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Original Article

Molecular identification of probiotic lactic acid bacteria isolated from raw Buffalo milk

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Abstract

Background and Objectives: Lactic acid bacteria (LAB) are a group of probiotic bacteria with health promoting effects on the human gastrointestinal tract. Dairy products, particularly raw milks, are considered a rich source of probiotics. This study aimed to carry out the molecular identification of LAB with probiotic properties isolated from raw buffalo milk in Behbahan city, Iran.

Materials and Methods: Raw milk samples were cultured on MRS agar, and isolates were screened based on phenotypic and biochemical characteristics. Probiotic properties including acid tolerance, simulated gastric juice (pepsin and trypsin), and bile salts (0.3%), as well as physiological characteristics such as hydrophobicity, auto and coaggregation, were examined. Antibiotic susceptibility and antimicrobial activity of the isolates against four pathogenic bacteria were also evaluated. Finally, selected isolates were identified by 16S rRNA gene sequencing.

Results: From 28 isolated strains, 17 exhibited typical LAB characteristics, i.e., Gram positive coccobacilli, catalase and oxidase negative). Four isolates showed the ability to grow under acidic conditions (pH 2.5 and 4), in simulated gastric juice (pepsin and trypsin), and in the presence of bile salts (0.3%). The isolate, D20 was susceptible to ampicillin, vancomycin, and penicillin and displayed the highest autoaggregation (52.27%). The isolate, B7 showed the highest coaggregation with *E. coli* (31.72%) and the highest antibacterial activity against *B. cereus* (inhibition zone diameter: 22 mm). The isolate, A3 exhibited the highest hydrophobicity (39.1%) as well. Sequencing results revealed that three isolates belonged to *Enterococcus faecium* and one isolate belonged to *Enterococcus faecalis*.

Conclusion: According to the results, raw Buffalo milk from Behbahan city is a rich source of probiotic bacteria. This study can provide comprehensive information for the establishment of probiotic culture collections in the country.

Keywords: Probiotic, Lactic acid bacteria, *Enterococcus*, Raw Buffalo milk.

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Introduction

Raw milk is rich in nutrients that can provide ideal nutritional conditions for many microorganisms (1). Raw cow milk is a potential source of lactic acid bacteria (LAB) (2). LAB are candidate probiotic bacteria that are widely distributed in nature and can be used in the food industry (3). In 2001, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as: "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". This definition emphasizes that probiotics can improve host health by affecting the microbial balance, especially in the gastrointestinal (GI) tract (4). A probiotic isolate should possess specific characteristics such as resistance to acidic pH of the stomach, tolerance to bile salts, and the ability to adhere to epithelial cells. It should also exhibit antimicrobial, anticancer, detoxifying effects, and immune system enhancement. Therefore, before using bacteria as probiotics, their adhesion to appropriate surfaces and survival in the GI tract of humans and animals must be confirmed through in vitro evaluation (5).

Lactic acid bacteria are Gram positive, nonsporulating, nonmotile, rod-shaped or cocci, and are capable of tolerating acidic conditions. The main function of this group is the production of lactic acid, which leads to acidification of food. Hence, they are used as starter cultures in the food industry. These bacteria improve the taste, texture and nutritional value of fermented foods. In addition, they have beneficial health effects and can be used as probiotics. They also prevent the growth of pathogenic and spoilage microorganisms, thus acting as a biological preservative for the product (6).

Common genera of lactic acid bacteria include *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Weissella*, *Pediococcus*, and *Leuconostoc* (7).

The genus *Enterococcus* is one of the main genera of lactic acid bacteria, comprising more than 50 species and subspecies. They are found in various habitats such as the GI tract of humans and animals, plants, soil, water, and foods of animal origin, especially cheeses and fermented meats. Although enterococci have beneficial effects for fermented food production, they are also known as opportunistic pathogens that can cause nosocomial infections such as bacteremia, urinary tract infections, and endocarditis. Two enterococcal species, *Enterococcus faecalis* and *Enterococcus faecium*, are the main causative agents of these infections. They exhibit high levels of pathogenicity and transfer of antibiotic resistance genes, particularly vancomycin resistance genes (8). However, the use of *Enterococcus faecium* as a probiotic in the treatment of diarrhea in humans is considered as an alternative to antibiotic therapy. The probiotic effect of *Enterococcus faecium* is the reduction of cholesterol absorption into the bloodstream from the gastrointestinal tract (9).

In a study on probiotic, *enterococci* bacteria have been isolated form goat milk, traditional cheese, and breast milk. Results showed that the isolated bacteria exhibited the ability to tolerate acid and bile salts and showed significant antimicrobial activity against pathogenic bacteria such as *Salmonella typhi* (native), *Pseudomonas aeruginosa* (RTCC 1483), *Klebsiella pneumoniae* (RTCC 1254), *Escherichia coli* (RTCC 1174), and *Staphylococcus aureus* (RTCC 1240) (10). In another study aimed at investigating the antibiotic resistance in *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus durans* isolated from traditional cheeses, it was concluded that *E. faecalis* strains were more resistant than *E. durans* and *E. faecium*. Therefore,

identification of *Enterococcus* sp. and evaluation of antibiotic resistance are important to prevent potential risks to public health caused by contamination of milk and other dairy products (11).

Dairy products produced from local raw milk are an important part of the daily diet. The nature of these products varies depending on the specific native microflora of the region, which reflects the climatic conditions of that area (12). Due to the high climatic and nutritional diversity in Behbahan, a city in the southwest of Iran, it is expected that unique strains of probiotic bacteria present in the raw milk of this region. Identification of these strains can lead to a better understanding of the microbial composition of raw milk in Behbahan. Besides, native identified probiotic strains have a high potential to improve the quality and nutritional value of dairy products produced in this region. The aim of this study was to carry out the molecular identification of lactic acid bacteria with probiotic properties from raw milk in Behbahan city.

Materials and Methods

A) Isolation and identification of lactic acid bacteria: In this study, Five raw Buffalo milk samples (100 mL each) were collected from dairy farms near the Behbahan city in sterile bottles, and placed into a cooling box on dry ice and immediately transformed to lab at 4-6 degree of Celsius. For isolation,0.1 ml of each milk sample was surface cultured on De Man, Rogosa and Sharpe (MRS) agar (Ibresco, Iran). The plates were incubated at 37 °Celsius for 24-48 hours. Selected colonies were sub-cultured by the streak plate method on MRS agar to obtain pure strains (13). Subsequently, the purified isolates were subjected to Gram staining, catalase, oxidase, potassium hydroxide test and

B) Preparation of bacterial suspensions: The target strains were cultured in an appropriate liquid medium, namely MRS broth (Ibresco, Iran), under optimal conditions at 37 °C for 24 hours. The bacterial culture was centrifuged at 5000 rpm for 10 minutes, and the supernatant was discarded. The resulting pellet was washed with phosphate buffered saline (PBS, pH 7), centrifuged again, and the supernatant was discarded. The final suspension was adjusted with PBS to a turbidity equivalent to 0.5 McFarland standard (14).

C) Evaluation of probiotic potential (All tests were performed in triplicates) Acid Tolerance test: To assess the bacterial tolerance to acidic conditions, 100 µL of the bacterial suspension equal to 0.5 MacFarland were inoculated into 10 mL of MRS broth tubes adjusted to pH 2.5 and pH 4. For counting at time zero- and 3-hours intervals with acidic conditions, the inoculated medium was serially diluted with physiological normal saline serum from 10^{-1} to 10^{-8} test tubes, pour-plated with MRS broth media, and incubated at 37 °Celsius for 24-48 hours. Following incubation, in order to count the bacterial survival rates at indicated times, the number of colonies were counted. The same procedure was followed for acid tolerance while the bacterial suspensions were adjacent to acidic conditions for 3 hours. The counted colonies should not be less than 10^6 CFU/mL (14).

D) Resistance to simulated gastric juice: Simulated gastric juice media were prepared according to standard procedures: one containing pepsin (2 g sodium chloride and 2.3 g pepsin dissolved in 1000 mL distilled water, adjusted with concentrated HCl to pH 2-2.3), and another containing trypsin (2 g sodium chloride and 2.5 g trypsin dissolved in 1000 mL distilled water, adjusted with concentrated HCl to pH 2-2.3).

Control media were adjusted with 5 N NaOH to pH 6.5-7. After autoclaving, 2% (v/v) of the bacterial suspension was inoculated into the pepsin-containing, trypsin-containing, and the control media. For counting at time zero, the inoculated media were serially diluted from 10^{-1} up to 10^{-8} tubes, pour plated with MRS Agar media, and incubated at 37 °Celsius for 24-48 hours. For counting after 3 hours, the inoculated media were incubated for 3 hours. After incubation, serial dilution and pour plating were repeated, followed by incubation at 37 ° Celsius for 24-48 hours. The number of surviving colonies was counted and compared with the control group. The number of viable bacteria after treatment with the simulated gastric juices should not be less than 10^6 CFU/mL (14).

E) Bile salt resistance: Freshly prepared bacterial suspension (100 µL) was added to an Nutrient broth medium containing 0.3% bile salt (bile oxalate) as well as to a medium without bile salt (control). The optical density of both media was measured at 600 nm at time zero and after 8 hours of incubation. The growth inhibition coefficient was calculated using the following formula (1,14):

$$C_{inh} = \frac{(T8-T0)\,Control - (T8-T0)\,Treatment}{(T8-T0)\,Control} = (1)$$

where C_{inh} is the inhibition coefficient, T8 control is the absorbance in the medium without bile after 8 h incubation, T0 control is the absorbance in the medium without bile before incubation, T8 treatment is the absorbance in the bile-containing medium after 8 h incubation, and T0 treatment is the absorbance in the bile-containing medium before incubation. According to the Iranian National Standard, the inhibition coefficient should be ≤ 0.4.

F) Cell Surface Hydrophobicity: Bacterial adhesion to xylene was used to evaluate the hydrophobicity of the strains. Overnight cultures of the target bacteria in MRS broth were prepared. The cultures were centrifuged at 5000 rpm for 15 min, and the pellets were washed twice with PBS. 0.5 McFarland suspensions were then prepared in PBS, and their absorbance were read at 600 nm (A0). Then, 2 mL of the bacterial suspensions were mixed with 2 mL of xylene and vortexed for 3 min. The mixtures were left at room temperature for 1 h to allow phase separation. After this period, the absorbance of the aqueous phase were read at 600 nm (A1). The percentage of hydrophobicity were calculated as follows (2,15):

$$\text{Hydrophobicity (\%)} = [1-(A1/A0)] \times 100 = (2)$$

where A0 is the initial absorbance and A1 is the final absorbance.

G) Auto and coaggregation test: Autoaggregation is essential for probiotics to adhere to epithelial cells and mucosal surfaces and consequently to colonize the gastrointestinal tract, allowing them to create a barrier against undesirable bacteria. To perform this test, a 24h culture of the target bacterial isolates in MRS broth at 37 °Celsius equal to 0.5 MacFarland suspensions were prepared. The bacterial cultures were centrifuged at 5000 rpm for 10 min, and the pellet were washed twice with PBS. Five mL bacterial suspensions in PBS were then prepared. The tubes were vortexed for homogenization and incubated at 37 °Celsius for 5 h. The absorbance of the bacterial suspensions were measured at 600 nm at time zero (A0) and after 5 h (A5) (3,16). The autoaggregation percentage were calculated using the following formula:

$$\text{Auto-aggregation (\%)} = 1- (A5/A0) \times 100 = (3)$$

where A0 is the initial absorbance and A5 is the absorbance at 5 h.

To evaluate the coaggregation ability of the

probiotic bacteria with pathogens, two control tubes containing 4 mL of the probiotic isolate suspension and 4 mL of the pathogen suspensions (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, and *Bacillus cereus*) separately were used. In another tube, 2 mL of the probiotic suspensions and 2 mL of the pathogen suspension were mixed. The tubes were incubated at 37 ° Celsius for 5 h, and then the absorbance of the suspension surface were measured at 600 nm. The coaggregation percentage were calculated using the following formula (4,16):

$$\text{Coaggregation \%} = \frac{(Ax+Ay)/(2)-A(x+y}{(Ax+Ay)/2} = (4)$$

where Ax and Ay are the absorbances of the probiotic and pathogen suspensions, respectively, and A(x+y) is the absorbance of the mixed suspension.

H) Antimicrobial activity assay: After isolation and identification of lactic acid bacteria with probiotic potential, the antibacterial activity of the cell-free supernatant (obtained by centrifugation) was evaluated against four pathogenic strains: *Escherichia coli* ATCC 25923, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* ATCC 14028, and *Bacillus cereus* ATCC 11778 (Zist kavosh Co., Iran) using the well diffusion method. First, the selected probiotic strains were incubated in MRS broth at 37 °Celsius for 24 h. A 0.5 McFarland suspension was prepared and 2% (v/v) was inoculated into MRS broth and placed in a shaker incubator. Simultaneously, a 24h culture of the pathogenic bacteria was prepared in Muller Hinton broth media (MHB, Ibresco, Iran) at 37 °Celsius. A suspension with a concentration of 1.5×10^8 CFU/mL (0.5 McFarland) was prepared in sterile physiological saline. 100 µL of this suspension was spread onto Muller Hinton Agar media (MHA ,Ibresco, Iran). Wells were made in each plate using a sterile Pasteur pipette. 100 µL of the probiotic supernatants were added into the wells. The plates were then incubated at 37 °Celsius for 24 h. Sterile distilled water was used as a negative control. The inhibition zone diameters produced by each probiotic strain were measured. Formation of a clear zone around the well indicates antimicrobial activity of the selected microorganisms (17).

I) Antibiotic susceptibility: Antibiotic susceptibility testing method were performed by using Kirby Bauer disk diffusion method. 0.5 MacFarland suspensions of the selected strains were prepared in MRS Broth media as the new bacterial inoculums. Then, the cultures were stroked on Muller Hinton Agar media (MHA, Ibresco, Iran) using sterile swabs and working near a flame. Disks of common antibiotics (amoxicillin: AMX25, ampicillin: AM10, vancomycin: V30, ciprofloxacin: CP5, trimethoprim sulfamethoxazole: SXT, penicillin: P10) (Padtan Teb Co., Iran) were placed on the plates at appropriate distances from the margin of plates. The plates were incubated at 37 ° Celsius for 24-48 h. After the incubation time, the zone inhibition diameters were measured and compared with Clinical and laboratories standards institute (CLIS) 2026 chart (18).

J) Molecular identification of the isolates by 16S rRNA Sequencing: Pure cultures were prepared from the lactic acid bacteria that had been isolated and showed desirable probiotic properties. Genomic DNA was extracted using a DNA extraction kit (SinaClon, Iran) according to the manufacturer's protocol. The 16S rRNA gene was amplified using the universal primer pair F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1492 (5′-TACCTTGTTAGGACTTCACC-3′) in a final volume of 25 µL. The thermal cycler program was as follows: initial denaturation at 94 °C for 3 min; 30 cycles of denaturation at

94 °Celsius for 30 s, annealing at 59 °C for 30 s, extension at 72 °Celsius for 30 s; and final extension at 72 °Celsius for 8 min. Sterile distilled water was used as a no template control (NTC) alongside the samples. The PCR products were electrophoresed on a 1% agarose gel. The gel was then visualized under UV light on a transilluminator, and a positive band (1500 bp) was observed and photographed. Finally, 25 µL of the desired PCR product along with the forward and reverse primers were sent to Genetic Codon Iran Company for sequencing. The obtained 16S rRNA gene sequences were compared with sequences available in the GenBank database using BLAST (19).

Results

A) Biochemical and morphological characteristics: A total of 28 isolates were obtained from five raw Buffalo milk samples. Based on colony morphology (Creamy, Milky, cloudy, soft to semi soft, opaque and shiny colonies), and the results for gram staining and primary biochemical tests, 17 isolates were identified as Gram positive coccobacilli, Catalase negative, Oxidase negative, and KOH negative. These 17 isolates were selected for further probiotic evaluation tests and coded accordingly.

B) Selection of strains with probiotic potential, Acid Tolerance Test: Among the 17 isolates, strains A1, A3, B7, D20, and E28 were able to grow at both pH 2.5 and pH 4 as it has been represented at the Table 1. These isolates were selected as superior strains and subjected to further tests.

C) Resistance to simulated gastric juice: The results for resistance to stimulated gastric juice of the isolated strains has been shown at the Table 2. Among the five acid-resistant strains, superior strains(isolates A1, A3, B7, and D20) were able to grow in both simulated gastric juice media (containing pepsin and trypsin).

Table 1. Acid Tolerance tests for the isolates.

Isolates	Initial colony count (CFU/ml)	Colony count after acid treatment, 3 h incubation at 37 °C (CFU/ml)	
		pH 2.5	pH 4
A1	$10^9\times3$	$10^6\times3$	$10^7\times5$
A2	$10^{11}\times1$	-	$10^6\times5$
A3	$10^8\times6$	$10^6\times4$	$10^7\times5$
A4	$10^9\times3$	-	$10^4\times4$
A5	$10^{10}\times2$	-	$10^7\times2$
A6	$10^{11}\times5$	-	$10^7\times3$
B7	$10^9\times3$	$10^7\times6$	$10^7\times6$
B8	$10^7\times4$	-	$10^6\times8$
B9	$10^{10}\times2$	$10^4\times3$	$10^6\times2$
B10	$10^7\times4$	-	$10^6\times8$
B11	$10^9\times7$	$10^5\times4$	$10^6\times5$
C12	$10^9\times2$	$10^5\times4$	$10^6\times8$
C13	$10^8\times6$	$10^5\times1$	$10^6\times3$
D20	$10^8\times8$	$10^7\times2$	$10^8\times2$
E24	$10^9\times3$	-	$10^5\times5$
E26	$10^7\times2$	-	$10^5\times3$
E28	$10^9\times5$	$10^6\times4$	$10^7\times3$

Table 2. Resistance of the isolates in simulated gastric juice (CFU/ml).

Isolates	Initial colony count (CFU/ml)	Colony count after treatment with pepsin and trypsin 3 (h incubation at 37 °C) (CFU/ml)	
		Pepsin	Trypsin
A1	$10^8\times3$	$10^7\times1$	$10^6\times4$
A3	$10^9\times2$	$10^8\times5$	$10^7\times2$
B7	$10^9\times1$	$10^7\times3$	$10^6\times4$
D20	$10^9\times2$	$10^7\times3$	$10^6\times4$
E28	$10^8\times3$	$10^6\times4$	$10^5\times2$

D) Bile salt resistance: The four superior strains were evaluated for resistance to bile salts (0.3% bile oxalate). Strains A1 and D20 showed good resistance with inhibition coefficients of 0.076 and 0.09, respectively, while strains A3 and B7 showed moderate resistance with inhibition coefficients of 0.394 and 0.352, respectively.

E) Cell surface hydrophobicity: Strain A3 exhibited the highest hydrophobicity (39.1%) and strain D20 exhibited the lowest hydrophobicity (22.5%).

F) Auto and coaggregation: As it has been demonstrated at the Figure 1., Strain D20 showed the highest autoaggregation rate (52.27%) while strain A1 showed the lowest autoaggregation rate (14.28%) among the selected strains.

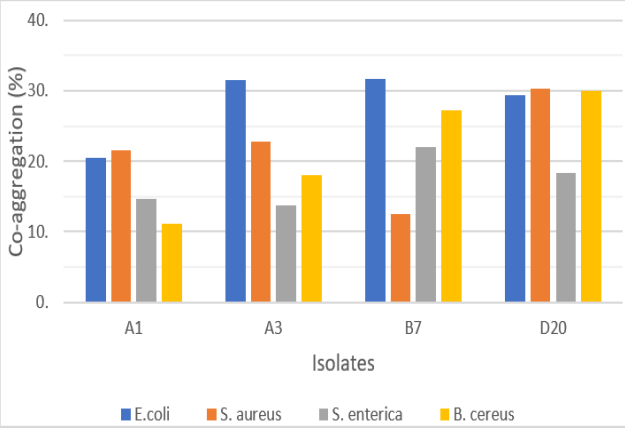

Fig 1. Co-aggregation of the LAB isolates with pathogenic bacteria.

The highest coaggregation rates, referring to the Figure 1., were as follows:

- With *E. coli*: strain B7 (31.72%)
- With *S. aureus*: strain D20 (30.32%)
- With *S. enterica*: strain B7 (21.99%)
- With *B. cereus*: strain D20 (29.94%)

G) Antibacterial activity and antibiotic susceptibility: As it has been indicated at the Table 3, strain B7 exhibited the highest antimicrobial activity against *B. cereus* with an inhibition zone diameter of 22 mm. Only strain B7 showed antimicrobial activity against *S. enterica* (inhibition zone diameter: 8 mm). Against *S. aureus*, strains B7 and A3 showed inhibition zones of 11 mm and 10 mm, respectively. Against *E. coli*, strain D20 showed the highest antimicrobial activity with an inhibition zone diameter of 11 mm.

H) Antibiotic Susceptibility Results: As it can be seen at Table 4, According to the CLSI 2026 guidelines, the inhibition zone diameters for the selected strains against common antibiotics were as follows:

- Ampicillin (≥17 mm): susceptible - strains A1 and D20
- Vancomycin (≥17 mm): susceptible - strains A3 and D20
- Penicillin (≥15 mm): susceptible - strains A1 and D20

Table 3. Antibacterial activity of the LAB isolates against 4 pathogenic strains.

Isolates	Bacterial Pathogens			
	Staphylococcus aureus	***Salmonella enterica***	***Bacillus cereus***	***Escherichia coli***
	Diameter of inhibition zone (mm)			
A1	8	-	14	9
A3	10	-	18	9
B7	11	8	22	8
D20	8	-	13	11

I) Molecular identification of strains: After DNA extraction from the four selected strains (isolates A1, A3, B7 and D20) which had shown acceptable probiotic features according to the previous probiotic tests results and amplification of the 16S rRNA genes using universal primers in a thermal cycler, agarose gel electrophoresis were performed to verify the quality of the PCR products. Subsequently, the PCR products of the selected strains, along with forward and reverse primers, were sent to Genetic Codon Company, Iran, for sequencing. According to the Table 5, results were reported as follows and deposited in the NCBI database.

Table 4. Antibiotic susceptibility results of the LAB isolates.

Isolates	Antibiotics					
	Amoxicillin 25	**Ampicillin 10**	**Vancomycin 30**	**Ciprofloxacin 5**	**Trimethoprim Sulfamethoxazole**	**Penicillin 10**
	Diameter of inhibition zone (mm)					
A1	18	18	14	-	28	18
A3	16	8	18	18	22	-
B7	-	-	-	14	20	-
D20	20	20	18	20	20	20

Table 5. Identification of the LAB strains with probiotic properties.

Isolates	Species	Strain	NCBI accession No.
A3	*Enterococcus faecalis*	Probionaz	PQ893665
D20	*Enterococcus faecium*	Ho1224	PQ893691
B7	*Enterococcus faecium*	AR1224	PQ893690
A1	*Enterococcus faecium*	Fa1224	PQ893689

Discussion

Raw cow milk is considered an excellent source of probiotic bacteria. For probiotic bacteria in foods to be beneficial to the host, they must be able to pass through the stomach and reach the small intestine in sufficient numbers (10^6-10^7 CFU/mL or mg) to be effective. Furthermore, probiotics must be able to tolerate the harsh conditions of the stomach and intestine (including acid, bile, and enzymes) and adhere to the intestinal epithelium. These characteristics may affect their potential for commercial applications (20). In a study on *Lactobacillus* spp. strains isolated from kefir samples, none of the isolates survived at pH 2, but they were able to tolerate moderate pH levels (pH 3 and pH 4) (21). In another study on *Enterococcus* species isolated from raw goat milk, all tested isolates survived at pH 2 and pH 4, with only isolate 23 failing to survive at pH 2 (22). In the present study, among the isolates exhibiting lactic acid bacteria characteristics, five isolates were able to grow at both pH 2.5 and pH 4. Furthermore, in a study investigating the probiotic properties of *Enterococcus durans* LAB18s, the results showed that the *E. durans* LAB18s isolate was not resistant to simulated gastric juice at pH 2 (23). In the present study, all except one of the isolated strains were able to grow in the simulated gastric juice medium.

The presence of polysaccharides on the outer cell membrane may confer resistance to bile salts in *Lactobacillus* strains (24). A study on *Enterococcus faecium* strains isolated from camel milk concluded that the isolated strains showed good tolerance to bile salts (25). In this study, the inhibition coefficient of the isolated strains was less than 0.4, indicating microbial resistance in the presence of bile salts. The ability of bacterial strains to adhere to epithelial cells and mucosal surfaces is another important characteristic for selecting potential probiotic strains. Cell surface hydrophobicity is defined as the ability of a strain to adhere to hydrocarbons (26). In this study, hydrophobicity values ranged from 22.5% to 39.1%. In a study on lactic acid bacteria isolated from mare milk, cell surface hydrophobicity percentages ranged from 13.6% to 56.2% (27). Differences in bacterial cell surface hydrophobicity are influenced by various parameters such as the chemical composition and structural properties of the bacteria (type of amino acids, composition of proteins, polysaccharides, and lipid compounds in bacterial cells), the growth phase of the bacteria, and environmental factors (28). In the present study, the co-aggregation of the isolated strains with pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, and *Bacillus cereus*) was also investigated. The highest co-aggregation rate was reported with *E. coli* (31.72%). In a study investigating the probiotic properties of enterococci isolated from traditional dairy products, isolates 11, 20, and 32 showed higher co-aggregation ability compared to other isolates (29). Probiotic microorganisms that have the ability to co-aggregate with pathogens may be better able to eliminate undesirable bacteria, as they can produce antimicrobial substances in close proximity to them. Co-aggregation of potential probiotic strains with bacterial pathogens varies and depends on the species or strain (30). Furthermore, in this study, the highest auto-aggregation value after 5 hours of incubation was 52.27%. In a study evaluating the probiotic properties of lactic acid bacteria isolated from camel milk, the highest auto-aggregation of *Lactobacillus casei*-03 after 4 hours of incubation was 28.65% (31). In another study evaluating the probiotic potential of *Enterococcus hirae* G24, the highest auto-aggregation rates of isolate G24 at 4 and 6 hours after incubation were 44% and 55%, respectively (32). Lactic acid bacteria exert their antimicrobial activity through reducing pathogen translocation, inhibiting pathogen adhesion, and producing antimicrobial compounds (33). In this study, inhibition zones were evaluated against four pathogens: *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica*, and *Escherichia coli*. The strongest effect was observed with isolate B7 against *Bacillus cereus* (inhibition zone diameter: 22 mm). In a study on enterococci isolated from camel milk, *Enterococcus durans* showed inhibition zone diameters ranging from 10.42 to 14.62 mm against pathogenic bacteria (*Staphylococcus aureus* CIP 483, *Bacillus subtilis* CIP 5262, *Listeria innocua* CIP 864, *Escherichia coli* CIP 53126, *Salmonella enterica* CIP 8039, and *Pseudomonas aeruginosa* CIP 82118) (34). In another study investigating the probiotic properties of enterococci isolated from cheese, the inhibition zone diameters of the strains against pathogenic bacteria (*Escherichia coli* ATCC 35218, *Salmonella enterica* ATCC 13076, *Salmonella Typhimurium* MU80, *Bacillus cereus* RSKK 863, and *Escherichia coli* O157:H7) ranged from 2.5 to 7.8 mm (35). The antimicrobial activity of lactic acid bacteria against pathogens depends on the species and strain. The antimicrobial activity of these bacteria

is mainly due to the production of one or more active metabolites during their growth, such as organic acids, hydrogen peroxide, and bacteriocins (36). An important aspect to consider is the evaluation of antibiotic susceptibility of lactic acid bacterial strains for food safety and security (37). Regarding antibiotic resistance, potential probiotics must be susceptible to antibiotics. Antibiotic-susceptible probiotics do not play a role in the horizontal transfer of antibiotic resistance genes to pathogens. Furthermore, inconsistencies in susceptibility among isolates may be due to differences in species and strains (38). In a study on *Enterococcus faecium* strains isolated from breast milk, *E. faecium* strains TM13, TM15, TM17, TM18, and TN3 were susceptible to vancomycin (39). In the present study, isolates A3 and D20 were susceptible to vancomycin. Finally, four isolates with favorable probiotic properties were sequenced using molecular methods. The results showed that the four superior strains isolated from raw milk in Behbahan county belonged to the genus *Enterococcus*.

Conclusion

This study demonstrated that raw milk from Behbahan county is a rich source of lactic acid bacteria with probiotic properties. Therefore, the identification and isolation of native lactic acid bacteria with probiotic characteristics could be effective in improving the country's food chain. The isolated strains exhibited favorable probiotic properties under *in vitro* conditions. However, more extensive research is necessary to evaluate their efficacy as probiotic bacteria under *in vivo* conditions to confirm their true potential for use in the food and pharmaceutical industries.

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Statements and Declarations

I hereby declare the information given above and in the enclosed document is true to the best of my knowledge and belief and nothing has been concealed therein.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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