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Lactobacillus Species from Iranian Jug Cheese: Identification and Selection of Probiotic Based on Safety and Functional Properties

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Abstract

Background and Objective: Traditional fermented products are appropriate sources for the isolation of indigenous bacteria with probiotic characteristics and potential similar or better than commercial probiotics. In this study, *Lactobacillus* species were isolated from jug cheese, a type of Iranian traditional cheese, and their potential probiotic characteristics were studied.

Material and Methods: Study of the probiotic species included hemolytic activity, antibiotic susceptibility, inhibitory activity against pathogenic bacteria, low pH and bile salts tolerance, viability in gastrointestinal tract conditions and adhesion ability to HT-29 cells.

Results and Conclusion: Results showed that the isolates included no hemolytic activity and were susceptible or intermediate susceptibility to most antibiotics. Of four isolates, *Lactobacillus plantarum* KMJC4 showed the strongest antibacterial activity (MIC = 6.25 mg ml^{-1}) against *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus* and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium*. All the isolates, except *Lactobacillus curvatus* KMJC3, preserved their viability after transition through the simulated gastrointestinal tract conditions above 10^6 CFU ml^{-1}. *Lactobacillus acidipiscis* KMJC2 and *Lactobacillus plantarum* KMJC4 showed the lowest and the highest adhesion rates to HT-29 cells with 3.55 and 6.80 Log_{10} CFU ml^{-1} (42.51 and 71.35%), respectively. *Lactobacillus plantarum* KMJC4 included a better bacterial inhibitory activity and adhesion to HT-29 cells than that *Lactobacillus rhamnosus* GG did as control. *Lactobacillus brevis* KMJC1 demonstrated appropriate probiotic characteristics such as antibacterial activity, viability in low pH, bile salts and gastrointestinal tract conditions and adhesion capability to HT-29 cells. In conclusion, *Lactobacillus plantarum* KMJC4 and *Lactobacillus brevis* KMJC1 were introduced as probiotic capable strains. Based on the results from the current *in vitro* study, finding probiotics with similar or better characteristics than commercial probiotics within indigenous bacteria is quite possible. *In vivo* assessment of the bacteria can be considered in future studies, investigating using possibilities of these bacteria in food industries to produce functional fermented foods and in pharmaceutical industries in form of probiotic capsules.

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1. Introduction

Probiotics are living microorganisms that include positive effects on human health when consumed in sufficient quantities. To report a microorganism as probiotic, it should include safe characteristics including nonpathogenicity and antibiotic susceptibility [1]. Functional characteristics, including survival during gastrointestinal tract (GIT) passage and adhesion ability to epithelial surfaces, are *in vitro* assays for the screening of potential probiotic strains [2]. Furthermore, antimicrobial activity of probiotics for the prevention of colonization and infection of GIT pathogens is an essential criterion for the selection of novel probiotics [3]. Members of lactic acid bacteria (LAB) such as *Lactobacillus* strains belong to probiotics [4]. Finding novel bacterial strains from foods with potential

probiotic characteristics is one of the major targets in food microbiology. Scientific societies have recently shown great interests in fermented food products as good sources for the isolation of novel probiotic strains. It has been reported that the origin of probiotic bacteria may affect their probiotic characteristics [5]. Therefore, finding new strains of LAB can be useful. Since various strains of LAB include unique characteristics; thus, successful production of a functional fermented food depends on specific strains of LAB [6]. Considering that most potentially probiotic microorganisms are involved in fermentation of various types of foods, it is expected that traditional fermented dairy products are appropriate sources to find novel *Lactobacillus* strains with potential probiotic characteristics [7].

Jug cheese is a traditional cheese. It is often made from cow milk or a mixture of sheep and cow milks in Western Azerbaijan and Kurdistan Provinces, Iran. After coagulation and draining the whey, it is crushed by hands and pressed in clay jugs. Jugs are stored in ground holes for several months, usually 3-6 months, as ripening period [8]. Long ripening period allows propagation of several bacteria. In ripened cheeses, bacteria with a higher growth rate and more viability are predominate. Nowadays, there is a limited knowledge about the probiotic characteristics of LAB isolated from jug cheeses. Therefore, investigation of LAB with probiotic potential in jug cheeses seems important. The aim of this study was to identify *Lactobacillus* species isolated from jug cheeses to investigate the bacterial probiotic characteristics and select the best isolate(s) for the production of functional fermented foods.

2. Materials and Methods

2.1. Isolation and initial identification of predominant *Lactobacillus* species

Specifications of cheese samples are shown in Table 1. Briefly, 10 g of cheese samples were weighed aseptically in sterile stomacher bags and mixed with 90 ml of sterile sodium citrate solution [2% (w v^{-1}] at 45 °C. Samples were homogenized using Laboratory Blender 400 Stomacher (Seward Laboratory, London, UK) for 5 min. Then, 100 µl of the homogenized samples were spread on de Man, Rogosa and Sharpe (MRS) agar plates (Merck, Germany). Plates were incubated at 37 °C for 24 h under anaerobic conditions using Gas Pak (Anaerocult A, Merck) [9]. Then, colonies were studied based on their morphology and catalase and Gram staining features.

2.2. Molecular identification

Amplification of 16S rRNA gene (1500 bp) was carried out using a pair of universal primers of 27F: 5´-AGAGTTTGATCCTGGCTCAG-3´ and 1492R: 5´-GGTTACCTTGTTACGACTT-3´. The PCR reaction was prepared in a final volume of 30 µl, including 15 µl of the PCR master mix (Macrogen, South Korea), 0.45 µl of each primer with concentration of 10 pM, 11.1 µl of deionized water and 3 µl of DNA template at concentration of 100 ng μl^{-1}, and thermally processed using N15128 Thermal Cycler (Corbett Research, Australia). The PCR thermal cycling included initial denaturation at 95 °C for 5 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min [7]. Then, PCR products were electro-phoresed on 1.5% agarose gel at a constant voltage of 90 V for 50 min. Sequencing of the PCR products was carried out by Macrogen, South Korea. Results were analyzed using BLAST online tool and compared to deposited sequences in GenBank (NCBI) to characterize the isolated bacteria.

2.3. Safety assessments

2.3.1. Hemolytic activity

Hemolytic activity of the isolates was assessed for signs of β-hemolysis (complete hydrolysis of blood cells and clear zones around the colonies), α-hemolysis (partial hydrolysis of blood cells and greenish zones around the colonies) or γ-hemolysis (no zones around the colonies) using sheep blood agar plates (Merck, Germany) [10].

2.3.2. Susceptibility of the isolates to antibiotics

Antibiotic susceptibility of the isolates was assessed against nine antibiotics of penicillin, ampicillin, tetracycline, chloramphenicol, erythromycin, gentamycin, streptomycin, vancomycin and kanamycin using disc diffusion method (Padtan Teb, Iran) [11]. All isolates were cultured anaerobically in MRS broth for 24 h at 37 °C. Then, 50 µl of each culture were spread on MRS agar and antibiotic discs were transferred onto the agar surface using sterilized forceps. After 24 h of incubation, diameter of the inhibition zone (in mm) around the discs was recorded and interpreted as susceptible, intermediate susceptibility and resistant based on the guidelines from Clinical and Laboratory Standards Institute (CLSI) [12] and Charteris et al. [13].

2.4. Preparation of the bacterial supernatants

Suspensions of the bacterial cultures at the end of the log phase were centrifuged at 2147 g for 15 min at 4 °C and cell-free supernatants (pH 4.5) were collected. By adding 5 N NaOH and adjustment to pH 7.0, antimicrobial effects of the organic acids were removed and the neutralized cellfree supernatants were achieved. Then, cell-free supernatants and neutralized cell-free supernatants were sterilized using 0.22-µm filters and freeze-dried. Freeze-dried samples were stored at -20 °C until use [14].

Table 1. Sequencing results of the PCR products for the characterization of *Lactobacillus* spp. isolated from jug cheeses*

Identified species	Isolates code	Milk source	Ripening period (months)	Sample collection location
Lactobacillus brevis	KMJC1	cow's and sheep's milk, 70:30	6	Boukan, Iran
Lactobacillus acidipiscis	KMJC2	cow's and sheep's milk, 70:30	6	Boukan, Iran
Lactobacillus curvatus	KMJC3	cow's and sheep's milk, 70:30	3	Boukan, Iran
Lactobacillus plantarum	KMJC4	cow's milk, contains some vegetables	3	Salmas, Iran

* An Iranian traditional cheese

2.5. Functional assessments

2.5.1. Antibacterial assay

Minimum inhibitory concentration (MIC) of the cell-free supernatants was assessed against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19115), *Bacillus cereus* (ATCC 11778) and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* (ATCC 14028) [15]. Freeze-dried cell-free supernatants were diluted in 2-folds in Muller-Hinton broth (MHB) (BioLab, Hungary) ranging 1.56-50 mg ml^{-1}. Then, 180 µl of diluted cell-free supernatant and 20 µl of each bacterial strain in a final concentration of 10^5 CFU ml^{-1} were added to all wells in the plate. The lowest concentration of cell-free supernatants that showed no macroscopical bacterial growth was reported as MIC. To assess minimum bactericidal concentration (MBC), 10 µl from MIC assay were spotted on MHA plates and incubated at 37 °C for 24 h. Observation of no growth on MHA was recorded as MBC. A commercial probiotic strain, *Lactobacillus* (*L.*). *rhamnosus* GG (ATCC 53103), was used as positive control.

2.5.2. Viability of the isolates in acid, bile salts and simulated gastrointestinal tract conditions

2.5.2.1. Viability of the isolates at low pH and in bile salts

Briefly, 24-h bacterial cultures were inoculated in MRS broth adjusted to pH 3.0 by 4 N HCl and MRS broth containing 0.3% (w v^{-1}) of bile salts (Merck, Germany). Suspensions were incubated at 37 °C for 2 h under acidic conditions and for 2 and 3 h in bile salts. After preparation of the bacterial serial dilutions in sterile 0.85% saline solution, 1 ml of each dilution was mixed with MRS agar and pour plated. Colonies were counted after 48 h of incubation of plates at 37 °C [7]. A commercial probiotic strain, *L. rhamnosus* GG, was used as positive control.

2.5.2.2. Viability of the isolates under simulated gastrointestinal tract (GIT) conditions

Viability of the isolates under simulated GIT conditions was assessed based on a protocol from Mahmoudi et al. [7]. Liquid culture of each bacterium at stationary phase (10^8-10^9 CFU ml^{-1}) was inoculated into MRS broth containing a filter-sterilized solution of pepsin (3 mg ml^{-1}) (Sigma, USA) adjusted to pH 3.0 by adding 4 N HCl. Samples were incubated at 37 °C for 2 h (simulated gastric juice conditions). Then, samples were adjusted to pH 6.5 by adding a filter-sterilized solution of 4 N NaOH. Furthermore, a filter-sterilized solution of 0.3% w v^{-1} bile salts and 0.1% w v^{-1} pancreatic solution (Sigma, USA) were added to samples. The pancreatic solution was prepared in 0.1 M sodium bicarbonate solution. Then, samples were incubated at 37 °C for 2 and 3 h (quick and slow intestinal digestion simulations). Sampling was carried out before and after introducing simulated gastric and intestinal conditions. Bacterial viability was assessed using pour plate method on MRS agar. Commercial probiotic strain of *L. rhamnosus* GG was used as positive control.

2.5.3. Adhesion assay

Human colon cancer cell line of HT-29 (Pasteur Institute of Iran, Tehran, Iran) was used to assess adhesion ability of the isolates. Briefly, HT-29 cells were cultured in RPMI-1640 media (Gibco, USA) supplemented with 10% FBS and 1% antibiotic (penicillin/streptomycin) (Gibco, USA) and incubated at 37 °C under 5% CO_2 and humidified atmosphere. First, HT-29 cells were seeded in 24-well plates at 2 × 10^5 cell $well^{-1}$ until a confluent monolayer was achieved. After removing the media, monolayers were washed twice with PBS to remove antibiotics. Then, 1 ml of the bacterial suspension (10^8-10^9 CFU ml^{-1}) was added to the monolayers. After 4 h of incubation at 37 °C, wells were washed twice with PBS to remove non-adhered bacterial cells Monolayers and adhered bacteria were then detached by adding 500 µl of 0.25% EDTA-trypsin solution (Gibco, USA) to each well. Adhered bacteria were counted on MRS agar and bacterial adhesion was expressed as the percentage of adhered bacteria to the total number of bacteria added [4,10]. Commercial probiotic strain of *L. rhamnosus* GG was used as positive control.

2.6. Statistical analysis

All assays were carried out in a randomized complete design and data was analysed using SAS software v.9.0. Duncan test was used to compare significant differences between the mean values at 5% level. Results were presented as the mean ±SD (standard deviation) of three replicates.

3. Results and Discussion

3.1. Isolation and molecular identification of *Lactobacillus* species from jug cheeses

Of 52 Gram-positive catalase-negative isolates from jug cheeses, 11 predominant rod-shaped bacteria were selected for molecular identification based on their microscopic characteristics. Of the 11 predominant *Lactobacillus* spp., four *L. brevis* KMJC1, *L. acidipiscis* KMJC2, *L. curvatus* KMJC3 and *L. plantarum* KMJC4 with the highest similarities (97-98%) were selected for further studies. Sequencing results and specifications of the collected cheeses are presented in Table 1. Similar to this study, other studies identified *L. curvatus* from Lighvan (Liqvan) (an Iranian traditional cheese made from raw ewe or goat milk) [16] and homemade Azerbaijani cheeses [17], *L. brevis*, *L. plantarum* and *L. acidipiscis* from artisanal Minas cheeses [18] and *L. plantarum* and *L. acidipiscis* from Mexican cheeses [19].

3.2. Safety assessments

3.2.1. Hemolytic activity

Since hemolysis is a common factor of pathogenic microorganisms, one of the major bacterial safety assessments is bacterial hemolytic activity on blood agar. Absence of hemolytic activity is one of the safety prerequisites to select a microbial strain as probiotic [1]. Based on the current results, no hemolysis was seen on sheep blood agar and all isolates were gamma hemolytic. Therefore, these bacteria were non-pathogenic. These results were similar to results from other studies [10,14,20] as described for *L. brevis* and *L. plantarum*. Nevertheless, results from the present study were in contrast to results from other studies on *L. curvatus* [21]. This might be due to the various bacterial strains used in various studies.

3.2.2. Susceptibility of the isolates to antibiotics

Table 2 describes that all isolates were susceptible or intermediate susceptibility to polyketides (tetracycline), β-lactams (penicillin, ampicillin), amphenicols (chloramphenicol) and macrolides (erythromycin). Similarly, studies have reported that *L. plantarum*, *L. brevis* and *L. curvatus* strains are susceptible to erythromycin, tetracycline, chloramphenicol, penicillin and ampicillin [11,22,23]. Antibiotic susceptibility is one of the major indicators for the selection of probiotic strains. Thus, LAB carrying trans-mitssible antibiotic resistance genes (e.g. plasmids) are not probiotics. Since bacterial species are susceptible to antibiotics, they may acquire antibiotic resistance via horizontal transfer of the resistance genes from other species [24].

Subsequently, pathogens become resistant to antibiotics and drugs include no effects on treatments. In fact, LAB carrying transmissible genes are not safe for use in foods. In this study, all isolates were resistant to glycopeptides (vancomycin) and aminoglycosides (kanamycin, streptomycin and gentamycin), while *L. brevis* KMJC1 and *L. acidipiscis* KMJC2 were susceptible to gentamycin. Most *Lactobacillus* species carry vancomycin-resistant genes on their chromosomes. Such an intrinsic resistance is due to the presence of D-Al-D-Lac instead of D-Ala-D-Ala dipeptide in their peptidoglycan, which is the effective site of the antibiotic [25]. Resistance to aminoglycosides has previously been reported in *Lactobacillus* spp. isolated from various fermented foods such as *L. brevis* [14] and *L. plantarum* from fermented olives [10,23]. Intrinsic resistance to aminoglycoside antibiotics in *Lactobacillus* spp. could be due to the lack of electron transport through cytochromes, which mediate antibiotic uptakes [13] as well as changes in cellular membrane permeability [24].

3.3. Functional assessments

3.3.1. Antimicrobial characteristics

Based on Table 3, all *Lactobacillus* spp. isolated from jug cheeses were able to inhibit growth of pathogenic bacteria. The MIC values of cell-free supernatants (pH 4.5) ranged 6.25-25 mg ml^{-1}, and the MBC values ranged 12.5-50 mg ml^{-1} against the highlighted Gram-negative and Gram-positive pathogenic bacteria.

Table 2. Antibiotic susceptibility schemes of *Lactobacillus* spp.

		Diameter of inhibition zone (mm)			
Antibiotic	Disk content	*L. brevis* KMJC1	*L. acidipiscis* KMJC2	*L. curvatus* KMJC3	*L. plantarum* KMJC4
Tetracycline	30 µg	20±0.00 (S) *	34±0.10 (S)	27±0.00(S)	15±0.00 (I)
Penicillin	10 µg	21±0.00 (S)	35± 0.00 (S)	27±0.00 (S)	19±0.15 (S)
Ampicillin	10 µg	29±0.25 (S)	31±0.06 (S)	20±0.00 (S)	25±0.00 (S)
Chloramphenicol	30 µg	27±0.12 (S)	31± 0.06 (S)	24±0.10 (S)	23±0.15 (S)
Vancomycin	30 µg	0 (R)	0 (R)	0 (R)	0 (R)
Streptomycin	10 µg	0 (R)	0 (R)	0 (R)	0 (R)
Gentamycin	10 µg	14±0.40 (S)	15±0.25 (S)	0 (R)	0 (R)
Kanamycin	30 µg	0 (R)	0 (R)	0 (R)	0 (R)
Erythromycin	15 µg	28±0.28 (S)	30±0.00 (S)	19±0.10 (I)	20±0.20 (I)

*(S) = Susceptible, (I) = Intermediate susceptibility, (R) = Resistant, *L.*: *Lactobacillus*. Tetracycline: R ≤ 14 mm; I: 15-18 mm; S ≥ 19 mm. Penicillin: R ≤ 14 mm; I: -; S ≥ 15 mm. Ampicillin: R ≤ 16 mm; I: -; S ≥ 17 mm. Chloramphenicol: R ≤ 12 mm; I: 13-17 mm; S ≥ 18 mm. Gentamycin: R ≤ 12 mm; I: -; S ≥ 13 mm. Erythromycin: R ≤ 13 mm; I: 14-22 mm; S ≥ 23 mm. Values were expressed as mean ± SD of three separate experiments.

The lowest concen-tration of MIC was seen in *L. plantarum* KMJC4, while the widest MBC spectra were recorded for *L. brevis* KMJC1 and *L. rhamnosus* GG. Of the investigated *Lactobacillus* spp., *L. plantarum* KMJC4 showed the strongest inhibitory activity against all the highlighted microorganisms, com-pared to *L. rhamnosus* GG. These results were similar to results of Nami et al. [4]. They reported that *L. plantarum* DP3 included the strongest inhibitory activity against pathogenic bacteria. Considering that neutralized cell-free supernatants (pH 7.0) included no antimicrobial effects (results not shown), it can be concluded that antimicrobial activity of the cell-free supernatants was pH dependent. Antimicrobial activity of the probiotics could be linked to the production of organic acids, bacteriocins or H_2O_2 [14]. Other studies [10] attributed this antibacterial activity to the organic acid production. Bacterial inhibitory effects of the organic acids are majorly due to undissociated forms of acids. They can penetrate microbial cell membranes, dissociate in alkaline cytosols and liberate hydrogen ion, decreasing intracellular acidity and hence leading to the death of pathogens [26]. In addition to the production of organic acids, studies reported that activity of bacteriocins produced by *Lactobacillus* species was pH dependent. Moreover, they reported that the antimicrobial activity of bacteriocins was stable at acidic values; however, the chemical activity decreased significantly at alkaline pH [27,28].

3.3.2. Viability of the isolates at low pH, in bile salts and under simulated gastrointestinal tract conditions

3.3.2.1. Viability of the isolates at low pH and in bile salts

As shown in Table 4, no significant decreases ($p > 0.05$) were found in the number of bacteria under acidic conditions after 2 h of incubation at pH 3.0, meaning that the bacterial viability was more than 90%. Similar results were reported by other studies [5,10,14,23]. Resistance of lactobacilli to low pH is due to the presence of F0F1-ATPase, which can increase intracellular pH when extracellular pH is low [29]. Major effects of the bile salts on sensitive cells are due to the dissolution of the lipid bilayer structure in bacterial cell membranes, which results in releases of contents inside the cells and cell death [30]. Therefore, bile salts tolerance of the probiotics is an important characteristic for their survival in the intestine. As shown in Table 5, all isolates were able to tolerate bile salts more than 6.0 Log_{10} CFU ml^{-1}. However, a significant decrease ($p < 0.05$) was seen in the bacterial viability after 3 h of incubation, except for *L. brevis* KMJC1 and *L. rhamnosus* GG. Results provided by other researchers [22,23,31,32] demonstrated that *Lactobacillus* strains preserved their viability in presence of 0.3% (w v^{-1}) bile salts.

Table 3. Inhibitory activity of *Lactobacillus* spp. cell-free supernatants against pathogenic bacteria

Pathogenic bacteria	MIC (mg ml^{-1})					MBC (mg ml^{-1})				
	L. brevis KMJC1	*L. acidipiscis* KMJC2	*L. curvatus* KMJC3	*L. plantarum* KMJC4	*L. rhamnosus* GG	*L. brevis* KMJC1	*L. acidipiscis* KMJC2	*L. curvatus* KMJC3	*L. plantarum* KMJC4	*L. rhamnosus* GG
E. coli ATCC 25922	12.5	12.5	12.5	6.25	12.5	25	25	25	12.5	12.5
S. aureus ATCC 25923	25	12.5	12.5	6.25	12.5	50	50	-	-	12.5
L. monocytogenes ATCC 19115	12.5	12.5	12.5	6.25	12.5	25	-	-	-	12.5
B. cereus ATCC 11778	6.25	12.5	12.5	6.25	12.5	25	-	-	-	12.5
S. enterica subsp. *enterica* serovar *Typhimurium* ATCC 14028	12.5	12.5	12.5	6.25	12.5	25	25	25	12.5	12.5

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.
- : without MBC (Growth showed). A commercial probiotic strain, *L. rhamnosus* GG was used as a positive control. Values are presented in triplicates. , *L. = Lactobacillus, E. coli= Escherichia coli, S. aureus= Staphylococcus aureus, B. cereus= Bacillus cereus, S. enterica= Salmonella enterica*

Table 4. Viability of *Lactobacillus* spp. after 2 h of incubation at pH 3.0*

Time (h)	*Lactobacillus* spp.				
	L. brevis KMJC1	*L. acidipiscis* KMJC2	*L. curvatus* KMJC3	*L. plantarum* KMJC4	*L. rhamnosus* GG
0	9.44±0.07 [a]	8.55±0.10 [a]	8.05±0.10 [a]	9.26±0.03 [a]	9.32±0.02 [a]
2	9.42±0.12 [a]	7.88±0.34 [a]	7.92±0.05 [a]	9.26±0.10 [a]	9.22±0.06 [a]

*The viability of each bacterium (Log_{10} CFU ml^{-1}) was measured with itself during time (2 h). A commercial probiotic strain, *Lactobacillus rhamnosus* GG was used as a positive control. Values were expressed as mean ± SD of three separate experiments. Mean values were not statistically significant in Duncan test ($P > 0.05$). *L=Lactobacillus*

3.3.2.2. Viability of the isolates under simulated gastrointestinal tract (GIT) conditions

As shown in Table 6, no significant differences were found in the number of bacteria under simulated gastric juice conditions. Viability of *Lactobacillus* spp. was not affected by simultaneous effects of low pH and pepsin, as previously reported by other researchers [5,14,21,23]. Therefore, bacteria entered the intestinal tract at large numbers, approximately 7.8-9.4 Log_{10} CFU ml^{-1} without tolerating stress. After transition through the gastric juice, probiotics should be able to resist pancreatic enzymes and bile salts secreted into the small intestine. In this study, simultaneous effects of bile salts and pancreatic enzymes were assessed at two time intervals (2 and 3 h). Significant decreases ($p < 0.05$) were seen in bacterial counts before and after exposure to simulated GIT conditions. All *Lactoba-cillus* spp. preserved their viability at 6.0-9.3 Log_{10} CFU ml^{-1} after 2 h of exposure to intestinal conditions. After 3 h, bacteria preserved their viability above the recommended levels (10^6-10^7 CFU ml^{-1}) as probiotics [33]. The only exception was *L. curvatus* KMJC3, which decreased signi-ficantly from 7.98 to 3.54 Log_{10} CFU ml^{-1} ($p < 0.05$). Based on the results from bile salts and intestinal digestion assays, pancreatic enzymes included harmful effects on the viability of *L. curvatus* KMJC3. In slow intestinal digestion simulation (3 h of exposure), the transit time increased exposure of *L. curvatus* KMJC3 to intestinal components. Furthermore, synergistic effects of the bile salts and pancreatic enzymes resulted in a significant decrease of approximately 4.5 Log_{10} CFU ml^{-1}. Probiotics must be resistant against action of pancreatic enzymes that may affect their viability/activity through effects on cell wall or cell membrane components [31]. A minimum viability of 6.0-7.0 Log_{10} CFU ml^{-1} is essential for bacterial strains to promote their therapeutic effects as probiotics [33]. Therefore, probiotics must be able to tolerate secretions in the GIT, including acids, bile salts, pepsin and pancreatic enzymes, to attach to intestinal epithelial cells and exert their health benefits to the host. Other *Lactobacillus* spp. survived at high levels (approxi-mately 7.6-9.1 Log_{10} CFU ml^{-1}), passing through simulated GIT. Similar to the present study, Angelescu et al. [34] designed a simulated GIT. They reported that *L. plantarum* BR9 and *L. plantarum* CR1 isolated from fermented beverages, water kefir and braga, showed a viability rate of more than 70% under simulated GIT. In another study, Jiang et al. [11] set up a simulated gastric-duodenal-intestinal transit system to further assess viability of *L. plantarum* WLPL04 isolated from human breast milk. They reported that the bacterium preserved its viability under simulated GIT (1.30×10^8 CFU ml^{-1}). Moreover, *L. plantarum* strains isolated from fermented olives survived under strain-dependent gastric and intestinal conditions [23].

3.3.3. Adhesion ability of the isolates to HT-29 cells

As shown in Fig. 1, adhesion abilities to HT-29 cells were significantly different ($p < 0.05$) within *Lactobacillus* species. The *L. acidipiscis* KMJC2 demonstrated the lowest adhesion rate to HT-29 cells ($p < 0.05$), approximately 3.5 Log_{10} CFU ml^{-1}. The *L. plantarum* KMJC4 significantly ($p < 0.05$) adhered to HT-29 cells (71.35%), compared to that

L. rhamnosus GG did (62%). The *L. plantarum* KMJC4 and *L. brevis* KMJC1 adhered to HT-29 cells at 6.0-7.0 Log_{10} CFU ml^{-1}; thus, they were able to colonize the intestine. Similarly, other researchers reported that *L. brevis* and *L. plantarum* included a higher adhesion ability than that *L. rhamnosus* GG did as a probiotic reference [6,10,22].

Table 5. Viability of *Lactobacillus* spp. in presence of 0.3% (w v^{-1}) bile salts after 3 h of incubation*

Time (h)	*Lactobacillus* spp.				
	L. brevis KMJC1	*L. acidipiscis* KMJC2	*L. curvatus* KMJC3	*L. plantarum* KMJC4	*L. rhamnosus* GG
0	9.3±0.02[a]	8.52±0.17[a]	8.00±0.07[a]	9.22±0.02[a]	9.33±0.02[a]
2	9.26±0.10[a]	8.19±0.18[ab]	6.98±0.18[b]	9.18±0.09[a]	9.32±0.08[a]
3	9.24±0.04[a]	8.10±0.23[b]	6.74±0.07[c]	9.04±0.04[b]	9.24±0.02[a]

* The viability of each bacterium (Log_{10} CFU ml^{-1}) was measured with itself during time (3 h). A commercial probiotic strain, *Lactobacillus rhamnosus* GG was used as a positive control. Values were expressed as mean ± SD of three separate experiments. Mean values with lowercase letters (a-c) show significant differences in Duncan test ($P < 0.05$) and means with the same letters are not significantly different ($P > 0.05$) by Duncan test. *L= Lactobacillus*

Table 6. Viability of *Lactobacillus* spp. during passage through the simulated gastrointestinal tract*

Time (h)	*Lactobacillus* spp.				
	L. brevis KMJC1	*L. acidipiscis* KMJC2	*L. curvatus* KMJC3	*L. plantarum* KMJC4	*L. rhamnosus* GG
0	9.44±0.06[a]	8.53±0.17[a]	7.98±0.12[a]	9.42±0.02[a]	9.42±0.04[a]
2	9.41±0.14[a]	8.72±0.21[a]	7.88±0.10[a]	9.30±0.11[ab]	9.37±0.05[ab]
4	9.18±0.07[b]	7.89±0.40[b]	6.21±0.19[b]	9.26±0.10[b]	9.31±0.10[b]
5	9.11±0.09[b]	7.63±0.31[b]	3.54±0.18[c]	9.10±0.08[c]	9.14±0.02[c]

*The viability of each bacterium (Log_{10} CFU ml^{-1}) was measured with itself during time (5 h). A commercial probiotic strain, *Lactobacillus rhamnosus* GG was used as a positive control. Values are expressed as mean ± SD of three separate experiments. Mean values with different lowercase letters (a-c) are significantly different in Duncan test ($P < 0.05$) and means with the same letters are not significantly different ($P > 0.05$) by Duncan test. *L = Lactobacillus*

Fur-thermore, *L. plantarum* KMJC4 and *L. brevis* KMJC1 showed a higher adhesion ability to HT-29 cells (71.35 and 67.81%, respectively), compared to adhesion ability of *L. plantarum* DP3 and *L. brevis* DP30 to Caco-2 cells (33.2 and 30.4%, respectively) [4]. Differences in ability of bacteria to adhere to colon cells might be associated to the bacterial strain, type of cell line, number of seeded cancer cells in each well, monolayer formation (confluence > 90%), lack of free space in each well of the plate and exposure time of the cancer cells to bacteria during incubation.

Aadhesion ability to epithelial cells and colonization in the intestine are the most important functional characteristics of probiotics because adherent probiotics are able to inhibit colonization of the pathogenic bacteria and promote the host health conditions [2,4,10]. The most commonly known mechanisms of LAB attachment to epithelial cells include mucus binding proteins, lipoteichoic acid, extracellular polysaccharides, flagella, pili and S layer. These not only form binding bridges between the bacteria and intestinal cells, but also play important roles in increased ability of LAB to compete with pathogens to bind and colonize epithelial cells of the intestines [35]. Liu et al. [36] reported that *L. plantarum* strains significantly inhibited invasion and adhesion of enteroinvasive *E. coli* (EIEC) to HT-29 cells. Tuo et al. [37] reported that differences in adhesion characteristics of the *Lactobacillus* strains to HT-29 cells could be linked to differences in the bacterial cell surface proteins. Interestingly, *L. curvatus* KMJC3 was eliminated in adhesion assessment due to its non-resistance characteristic to simulated GIT.

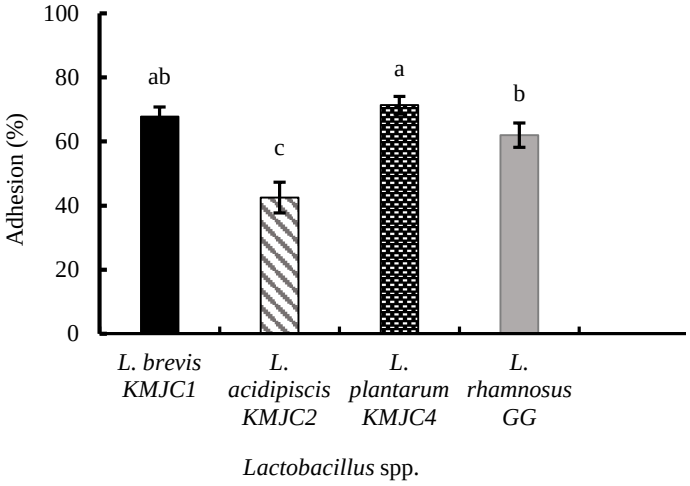

Figure 1. Adhesion ability of *Lactobacillus* spp. isolated from jug cheese to HT-29 cells (2×10^5 cells well^{-1}) was expressed as a percentage of the Log_{10} CFU ml^{-1} ratio of the adherent bacteria to Log_{10} CFU ml^{-1} of the added bacteria, compared to a commercial probiotic strain of *Lactobacillus rhamnosus* GG. Values are expressed as mean ±SD (standard deviation) of three experiments. Columns with no similar letters are statistically different ($p < 0.05$) in Duncan test.

4. Conclusion

Results revealed that all isolates were safe and susceptible or intermediate susceptibility to penicillin, ampicillin, tetracycline, chloramphenicol and erythromycin. Four isolates showed antibacterial characteristics; of which, *L. plantarum* KMJC4 showed the strongest inhibitory activity (MIC = 6.25 mg ml^{-1}) against pathogenic bacteria, compared to *L. rhamnosus* GG as control (MIC = 12.5 mg ml^{-1}). *L. brevis* KMJC1 and *L. plantarum* KMJC4 showed excellent tolerance under simulated GIT conditions (viability of approximately 9.0 Log_{10} CFU ml^{-1}) and significant adhesion ability to HT-29 cells (especially *L. plantarum* KMJC4), compared to *L. rhamnosus* GG. This can be considered as probiotic potential characteristics of the bacteria. Furthermore, *L. curvatus* KMJC3 was not resistant to simulated GIT conditions. The *L. acidipiscis* KMJC2 was not efficiently able to adhere to HT-29 cells. Based on the results, *L. brevis* KMJC1 and *L. plantarum* KMJC4 included potential *in vitro* probiotic characteristics, similar or better than *L. rhamnosus* GG. In conclusion, these two bacteria are introduced as good candidates for the investigation of their potential probiotic characteristics *in vivo* and study their health effects and uses in production of functional fermented foods.

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6. Conflict of Interest

The authors report no conflict of interest.

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گونه‌های *لاکتوباسیلوس* حاصل از پنیر کوزه ایرانی: شناسایی و انتخاب زیست‌یار بر مبنای ویژگی‌های ایمنی و فراسودمندی

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چکیده

سابقه و هدف: فرآورده‌های تخمیری سنتی منابعی مناسب برای جداسازی باکتری‌های بومی با توانایی و ویژگی‌های زیست‌یاری[1] مشابه یا بهتر از زیست‌یارهای تجاری می‌باشند. در این مطالعه گونه‌های *لاکتوباسیلوس* از پنیر کوزه‌ای، نوعی پنیر سنتی ایرانی، جدا شدند، و ویژگی‌های بالقوه زیست‌یاری آنها مورد بررسی قرار گرفت.

مواد و روش ها: مطالعه گونه‌های زیست‌یار شامل فعالیت همولیتیکی، حساسیت به آنتی‌بیوتیک، فعالیت مهارکنندگی در برابر باکتری‌های بیماری‌زا، تحمل نمک‌های صفراوی و pH پایین، زنده‌مانی در شرایط مجرای گوارش و توانایی چسبیدن به سلول‌های HT-29 بود.

یافته‌ها و نتیجه‌گیری: نتایج نشان داد که جدایه های جداشده فعالیت همولیتیکی نداشتند و به اغلب آنتی‌بیوتیک‌ها حساس یا دارای حساسیت متوسط بودند. از چهار جدایه جداشده، *لاکتوباسیلوس پلانتاروم* KMJC4 قوی‌ترین فعالیت ضدباکتریایی (MIC= ۶/۲۵ mg ml^{-1}) را در مقابل *اشرشیاکلی*، *استافیلوکوکوس اورئوس*، *لیستریا مونوسیتوژنز*، *باسیلوس سرئوس* و *سالمونلا انتریکا* زیرگونه *انتریکا* سروار *تیفی موریوم* داشت. تمام گونه‌های جداشده، بجز *لاکتوباسیلوس کورواتوس* KMJC3، زنده‌مانی‌شان را پس از عبور از شرایط شبیه‌سازی شده مجرای گوارش به‌میزان بیش از 10^6 CFU ml^{-1} حفظ کردند. *لاکتوباسیلوس اسیدی‌پیسیس* KMJC2 و *لاکتوباسیلوس پلانتاروم* KMJC4 به‌ترتیب پایین‌ترین و بالاترین میزان چسبیدن به سلول‌های HT-29، ۳/۵۵ Log_{10} CFU ml^{-1} و ۶/۸۰ (۴۲/۵۱ و ۷۱/۳۵ درصد) را نشان دادند. *لاکتوباسیلوس پلانتاروم* KMJC4 فعالیت مهارکنندگی باکتریایی و چسبیدن به سلول‌های HT-29 بهتری نسبت به *لاکتوباسیلوس رامنوسوس* GG، به‌عنوان شاهد داشت. *لاکتوباسیلوس برویس* KMJC1 خواص زیست‌یاری مناسبی مانند فعالیت ضد باکتریایی، زنده‌مانی در pH پایین، نمک‌های صفراوی و شرایط مجرای گوارش و قابلیت چسبیدن به سلول‌های HT-29 را نشان داد. در نتیجه، *لاکتوباسیلوس پلانتاروم* KMJC4 و *لاکتوباسیلوس برویس* KMJC1 به عنوان سویه‌های با قابلیت زیست‌یاری معرفی شدند. براساس نتایج حاصل از مطالعه در شرایط برون تنی[2] یافتن زیست‌یارهایی با خصوصیات مشابه یا بهتر از زیست‌یارهای تجاری درمیان باکتری‌های بومی، کاملا میسر است. ارزیابی درون تنی[3] باکتری‌ها می‌تواند در مطالعات آینده به‌منظور بررسی امکان استفاده از این باکتری‌ها در صنایع غذایی برای تولید غذاهای تخمیر شده فراسودمند و در صنایع دارویی به صورت کپسول‌های زیست‌یار مد نظر قرار گیرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] probiotic
[2] In vitro
[3] in vivo