive to erythrocin; likewise, all the *Lactobacillus* strains tested by Tulumoglu et al. (2013) and Mathara et al. (2008) showed sensitivity to erythrocin. The antibiotic CPFX belongs to the fluoroquinolone class of antibiotics, and some studies have reported that *Lactobacillus* has natural resistance against quinolones. In the present study, most of the strains (95%)

had tolerance for CPFX, compared to only 28% of the strains in the study by de Almeida Júnior et al. (2015). Penicillin antibiotics have been widely applied in clinical practice over a long period of time; therefore, tolerance for it is a widespread problem. In this study, only a small percentage (15%) of the *Lactobacillus* strains were sensitive to penicillin, while all the strains studied by Tulumoglu et al. (2013), Mathara et al. (2008), and Zhang (2011) showed sensitivity to penicillin, which is inconsistent with the results from the present study. Tetracycline effectively inhibits both Gram-negative and -positive bacteria. In the present study, 70% of the *Lactobacillus* strains were sensitive to tetracycline. In the studies by Xanthopoulos et al. (2000) and Tulumoglu et al. (2013), all *Lactobacillus* strains isolated from infant feces were sensitive to tetracycline. However, Temmerman et al. (2003) and Essid et al. (2009) both found that most *Lactobacillus* strains show tolerance for tetracycline.

Surface properties vary for different *Lactobacillus* species, which can adhere to the intestinal mucosa via specific and nonspecific mechanisms. Cell hydrophobicity is a cell surface property that affects nonspecific adherence, and thus, can be used to evaluate the adherence ability of *Lactobacillus*. *Lactobacillus* isolated from the small intestine of swine was studied by Wadstroum et al. (1987) in terms of their ability to adhere to swine enterocytes; a positive correlation was found between the adherence ability and the surface hydrophobicity of *Lactobacillus*. The same conclusion was drawn by Holzapfel al. (1998). Many studies (Nostro et al., 2004; Solieri et al., 2014) have found that a hydrophobicity of above 70% is considered to be highly hydrophobic. In this study, a total of seven strains (35%) had hydrophobicities above 70%, indicating that some *Lactobacillus* strains isolated from traditional Tibetan Qula cheese had relatively high hydrophobicity. The highest hydrophobicity of the strains in this study was 92%; similarly, the highest hydrophobicity found in the study by Zhang (2011) was 92.15%.

In conclusion, seven *Lactobacillus* strains were selected as appropriate probiotic candidates in this study. Due to their probiotic properties tested, these strains might help to promote health of hosts, protect hosts from intestinal pathogens and maintain the natural balance of intestinal microflora during antibiotic treatments. However, additional studies are required to verify *in vivo* the effectiveness of selected strains.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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