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Screening, Isolation and Identification of Lactic Acid Bacteria From a Traditional Dairy Product of Sabzevar, Iran

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Background: Lactic acid bacteria (LAB) are a major group of probiotics. Isolation of these bacteria is difficult, because they have a complex ecosystem in fermented dairy products.
Objectives: The aim of this study was to detect *Lactobacillus* and *Lactococcus* in a conventional dairy product (Khameh) and study their probiotic characteristics.
Materials and Methods: To isolateLAB, samples were collected from four different villages. Afterwards, screening was performed in pH = 2.5. The selected strains were examined for their tolerance to acidic pH (3) and 0.3% bile salt. Moreover, the antimicrobial activity of the isolated strains against two pathogenic bacteria, *Salmonella typhimurium* and *Staphylococcus aureus*, was assessed using the disc plate method. Finally, the selected strains were identified by polymerase chain reaction (PCR) screening and sequencing.
Results: Among the isolated samples, two strains (*Lactobacillus* and *Lactococcus*) were highly resistant to unfavorable conditions and the L1 strain showed the highest antimicrobial activity.
Conclusions: This study showed that the conventional dairy product (Khameh) contained probiotic bacteria, which are capable of fighting against pathogenic bacteria and living in the digestive tract.

Keywords:Staphylococcus Aureus; Probiotic; *Salmonella*; *Lactobacillus*

1. Background

Probiotics are a subgroup of microorganisms with positive effects on the host health through improving the gut bacterial balance. These bacteria were first discovered by Mechincof in 1907 (1, 2). Probiotic bacteria should be resistant to gastric acidity and bile salts, so that they can reach to colon and create their desirable effects (1, 3). Lactic acid bacteria (LAB) are the most common types of probiotics. These bacteria have a long-term survival in fermented products (4). *Lactobacillus* is a Gram-positive, non-spore-forming, rarely motile bacteria, while *Lactococcus* is a Gram-positive, spherical and rarely motile bacteria, both of which are present in considerable amounts in dairy products (5, 6). Some beneficial effects of probiotics include enhancing the immune systemfunction, reducing the symptoms of lactose intolerance, and growth in acidic foods like free amino acids as well as in compounds such as Nisinin which they can have antibacterial activities (7-9). LAB make an acidic condition and prevent the growth of pathogens by converting the milk sugar (lactose) into lactic acid (10). Some pathogens such as *Staphylococcus aureus* are important in food hygiene, because they produce toxins and cause food poisoning (11). *Salmonella typhimurium* is another pathogen that causes gastroenteritis. Therefore, controlling these bacteria in food products is important for human health (12). Antibiotics have been used for treating bacterial diseases for a long time. However, the continuous usage of antibiotics has caused innumerous problems including microbial resistance. As a result, scientists are determined to find substitute solutions and probiotics are on the top of their list (13, 14). Since probiotics have chronic effects on health and can improve the digestive tract function, their consumption is advised (15). Although a lot of studies have been performed on traditional dairy products of Sabzevar, Iran, not enough research has been performed to isolate LAB from these products.

2. Objectives

The aim of this study was to isolate and identify LAB strains from a traditional dairy product of Sabzevar and study some functional properties of these bacteria such as acid and bile salt tolerance.

3. Materials and Methods

3.1. Sampling

In the present study, dairy product samples were collected from four villages in Sabzevar (Khorasan Razavi

province, Iran), including Torosk, Bid, Sadkharv and Darein villages. All the samples were collected under sterilized conditions. Then samples were homogenized in 90 mL of peptone. At the enrichment phase, 10 mL of homogenous solution was added to 100 mL Man, Rogosa, Sharpe (MRS) broth and it was then incubated in an anaerobic candelabrum containing CO_2provided by a gas pack for 24 hours in 37°C. Nistatin was added to the mediumto prevent yeast contamination. For enumeration of heterotrophic bacteria, 1 g of Khameh was dissolved in a tube containing 9mLphosphate buffered saline (PBS) buffer. Afterwards, a 10-fold serial dilution was made. After the last three final dilutions, a 100-µL sample was spread on each plate and the plates were incubated at 37°C for 24 hours. The total number of heterotrophic bacteria was calculated using the following equation:

Colony-forming unit (CFU) mL^{-1} = 10 × subtlety coefficient revers × average of the colonies sum in three plates

3.2. Isolation of Bacteria

After 24 hours of enrichment, the tube was centrifuged, the supernatant solution was thrown away and the sediment was incubated in 20 mL of buffer (pH = 2.5) for two hours under anaerobic conditions and CO_2. After incubation, the solution was centrifuged for 30 minutes in 5000 g. Then, the sediment was centrifuged with the abovementioned buffer again. Finally, the remaining 5mL was mixed with the sediment smoothly. The remaining suspension was spread on MRS agar and incubated for 72 hours under 37°C and anaerobic conditions with CO_2.

3.3. Acid and Bile Tolerance Assay

The enriched MRS broth was used to assess pH tolerance. The dilutions (10-5) were prepared in PBS buffer (pH = 7) and cultured using the spread method. Moreover, 1 mL of the enriched broth was incubated in 20 mL of buffer (pH = 3) for two hours under anaerobic conditions and after incubation, the 10-5 dilutions were cultivated. Bile tolerance assay was carried out in MRS broth in two different conditions: without bile salts and with bile salts (0.3 % Oxgall); then, the optical density (OD at 600 nm) in 0, 8, 16, 24 hours was measured.

3.4. Antimicrobial Activities of the Isolated Bacteria Against Pathogenic Bacteria

To measure the antimicrobial activity of the isolated bacteria, S. *typhimurium* ATCC14389 and *S.aureus* ATCC18973 pathogenic strains were prepared in Kerman University of Medical Sciences. Probiotic bacteria were cultured in MRS broth. After that, blank discs were placed in the probiotic bacterial extract for one hour. The discs were stored in 40°C to completely dry. The antimicrobial susceptibility of the pathogenic bacteria to probiotic bacteria was examined as recommended by the Bauer-Kirby disc diffusion method. Of 18-hour cultured samples of the abovementioned bacteria, 500 µL was adjusted to 10^8 CFU/mL, poured and uniformly spread on Muller Hinton Agar. Later sterile 6-mm blank paper disks (PadtanTeb Inc. Tehran, Iran) saturated with probiotic bacteria were placed on MHA. Chloramphenicol (2 mg/mL) was used as a positive control; Methyl sulphateoxide was used as a control reagent. Each of the discs was placed on the inoculated plates and the plates were incubated at 37°C for 18 hours. The diameters of the inhibition zones were measured in millimeters.

3.5. Molecular Identification of Probiotic Bacteria

Analysis of 16S rRNA was performed to determine the taxonomic characterizations of the isolated strains. Total DNA extraction of bacterial strains was performed using the cetyltrimethylammonium bromide (CTAB) method. The bacterial 16S rRNA loci were amplified using the forward domain-specific bacterial primer, Bac27_F (-AGAGTTTGATCCTGGCTCAG-) and the universal reverse primer Uni_1492R (-TACGYTACCTTGTTACGACTT-). The amplification reaction was performed in a total volume of 50 µL, consisted of 1x solution Q (Qiagen, Hilden, Germany), 1xQiagen reaction buffer, 1 µM of each forward and reverse primer, 10 µM dNTPs (Gobco, Invitrogen Co, Carlsbad, CA), and 2 U of QiagenTaq polymerase (Qiagen). Amplification for 35 cycles was performed in a thermal cycler (GeneAmp 5700, PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was keptat 95°C for five minutes (one cycle); 94°C for one minute and 72°C for two minutes (35 cycles), followed by 72°C for 10 minutes at the end of the final cycle. The 16S amplified sample was sequenced with a Big Dye terminator V3.1 cycle sequencing kit in an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). A similarity rank from the Ribosomal Database Project (RDP) and FASTA nucleotide database query were used to determine partial 16S rRNA sequences, to estimate the degree of similarity to other 16S rRNA gene sequences. Analysis and phylogenetic affiliates of the sequences were also performed.

4. Results

4.1. The Quantity of Heterotrophic Bacteria in Collected Samples

The quantity of heterotrophic bacteria was determined in collected samples and was considered as microbial load of the traditional dairy product. The results of heterotrophic bacteria count in goat milk samples gathered from different regions of Sabzevar are as follows: 4×10^6CFU/mL for Torosk, 9×10^5CFU/mL for Bid, 1×10^7CFU/mL-Sadkharv, and 1.2×10^7CFU/mL for Darein.

4.2. Isolation and Selection of Probiotic Bacteria

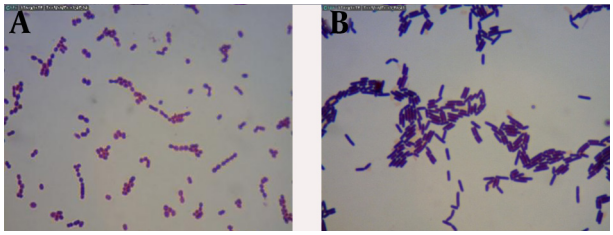
For these isolated strains Gram staining was performed,

revealing that two strains were Gram-positive and three were Gram-negative. The results of catalase test showed that two strains were catalase-negative and three were catalase-positive, which confirmed that they were pathogenic strains. Furthermore, other diagnostic tests were performed on the two strains. The results of biochemical tests are shown in Table 1; also, the microscopic images of the two selected strains are illustrated in Figure 1.

Table 1. The Results of Biochemical Tests

Characteristic Parameters	Gram Staining	Catalase Test	Temperature (15°C)	Temperature (45°C)	pH = 3	Sugar Utilization and Gas production	Arginine Hydrolysis	Growth in 6.5%NaCl
L1	(+)	(-)	(+)	(-)	(+)	(+)/(-)	(-)	(+)
L2	(+)	(-)	(+)	(-)	(-)	(+)/(-)	(+)	(-)
L3	(-)	(+)	-	-	-	-	-	-
L4	(-)	(+)	-	-	-	-	-	-
L5	(-)	(+)	-	-	-	-	-	-

Figure 1. Gram Staining of Selected Bacteria: a- L1 Strain and b- L2 Strain



a,L1strain; b, L2strain

4.3. Acid and Bile Tolerance Assays of Selected Isolated Probiotics

The results of acid tolerance for the two selected strains showed that L2 strain had maximum viability with minimum variance before and after the acid treatment, as its quantitybased on CFU/mL was 4×10^7 before the treatment and 1×10^7 after that. For strain L1, its quantitywas 12×10^7 before the acid treatment and 4×10^7 after that. These two strains had efficient bile slate tolerance rates. The results for bile salt tolerance are shown in Table 2. According to Table 2, L1 strain had the most viability, as its optical absorption increased from 0.947 to 0.337. The results of bile tolerance assayscomparison are illustrated in Figures 2 and 3.

4.4. Antimicrobial Effects of the Isolated Probiotic Bacteria

Figure 4 shows the antimicrobial activities of probiotic bacteria against two human pathogens. As shown in this figure LL1 strain had the most inhibitory effect on the two pathogens, as the zones of inhibition against *S. aureus* and *S. typhimurium* were 9 ± 1.3 mm and 12.7 ± 1.3 mm, respectively. However, the zone of inhibition for L2 strain against *S. aureus*and *S. typhimurium* were 8 ± 1.3 mm and 10.7 ± 1.3 mm, respectively.

Table 2. Absorbance of Probiotic Bacteria in 3% Bile Salts (600nm)

Isolated Strain	Absorbance in Different Incubation Times, h)			
	0	8	16	24
L1 without bile salts	0.255	0.375	0.614	0.947
L1 with bile salts	0.338	0.306	0.323	0.327
L2 without Bile salts	0.622	1.936	2.1	2.265
L2 with Bile salts	1.7	1.288	1.442	1.6

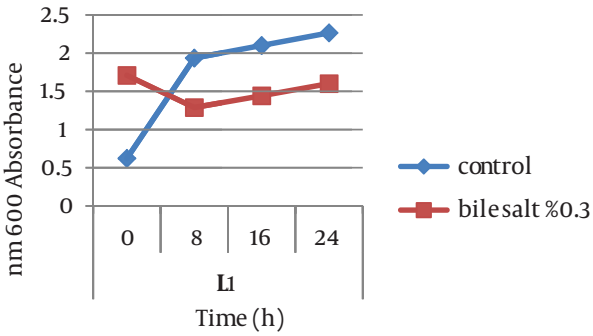


Figure 2. L1 Compared With Samples With 0.3% Bile Salts

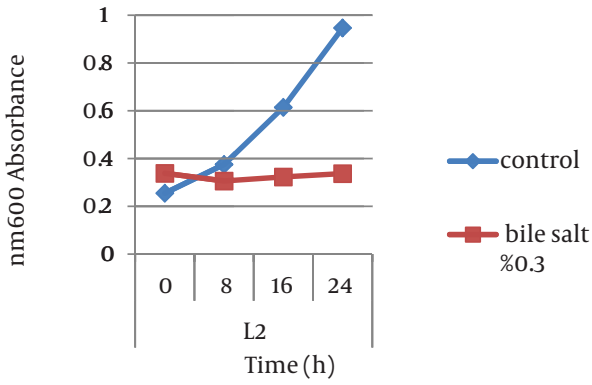
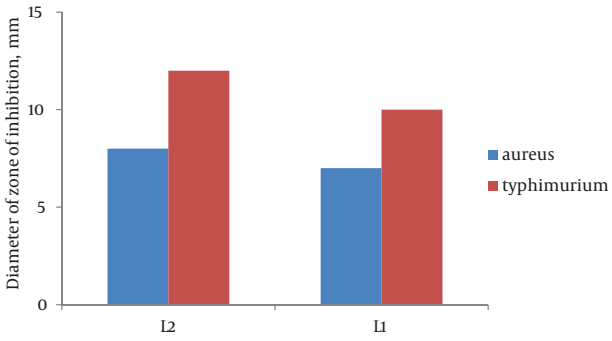


Figure 3. L2 Compared With Samples With 0.3% Bile Salts

Figure 4. Antimicrobial Effects



L1 and L2 against pathogens.

4.5. Molecular Identification

Molecular identification of the isolates was performed by amplifying and sequencing the 16S rRNA gene sequences and comparing the resultsto the database of known 16S rRNA sequences. The results of the identification procedure showed that the two isolated bacteria belonged to *Lactococcus*lactis (L1 strain) and *Lactobacillus plantarum* (L2 strain). The resulted sequences from the isolated strains were imported toMEGA-4 software with a standard strain sequence. Proximity of strains was attained in a phylogenetic tree formed by cluster W approach (Figure 5).

5. Discussion

LAB are the most proposed probiotics because oftheir beneficial effects on human health (16). Consumption of antibiotics for treatment and prevention from harmful bacteria causes not only drug resistance, but also disruption of helpful normal flora in gastrointestinal tract, which makes body susceptible to a variety of intestinal diseases such as diarrhea (17). Preventing the activities of pathogenic microorganisms by probiotics through production of lactic acid and organic acids reduces pH, which can greatly improve human protection against infections caused by common gastrointestinal pathogens (15). Lotfi et al. studied the effects of *L. plantarum* separated from yogurt on pathogens such as *S.aureus* and S. *typhimurium* and reported that the inhibitory zoneon Staphylococcus and *Salmonella* detected by disc diffusion method (through production oflactic acid) was 11.66 mm (16). In addition, Martin et al. isolated the same bacteria from milk. They showed the same inhibitory effects on these pathogens (18). In this research similar studies were performed, but the results hada subtle difference. Thokchom et al. isolated *Lactobacillus* and *Lactococcus* species from soy, which was capable of tolerating a pH = 2 and a bile concentration of 4000 ppm. Exploiting disc method revealed inhibitory effects on *S. parathyphi* MTCC735 and *S.aureus* MTCC740 (19). In the present study, some experiments carried out on this dairy product and some LAB bacteriawere isolated; then, the inhibitory effects of thesebacteria were assayed against specific pathogens. Some limitations of this work were difficulties in collection of the traditional dairy product, providing culture conditions suitable for LAB and difficulty performing desired screening tests. Lavanya, and Abdi et al. reported isolating *Lactococcus lactis* from traditional dairy products, which could only tolerate a bile concentration of0.3% and a pH of 4 (20, 21). The results related to the probiotics isolated fromthe dairy product (Khameh) in the present study are in harmony with the studies just mentioned. The Sabzevar traditional dairy product contained appropriate probiotic tags whichhada high potentialfor inhibiting the growth of pathogenic bacterial strains in human gastrointestinal tract and may have a very useful role in promoting health in consumers. The results of this study showed that the two screened strains in an acidic condition (pH = 2.5) couldalso tolerate some concentrations of bile salt. In this study, L1 strain showed

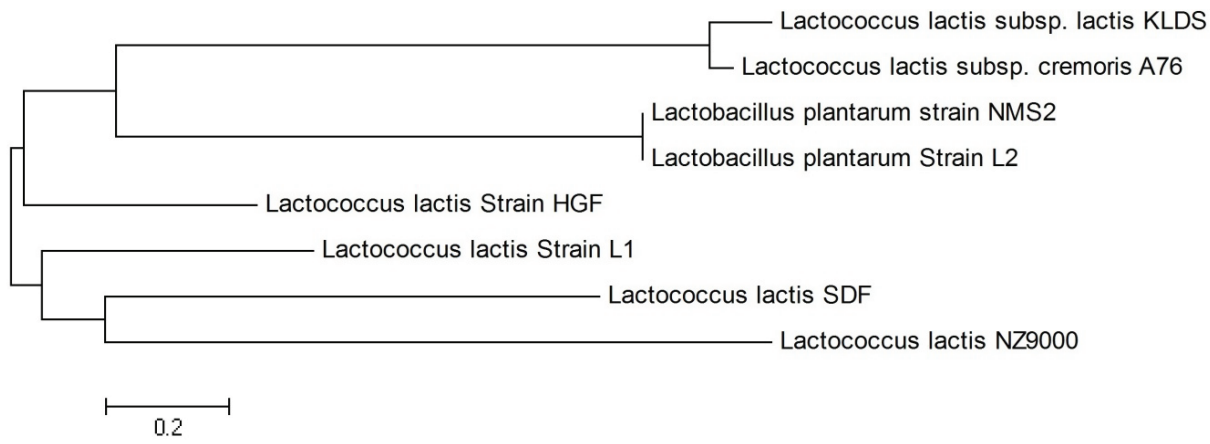


Figure 5. Phylogenetic Tree of the Isolated Strains

the most desirable result compared to other strains. This study emphasized on the significance of probiotics in curing *Salmonella* infections rather than antibiotics and indicated the importance of examining other substances in search of probiotics. Finally, encapsulation of the screened LAB for higher survival rates in invivo condition is suggested.

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Authors' Contributions

All the authors had equal roles in designing and performing the study, statistical analysis, and manuscript writing.

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