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Isolation of Probiotic Lactobacilli from Indigenous Yogurt and Cheese and Their Antagonistic Roles Against Foodborne Pathogens

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Abstract

Background: Probiotic bacteria are one of the useful dietary supplements for human health. The main reason for selecting probiotics is the lack of prolonged side effects.

Objectives: This study aimed to isolate lactobacilli from traditional yogurt and cheese samples collected in Neyshabur city, Khorasan Razavi, Iran, and to characterize them using specific biochemical and molecular assays.

Methods: The probiotic potency of bacteria was tested by resistance to acid, bile, NaCl, and organic acid production. Moreover, the antagonistic effects of the isolates were investigated against enteric pathogenic bacteria using the well diffusion method. Bacteriocin production was also investigated using the microtiter plate assay.

Results: Four *Lactobacillus* spp. with > 99% homology to *L. reuteri*, *L. plantarum*, and *L. acidophilus*, were isolated with probiotic potency. The quantitative measurements used in the study with the statistical analysis resulted in the interpretation of good effects against *Clostridium perfringens*, *Salmonella typhi*, *Staphylococcus aureus*, and *Listeria monocytogenes*. Our isolates exhibited bile salt hydrolase activity, excellent NaCl and acid tolerance (pH = 3), and bacteriocin production.

Conclusions: Our results showed that *Lactobacillus* strains isolated from Neyshabur traditional cheese could be considered good potential probiotic strains and had more antagonistic activity against human pathogens when compared to other samples. Their antibacterial activity was associated with both bacteriocin and organic acids production, but they should be further investigated for their human health benefits.

Keywords: Antagonistic Effects, Enteric Pathogens, Lactobacilli, Probiotic, Bacteriocin

1. Background

Probiotics are defined as alive, nonpathogenic yeasts or bacteria presumed to offer health benefits after consumption (1-3). The term probiotic is used for organisms and substances that affect the host animal by enhancing the intestinal microbial balance (4). At the beginning of the 20th century, probiotics were seemed to beneficially affect intestinal microbial balance by inhibiting microbial pathogens and toxin-producing bacteria (5). Intestinal lactic acid bacteria (LAB) are introduced as probiotics because of a range of health-beneficial properties (6). To date, the genus *Lactobacillus* is composed of more than 80 species. They are present in dairy products, such as cheese, yogurt, and fermented milk (7). These bacterial species have several benefits, such as boosting the immune system, inhibiting the growth of pathogenic microorganisms, reducing blood pressure and cholesterol, improving diarrhea and eczema, preventing cancer, relieving pain in the short term in children, and producing vitamins, especially in the case of vitamin deficiency including vitamin K, folic acid, and vitamin B12 (8-12).

The rise of multi-drug resistant bacteria has attracted the attention of scientists to the prophylactic and therapeutic uses of probiotics, and they have reconsidered them as alternatives to antibiotics (5, 13). Various probiotic bacteria, such as lactobacilli, *Bifidobacteria*, and *Streptococcus* species have been evaluated for the prevention or treatment of various infections and found to be safe (5, 14, 15). Recently, foodborne diseases have become one of the most common public health challenges worldwide (16, 17). As a result, preventing the spread of harmful bacteria along the food chain is critically important (13). The use of beneficial and friendly bacteria, including LAB, as a strategy to prevent or reduce the risks of foodborne diseases can improve food safety and consumers' health (5, 6, 17). Furthermore, some LAB strains may affect *Helicobacter pylori* infections that may lead to peptic ulcers and gastric cancer (18). In this regard, many researchers have studied the isolation of

probiotic bacteria and established a biobank to save them for future applications in the food industry and medicine. The probiotics have been isolated from food, cheese, yogurt, human milk, infant feces, vagina, etc. (13). Among food ingredients, yogurt and cheese are indicated as the main food sources of probiotics. In a study by Masoumikia and Ganbarov, several probiotic species with homology to *L. planetarium*, *L. rhamnosus*, and *L. casei* and high antibacterial effects were isolated (13). Al-Khafaji et al. worked on finding the effect or antagonism of LAB isolated from yogurt on Brucella isolates (19).

Due to the effects of probiotics as a supplement, researchers devoted much attention to this subject in the past few years. The guidelines have considered the safety evaluation of putative probiotic strains. The probiotic isolates must be checked for resistance to gastric acid, bile salt, antimicrobial activity, and isolation from natives' microbiome (13, 20).

From a long time ago, Neyshabur has been involved in animal husbandry and agriculture in Iran. Neyshabur (located in the northeast of Iran) could have potent sources of probiotics due to traditional lifestyles and the developed dairy industry (21).

2. Objectives

This study aimed to isolate LAB from fresh and unpasteurized traditional yogurt and cheese collected from Neyshabur and investigate the probiotic potency and antagonistic property of isolated *Lactobacillus* bacteria on *S. aureus*, *L. monocytogenes, S. typhi*, and *C. perfrigens*, causing infections in humans. Moreover, the isolates were characterized for bacteriocin production. This may lead to the development of cost-effective probiotic food additives that can improve health without posing any environmental health hazards.

3. Methods

3.1. Collection and Preparation of Yogurt and Cheese Samples

In total, four samples of fresh traditional dairy products (yogurt and cheese), were randomly Collected from different traditional markets of Neyshabur (a city in Khorasan Razavi, Iran) and named L_1 to L_4 (Table 1). All the samples were collected and prepared on the same day. The collected samples were transported immediately to the laboratory in a cold storage box at about 4°C to avoid perishing. The cheese and yogurt samples were mixed to suspend the bacterial content. Then, 2 g of each sample was dissolved in 18 mL of normal saline (1:10 w/v), and shaken at 600 rpm for 10 min.

3.2. Isolation of LAB

Fifty milliliters of the sample were centrifuged for at least 5 min at 1000 rpm, and 20 mL of phosphate-buffered saline (PBS) was added to the solid phase, including bacteria, to dissolve the precipitate in PBS. The resultant solution was incubated at 37°C for 2 h and subsequently centrifuged for 15 min at 400 rpm. After separating the cells from the solution, finally, the serially diluted samples were spread on MRS agar (Merck, Germany) and incubated at 37°C for 72 h in an atmosphere containing 10% CO_2 (7).

3.3. Biochemical and Molecular Identification of LAB Isolates

After incubation, the cells were biochemically analyzed. Gram-positive rods that were catalase- negative and non-motile were purified and stored at 4°C. To confirm the results of biochemical characterization, all isolates were analyzed for 16S rDNA. A Bioneer genomic extraction kit was used for the extraction of total DNA from the isolated strains according to the manufacturer's protocol. Amplification was performed using universal primers (F-5' AGAGTTTGATCCTGGCTCAG 3', R-5' AAGGTTACCTCACCGACTTC 3'), as previously reported (7, 17). The thermal cycler was programmed under the following conditions: 10 min at 94°C, 30 cycles of reaction (94°C for 1 min, 57°C for 1 min, 72°C for 1 min), followed by 5 min at 72°C. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel. Then, the purified products were sequenced (GATC Biotech, Germany), and submitted to the NCBI GenBank database (7, 17).

3.4. Probiotic Property

The probiotic characterization of isolated *Lactobacillus* was determined based on tolerance to pH, NaCl, bile salt concentration, and the level of producing organic acids (22-25).

3.4.1. Acid Tolerance Test

Each LAB strain was transferred (1%, v/v) into PBS, adjusted to pH 3 with 1 N HCl for an acidic environment and pH 7.2 as a control, and incubated in the anaerobic condition at 37°C (3 h). Then, 10-fold serial dilutions of each strain were provided with sterile PBS. From a 10^{-5} dilution of each sample, 100 μL was cultured on MRS agar plates and then incubated at 37°C for 24 h. After that, colonies on the plates were counted. The acid tolerance of isolates was identified using cell viability in acidic and control conditions by counting (26-28).

Table 1. Tolerance of Isolated Strains to Low pH[a]

Isolate	Sample	pH Value										
		8	7.5	7	6.5	6	5.5	5	4.5	4	3.5	3
L_1	Traditional Cheese	+	+	+	+	++	++	±	±	±	±	±
L_2	Kurdish Cheese	+	+	+	+	++	++	±	±	±	±	±
L_3	Traditional Yogurt	+	+	+	+	++	++	±	±	±	±	±
L_4	Traditional Yogurt	+	+	+	+	++	++	+	±	±	±	±

[a]Legend: +++ and ++ good growth, + visible growth, - no growth.

3.4.2. Bile Salt Tolerance Test

In a similar manner, an overnight culture of each LAB strain was inoculated (1%, v/v) into 10 mL of MRS broth supplemented with 0.3% (w/v) oxgall, and incubation was done at 37°C for 4 h. Then, 10-fold serial dilutions were provided with PBS. From a 10^{-5} dilution of each sample, 100 μL was cultured on MRS agar plates, and then incubation was performed at 37°C for 24 to 48 h. Then, bacterial cell counting was done. An MRS broth without oxgall was used as a control. Bile resistance was assessed by viable cell counting in control plates and MRS agar supplemented with oxgall (bile) (26-28).

3.4.3. NaCl Tolerance Test

For determining NaCl tolerance of the isolates, 10 tubes containing MRS broth with different concentrations of NaCl (1 - 10%) were prepared. Then, tubes were inoculated with 1% (v/v) of a fresh culture of lactobacilli and incubated at 37°C. After 24 h, the medium turbidity was (26-28)determined (26-28).

3.4.4. Quantification of Organic Acid Production

Sterilized skim milk (10% v/v) was inoculated with 1% of *Lactobacillus* active culture (29). The initial medium pH was adjusted to 6.6. The inoculated media were incubated at 37°C for 72 h, and sampling was done at 24, 48, and 72 h. Coagulated milk was separated from the liquid portion by filtration. The pH of the liquid fraction was recorded, and organic acid quantitative analysis was done by the titration method (0.1 N NaOH).

3.5. Screening of Antibacterial Activity and Bacteriocin Production of Isolates

In this study, the antibacterial activity of isolated Lactobacillus was investigated against four pathogenic bacteria, including *S. aureus* PTCC1112, *L. monocytogenes* PTCC1298, *S. typhi* PTCC1609, and C. *perfrigens* PTCC1765 using the well diffusion method. To evaluate the ability of isolated lactobacilli to antagonize the given strains, initially, the isolates were inoculated with MRS broth under an anaerobic atmosphere at 37°C for 24 h. Then, a homogenous suspension of target strains was prepared and cultured on Muller Hinton agar plates. After creating regular wells on agar, 100 μL of lactobacilli solution was added to each well and incubated at 37°C for 24 h. The antagonistic activity was evaluated by measuring the observed diameter of inhibition zones.

Bacteriocin activity was quantified using the 96-well microtiter plate method (30). For the screening of bacteriocin production, overnight cultures of isolated lactobacilli were centrifuged (4000 rpm for 5 min at 4°C) and the supernatants were filtered with 0.2 μm cellulose acetate filters to remove residual cells. The pH of supernatants was neutralized to 6.5 with NaOH (1 N). Then, 0.1 mL of an overnight culture of the target strain was inoculated into 10 mL of nutrient broth. Each well of microtiter plate was filled with 200 μL of MRS broth, 50 μL of cell-free supernatant (two-fold serially diluted), and 100 mL of the bacterial suspension of the pathogenic strain. After 12 h of incubation, the inhibitory effect was determined by the determination of bacterial growth using an ELISA reader (Bio-Rad, Germany). Culture without bacteriocin was used as a control. All the experiments were performed in triplicate.

3.6. Statistical Analysis

Each experiment was performed in triplicate. Statistical analysis was performed using SPSS (Version 18) software with one-way analysis of variance (ANOVA). The significance level was set at $P \leq 0.05$.

4. Results

4.1. Isolation of Probiotic Strains

In this study, four bacterial strains were obtained from samples including traditional yogurt and cheese. According to Gram-staining and biochemical tests, they were Gram-positive, non-motile, rod-shaped, and catalase and oxidase-negative, thus being specified as *Lactobacillus*.

4.2. Identification of Lactobacilli by 16s rDNA Pattern

The basic local alignment search tool (BLAST) was used for alignment of the sequencing results with deposited sequence data in the NCBI GenBank. The results showed 100% similarity of the isolates with *L. reuteri*, *L. plantarum*, and *L. acidophilus*.

4.3. Probiotic Characterization

4.3.1. Acid-Resistant Isolates

The results of specific biochemical tests (tolerance to acid, NaCl concentration, bile salt, and organic acid production) are shown in Tables 1 and 4, respectively.

After transferring the strains into PBS (1%, v/v) and adjusting to pH = 3 for an acidic environment, the screening and selection of four lactobacilli species, according to the acidic conditions, were performed (Table 1). Three out of four isolates were tested that showed poor tolerance to acidic conditions. Three isolates demonstrated good tolerance, and three isolates demonstrated very good tolerance. All the isolates showed good growth at pH 5.5 and 6.

4.3.2. Bile Tolerance Test

Concerning tolerance to oxgall (0.05 to 0.5 w/v) as bile salt, the results showed that all lactobacilli isolates showed good resistance to bile salts, by surviving under exposure to 0.3% bile salts in the MRS broth culture (Table 2). The L_4 sample exhibited better bile salt tolerance compared to others.

4.3.3. Tolerance to NaCl

Identified lactobacilli (isolates L_1 to L_4) from yogurt and cheese could tolerate 1 - 9% NaCl (Table 3). The isolates showed good growth under a 1% concentration of NaCl, and isolates L_1 and L_4 demonstrated better tolerance to NaCl than the others.

4.3.4. Organic Acid Production

In the titration assay, identified lactobacilli coagulated skim milk, indicating organic acids production (Table 4). The levels of organic acids produced varied among the isolates, ranging from 2.5% to 4% ($P < 0.05$). The highest acidity (4%) with a pH equal to 3.6 was related to the L_1 isolate, and the lowest amount of acidity (2.5%) with a pH of 4.2 corresponded to the L_2 sample.

4.4. Antibacterial Activity of Isolates

Antimicrobial effects of substances produced by four isolated lactobacilli were determined against *S. aureus*, *L. monocytogenes*, *S. typhi*, and *C. perfrigens*. Due to no growth diameter corresponding to four isolated lactobacilli from traditional yogurt and cheese samples, it was demonstrated that the isolated bacteria had an inhibitory effect on the pathogens of interest, and the growth diameter (zero) in these samples was significantly lower than that in controls (Table 5).

To investigate bacteriocin production by the four isolates, the microtiter plate assay was used. All the isolates could produce bacteriocin-like substances and exhibited antibacterial activity.

5. Discussion

In the current study, 4 LAB strains isolated from yogurt and cheese were further investigated for their probiotic characteristics and antimicrobial activity against enteric human pathogens. Based on morphological, biochemical, and molecular characteristics, the isolates were identified as *Lactobacillus* spp. (*L. reuteri*, *L. plantarum*, and *L. acidophilus*). Tolerance to acidic conditions is an important selection criterion to confirm the viability and activity of probiotic strains in the gastrointestinal tract (14, 28). Our results showed that the isolates of *Lactobacillus* spp. could grow in different pH, ranging from 3 to 8.

The pH range was chosen to exhibit the growth of our species in acidic and alkaline conditions and determine the optimal pH range. Previous studies demonstrated optimal acid tolerance for probiotic bacteria that could survive at pH 3.0(26, 31) . The results showed that isolated *Lactobacillus* spp. from yogurt and cheese samples could tolerate extreme acidic (pH 3 to 3.5) and basic (pH 7.5 to 8) environments, and therefore they could be considered to be acid-tolerant LAB strains. Maximum growth was observed at pH 5.5 to 6. The LAB strains may create unfavorable conditions for the survival of pathogens in humans and/or animals by their tolerance capacity correlated to the production of various antimicrobial agents (30, 32). It was reported that the survival of lactobacilli at pH of 2.0 to 3.0 as the stomach environment was variable and strain-dependent (14). Probiotic bacteria have a constant challenge to sustain the high bile-salt environments and their resistance to bile salt is the second most important criterion for persistence and activity in the gastrointestinal (GI) tract (15). Due to this phenomenon, a potential probiotic exhibited tolerance to bile salt at higher concentrations. In the current study, all acid-resistant LAB isolates were employed in the bile salt survival study. All strains successfully tolerated different concentrations of bile salt (Table 2).

As an inhibitory agent, NaCl can influence the growth of many types of microorganisms. In this study, tolerance to 1 - 9% salt concentrations was observed in isolated LAB from yogurt and cheese with an optimal growth rate at 1 to 2% NaCl concentrations. These results showed similarities

Table 2. The Growth Inhibitory of Oxgal (0.3%) as Bile Salt[a]

Isolates	Bile salts (% w/v)				
	0.05	0.1	0.15	0.3	0.5
L_1	+++	+++	++	±	±
L_2	++	++	++	+	+
L_3	+++	+++	++	±	±
L_4	+++	+++	+++	++	±

[a]Legend: +++ and ++ good growth, + visible growth, - no growth.

Table 3. Tolerance to Nacl of Isolated Lactobacilli [a]

Isolates	NaCl (%)									
	10	9	8	7	6	5	4	3	2	1
L_1	-	-	±	+	+	+	+	+	+	++
L_2	-	-	±	±	±	+	+	+	+	+
L_3	-	±	±	±	±	+	+	+	+	+
L_4	-	±	±	±	+	+	+	+	+	++

[a]Legend: +++ and ++ good growth, + visible growth, - no growth

Table 4. Organic Acids (%) and pH in Skim Milk Produced by Isolated Lactobacilli

Isolates	L_1	L_2	L_3	L_4
Organic acids (%)	4	2.5	3.5	3.2
pH	3.6	4.2	4	3.9

Table 5. Antagonistic Effect of Selected Isolates for Human Enteropathogenic Bacteria [a,b]

Isolates	Inhibition Zone (mm)			
	C. perfrigens	*S. typhi*	*L. monocytogenes*	*S. aureus*
L_1	-	-	9.67 ± 0.577	-
L_2	-	12± 1.000	10.33 ± 1.155	9.67 ± 0.577
L_3	-	11 ± 1.000	11.67 ± 0.577	9 ± 1.000
L_4	12 ± 1	13 ± 1.000	8.67 ± 0.577	-

[a]Values are expressed as (mean ± SD).
[b] The significance difference $P \leq 0.05$.

with other findings (18) that isolated lactobacilli from the GI tract of swine were tolerant to 4 - 8% NaCl.

Nowadays, with increasing the diseases and appearance of resistant bacteria to antibiotics, applying useful microorganisms for controlling the diseases is crucial (26, 33, 34). For this purpose, many studies have been performed, and their results indicated that isolated lactobacilli have antagonistic properties against some human pathogens. As a potential probiotic, their antimicrobial effect represents an alternative in the prevention and control of gastrointestinal infections (30). This study was performed for the investigation of antibacterial characteristics of probiotic bacteria isolated from traditional yogurt and cheese samples against Gram-negative pathogens. In the present study, all the isolates were further evaluated for their antibacterial activity against four selected foodborne pathogens, namely *S. aureus*, *L. monocytogenes*, *S. typhi*, and *C. perfrigens*, demonstrating a good antimicrobial activity against these bacteria. In the well diffusion test, the LAB isolates exhibited different inhibitory effects, and the inhibition zone ranged from 1 mm to $\geq$ 13 mm (Table 5). The results showed that most antibacterial activities were against *C. perfringens* and *S. typhi*, and the least effect was against *S. aureus*. Isolated LAB from traditional

samples successfully inhibited the growth of these enteric pathogens and had bacteriostatic effects, which is to some extent close to the results of other dairy products (8 - 12 mm) (33). It is noteworthy that our isolates had more antibacterial effects than isolates from other investigations and the zone of probiotics was bigger compared to others. The greater diameter of the no-growth zone and the higher antimicrobial effect are probably due to the production of more antimicrobial compounds and organic acids.

The ability to prevent the growth of bacterial pathogens could be explained by the production of antimicrobial substances (5, 26, 28). The antimicrobial effect of LAB has been mostly attributed to a variety of metabolites that can be produced, such as organic acids, hydrogen peroxide, ethanol, diacetyl, acetoin, carbon dioxide, and bacteriocins. Among these antibacterial compounds, organic acids, hydrogen peroxide, and bacteriocins are the strongest agents (5, 26, 30). This investigation indicated an increase in incubation time increased organic acid production, which caused a decrease in the pH of the media. The highest acidity (4%) and lowest pH (3, 6) were indicated after a 72h incubation of LAB isolated from yogurt and cheese. Generally, LAB isolated from cheese samples showed better probiotic properties, such as excellent tolerance to acid, bile salt, and NaCl, and had more organic acid production and antibacterial effects, followed by strains from traditional yogurt.

Siroli et al. reported that some of their strains produced bacteriocin, and antibacterial activities of the others probably were associated with organic acids production (35). In this regard, our results showed that the cell-free supernatant of all four isolates could inhibit the growth of pathogens, suggesting that the antibacterial activities of the strains were due to all of the organic acids, bacteriocin, and bacteriocin-like inhibitory substances. In particular, the antibacterial effect of bacteriocin substances was more against *S. aureus* and *S. typhi* than against other examined pathogens. None of the *Lactobacillus* strains isolated by Siroli et al. could produce bacteriocins against *L. monocytogenes*, *S. aureus*, and *E. coli* (35), and their antagonistic activity was due to other mechanisms.

5.1. Conclusions

The present study showed that traditional dairy products from Neyshabur, located in the northeast of Iran, can be used as a good source of probiotics. All isolated acid- and bile-resistant lactobacilli from yogurt and cheese samples could be considered good candidates for probiotics. These probiotic bacteria possessed varying degrees of inhibition towards enteric pathogens. Therefore, there are potential health benefits from eating yogurt and cheese, containing specific probiotic bacteria that confer some health benefits for human hosts. The probiotic LAB were isolated from traditional areas as indigenous microbiome, could be successfully stored in a Biobank for application in pharmaceutical and food industries, especially as a culture starter in the future. However, these isolated LAB should be investigated further, for in-vivo effects besides other potential probiotic bioactivities including anti-cancer, against certain bowel disorders, and anti-allergy properties. Because of the increasing use of probiotics as dietary supplements and therapeutic agents, clinicians need to be aware of the risks and benefits

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Footnotes

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