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Identification of lactobacilli from milk enzymatic clots and evaluation of their probiotic and antimicrobial properties

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Abstract

The aim of this study was to investigate the probiotic characteristics profile of the isolated lactic acid bacteria (LAB) isolated from cow milk enzymatic clots. Some criteria for probiotic potential such as fermentation profile, acid and bile tolerance, bile salt hydrolysis, antimicrobial activities, lactic acid and bacteriocin production assay, acidification and coagulation, and hemolytic properties were examined. 16S rDNA-based method was applied to confirm the identity of the isolated LABs. LAB E showed the highest resistance to acidic environments. In bile conditions, the number of LAB B and D colonies was also significantly reduced from about 10^7 to 10^2 after 1 hour and to less than 100 after 2 and 4 hours. None of the strains showed sensitivity against fungal and microbes except *Escherichia coli*. This study showed that LABs B, E, and G isolated from cow milk enzymatic clots presented good properties to consider as probiotics and to use for the production of probiotic dairies.

Keywords: lactic acid bacteria; probiotics; lactobacilli; milk enzymatic clots.

Practical Application: Using new discovered Iranian strains of *Lactobacillus* in producing probiotic foods and Supplement.

1 Introduction

There are some microorganisms living in gastrointestinal and vaginal tracts that host get benefits of them. These advantages are because of their specific metabolism. They can find in matrices of a verity of foods including milk, meat, vegetables, and cereals. However, over recent years, the terminology of probiotics has been updated and new definitions such as paraprobiotics (dead/inactivated cells of probiotics) and postbiotics (healthful metabolites of probiotics) have been added which mean that dead cells and also cell metabolites could exhibit significant effects on human's health. Moreover, Zendeboodi et al. (2020) proposed a more comprehensive definition for probiotics, covering all aspects of probiotic features and benefits. They defined three class of probiotics including (1) True probiotics (TP) meaning viable and active probiotic cells, (2) pseudo-probiotic (PP) mentioning to viable and inactive cells like spores, and (3) ghost probiotic (GP) referring to dead cells (see the review for more details) (Zendeboodi et al., 2020).

By adding probiotics to foodstuffs, the benefits of microorganisms can be taken in supplementary or therapeutic uses. Nowadays, probiotics are widely used as functional food supplements and medicines. These functional foods have been shown to lower the blood pressure, the blood sugar, the serum cholesterol, prevent cancer, regulate the immune system, improve lactose intolerance and reduce the use of antibiotics (Clare & Swaisgood, 2000; Khan, 2014; Lähteinen et al., 2010; Shinde et al., 2019). For instance, a review study presented by Murat Açik et al. (2020) showed that kefir as a probiotic-enriched food product exhibited anti-inflammatory, antioxidant and antimicrobial activity against pathogens and protective impacts on gut microbiota (Açik et al., 2020).

Lactic acid bacteria (LAB) including *Lactobacillus* and *Bifidobacterium* species are desirable microflora used as common probiotics. LABs ferment sugars and synthesize lactic acid, acetic acid, H_2O_2, and bacteriocin. These products preserve foods from spoilage by food borne pathogens (Angmo et al., 2016; Arena et al., 2017). Their availability as well as good health benefits has increased interest toward applying them in pharmaceutical and food industries.

LAB species can be found in many foods including different meat products and dairy products such as yogurt, curd, cheese and fermented milk, offering impressive features as probiotics applicable in food industry (Ambadoyiannis et al., 2004; Angmo et al., 2016; Bergamini et al., 2005; Dincer & Kivanc, 2020; Yazdi et al., 2017).

With the increasing interest to consume dairy products, more than thousands of tons of these products are being produced daily in country's dairy factories. Therefore, the need for lactic acid bacteria as initiators also increases. Statistical studies conducted in dairy factories showed that these industries in the country are dependent on the supply of these bacteria from other countries. Therefore, the isolation and preparation of native bacteria with accurate molecular identity in our country is needed.

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The aim of this study was to investigate the probiotic potential of LABs isolated from cow milk enzymatic clots collected from some villages of Varamin, Karaj and Damavand.

2 Materiald and methods

2.1 Sampling

13 milk samples were collected from cows of 13 farms located in different villages of Varamin, Karaj, and Damavand. Milk samples were collected under a sterile condition and stored in the sealed containers and then transported to the laboratory on ice according to AOAC (Association of Official Analytical Chemists, 2002).

2.2 Preparation of coagulated milk

To produce enzymatic clots, renin enzyme was added to all collected milk samples in a ratio of 0.055 g per 1 kg of milk and then the samples were incubated at 37 °C for a few hours until the clots were formed. Very small amount of these clots were cultured on MRS (Man Rogosa and Sharpe) agar or M17 and placed in a CO_2 container (under micro-aerophilic conditions).

2.3 Isolation and identification of lactobacillus strains

Bacteria that appeared to be lactobacilli (named as A, B, C, D, E, G, and H) were isolated from MRS agar and sub-cultured for 2-3 times at 30 °C for 24 to 48 h. In order to confirm and identify the isolated bacteria, Gram staining and catalase assays were performed according to available standard protocols (Ali & Chaouche, 2019). Gram-positive and catalase-negative colonies were considered for further tests.

2.4 Carbohydrate fermentation assay

The sugars (including arabinose, maltose, lactose, melsitose, galactose and sorbitol) were dissolved in the sterile water and added to the sterile phenol red broth culture medium at a ratio of 10%. We made a leachate from each bacterium and diluted it. From a dilution of 10^8, one drop was inoculated into the culture medium and placed in a 37 °C incubator for 24 to 48 hours. The positive bacteria for sugar fermentation test were recognized by color changes from red to yellow. The results of carbohydrate fermentation were compared to the biochemical standard table (Partovi et al., 2015; Mehmood et al., 2009).

2.5 Bacterial sensitivity to antibiotics

Disc diffusion method on MRS-agar plates was applied to determine bacterial sensitivity to antibiotics according to the Performance Standards for Antimicrobial Disk Susceptibility tests (2007). Briefly, the freshly prepared LAB cultures were seeded on MRS agar plates. Then, discs coated with antibiotics (ciprofloxacin, ampicillin and penicillin) were placed on the surface of the plates and were incubated at 37 °C for 48 h (Minelli et al., 2004; Rojo-Bezares et al., 2006; Rishabh et al., 2021).

2.6 Probiotic screening assays

Acidic pH tolerance

The broth MRS medium was adjusted to pH 2.00 with 1 N HCl. 10 µL of 24 hour-cultured strains was mixed with 240 µL of MRS medium and incubated at 37 °C for 2 h. The successive dilutions of bacteria were then prepared and cultured on MRS agar plate for 24-48 hours at 37 °C and finally the viability rate was determined through total viable count (Abushelaibi et al., 2017; Soni et al., 2020).

Bile tolerance

Oxgall types of bile were used to study bile tolerance of the isolated microorganisms according to the Gilliland and Walker method (1990). Briefly, each strain was inoculated into MRS broth containing 0.30% (w/v) of oxgall and incubated at 37 °C. Bacterial growth was assessed by counting methods in 0, 2, and 4 hours post incubation (Angmo et al., 2016; Braïek et al., 2018).

Resistance to the simulated gastric and intestinal conditions

To assess resistance to gastric conditions, fresh bacteria were cultured in MRS broth for 24 to 48 h to grow and make turbidity. Then they were centrifuged at 3000 rpm for 10 min and the bacterial pellet were then resuspended in PBS to reach a turbidity of 10^8 (0.5 McFarland). 4.5 mL gastric juice and 0.5 mL of bacterial suspension were mixed. After 30, 60 and 120 min incubation at 37 °C, the viable colonies were counted.

To assess resistance to intestinal conditions, 1 mL of bacterial suspension previously incubated for 60 minutes was added to 9 mL intestinal juice (1% pancratin and 0.3% bile at pH 7.5) and then incubated at 37 °C for 30, 60 and 120 min. Finally the bacterial growth was analyzed by counting method (Agostini et al., 2018; Archer & Halami, 2015; Charteris et al., 1998; Fang et al., 2015; Fernandes et al., 2013; Maragkoudakis et al., 2006; Shinde et al., 2019; Tokatlı et al., 2015; Vinderola & Reinheimer, 2003; Pinto et al., 2006; Zárate et al., 2000).

Bile salt hydrolase (BSH) activity

To determine the BSH activity of LAB isolates, MRS agar plates containing glycodeoxycholic acid, taurocholic acid and glycocholic acid (TDCA; Sigma, USA) either combined together or separate were prepared. The LAB isolates were then cultured on the prepared MRS plates and incubated at 37 °C for 72 h under anaerobic conditions. The precipitation zone around colonies is considered as the bile salt hydrolase activity of bacteria (Abushelaibi et al., 2017; Braïek et al., 2018).

Antibacterial and antifungal activity

Agar well diffusion method was performed using trypticase soy broth (TSB, BDDIFCO) medium. The TSB media were prepared according to manufacturer's instructions. *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus parasiticus* and *Geotrichum candidum* were used for evaluating the antibacterial and antifungal activity of the LAB isolates.

For antibacterial assay, some colonies were picked from pure bacterial cultures, inoculated into 5 mL of trypticase soy broth (TSB) (BDDIFCO), and incubated overnight at 37 °C. The concentration of the overnight-grown cultures was adjusted to a turbidity of 0.5 MacFarland standard. The TSB plates were inoculated by 100 µL of each prepared bacterial suspensions. Then, several deep holes were generated in plates using a sterile Pasteur pipette and the bottom of holes were sealed by one drop of cell-free medium. In parallel, the fresh cell suspensions were prepared for all LAB isolates by incubating at 37 °C for 48 h. After 48 h, LAB suspensions were centrifuged and the isolated cell-free medium related to each LAB suspension was transferred to each hole in the MHA plate and incubated at 37 °C for 24 h.

For antifungal assay, the fungal suspension with a turbidity of 0.5 MacFarland were prepared and then cultured on Yeast Glucose Chloramphenicol (YGC) agar as specific culture media for molds and yeasts and well diffusion assay was performed (Braïek et al., 2018; Rishabh et al., 2021).

Cell surface hydrophobicity (CSH) assay

To assess the surface hydrophobicity, the LAB isolates were inoculated in MRS broth and incubated at 37 °C for 24 h. Next day, the cell suspensions were centrifuged and the resulting cell pellets were resuspended in PBS to 10^8 CFU/mL. Then, 2 mL of each bacterial suspension were mixed with equal volume of hexadecane (Sigma-Aldrich) through vortexing for 2 min and incubated at room temperature (RT) for 1 h until different phases obtained. The aqueous phase was separated to record the absorption value of all samples at 600 nm wavelength. The cell surface hydrophobicity of the LAB isolates was calculated by the Equation 1 illustrated below:

$$Surface\ hydrophobicity(\%) = \frac{OD_{600}(A) - OD_{600}(AO)}{OD_{600}(A)} \times 100\% \quad (1)$$

Where, A= initial absorbance at 600 nm, and A0= final absorbance (Agostini et al., 2018).

Auto-aggregation and co-aggregation assays

For auto-aggregation, the LAB isolates were inoculated in MRS broth and incubated at 37 °C. 24 h post-incubation, the bacterial suspensions were centrifuged and then the cell pellets were were resuspended in PBS to 10^8 CFU/mL and then 4 mL of each bacterial suspension were transferred into glass tubes and vortexed for 10 sec. The bacterial suspensions were incubated in RT for 5 h and their absorption values at 600 nm wavelength was recorded after 0, 1, 2, 3, 4, and 5 h.

For co-aggregation, the bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Salmonella enteritidis* as well the LAB isolates were separately inoculated in MRS broth and incubated at 37 °C. After preparing a concentration of 10^8 CFU/mL, 2 mL of each LAB isolates (A, B, C, D, E, G, and H) were separately mixed with 2 mL of each *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Salmonella enteritidis* suspension and incubated in RT for 5 h. Again, their absorption values was recorded at 600 nm after 0, 1, 2, 3, 4, and 5 h. The aggregation activity of the LAB isolates was calculated by the following Equation 2 and 3:

$$Auto-aggregation\ activity = \left[1 - \left(A_t / A_0\right)\right] \times 100 \quad (2)$$

Where, At defined as the absorbance measured at time t = 1, 2 ,3, 4 and 5 h and A0 is the absorbance at t = 0 h

$$Coaggregation(\%) = \frac{\{[(Ax + Ay)/2] - A(x + y)\}}{\left(Ax + \frac{Ay}{2}\right)} \times 100 \quad (3)$$

Where, A_x is the pathogen absorbance, Ay is absorbance of probiotics, and A(x + y) is absorbance of their mixture (Braïek et al., 2018).

Hemolytic activity

To examine hemolytic activity, the isolated strains were cultured on Colombia blood agar (Himedia, Mumbai, India) containing 5% human blood and incubated at 37 °C for 48 h. Those colonies revealed a clear hemolysis zone were considered as β-hemolytic or complete hemolytic and those revealed a greenish halo were considered as α-hemolytic or partial hemolytic (Angmo et al., 2016; Braïek et al., 2018).

Indole assay

To measure the indole production capability of LAB isolates, the tryptophan broth media were inoculated by LAB colonies obtained from fresh cultures related to each isolate and were incubated at 37 °C for 24-48 h. to detect the positive indole samples, 2-3 drops of Kovac's reagent was added to each sample tube (Ali & Chaouche, 2019).

Solubility activity P

LAB isolates were inoculated at the center of Pikovskaya (PVK) agar plate and incubated at 37 °C for 48 hr. The plates were examined for halo zone around bacterial colonies (Pikovskaya, 1998).

Exopolysaccharide (EPS) production assay

To measure the ability of EPS production by LAB isolates, the isolated strains were grown in MRS broth at 37 °C for 48 h. Bacterial cells were precipitated by centrifuging at 8000 rpm at 4 °C for 10 min. one volume of supernatant was mixed with three volumes of cold absolute ethanol and incubated overnight at 4 °C. Next day, the precipitated EPS was separated from supernatant by centrifuging at 8000 rpm at 4 °C. The EPS pellet was dissolved in distilled water and immediately mixed with 1 mL 6% phenol and 5 mL 95% sulfuric acid. After mixing well, the absorption of samples was measured at 490 nm wavelength (Amatayakul et al., 2006a, b; McCready et al., 1950).

The total saccharide content of LAB isolates was also measured according to McCready et al. (1950). In brief, 200 µL of the concentrated extract was mixed with 3 mL of anthrone indicator and incubated in a hot water bath at 100 °C for 20 min.

The light absorption of samples was measured at 620 nm (McCready et al., 1950).

Lactic acid production assay

The LAB isolates were grown in MRS broth at 37 °C for 24 h. Next day, bacterial cells were separated from supernatant by centrifuging at 8000 rpm at 4 °C for 10 min. The pellets were resuspended in PBS to 10^8 CFU/mL. 0.1 mL of bacterial suspensions were mixed with 10 mL of 10% skim milk and incubated at 37 °C for 24 h. After 24 h, 5-6 drops of phenolphthalein reagent as an indicator were added and then the suspensions were tittered with 0.1 N NaOH until a pink color appears and persists for 30 sec. Finally, the volume amount of the consumed NaOH was recorded (Gawad, 2010; Kazeminia et al., 2019; Azizkhani & Tooryan, 2017).

Bacteriocin production assay

To survey the bacteriocin production ability of the LAB isolates, spot plate assay, disk diffusion and well diffusion methods were performed. Briefly, the fresh bacterial suspensions were prepared and diluted to a turbidity of 0.5 MacFarland. The prepared TSB plates were inoculated by 100 µL of the prepared bacterial suspensions. On the other hand, the fresh suspensions of LAB isolates were prepared by incubating at 37 °C for 48 h. The LAB isolates were separated from supernatant by centrifugation. For spot plate assay, each LAB supernatant were placed on different areas on the MHA plate drop by drop and incubated at 37 °C for 24 h. For disk diffusion assay, the paper disks were immersed in the cell-free supernatant of each LAB and placed on the surface of the inoculated MHA plate which finally were incubated for 24 h at 37 °C. For well diffusion assay, a number of wells was created in MHA plates and LAB supernatants were poured into wells in separate plates (Braïek et al., 2018).

Acidification and coagulation

The sterile 10% skim milks were prepared and divided into lab tubes. Then, the LAB isolate suspensions at a concentration of 10^8 CFU/mL were added to each tubes and incubated at 30 °C and 37°C for 24 h (Shangpliang et al., 2017).

2.7 Molecular identification of LAB isolates

The isolated LAB strains were identified using 16S rDNA-based PCR method. Briefly, DNA samples were extracted from freshly suspensions of LAB isolates using DNA extraction kit (Takapoozist Co., Iran) and 16S rDNA gene was amplified through PCR method using the designed PCR primer set of 27-F (5'-AGAGTTTGATCA/CTGGCTCAG-3') and 1525-R (5'-AAGGAGGTGA/TTCCAA/GCC-3'). The PCR program was run as follows: initial denaturation at 94 °C for 3 min, amplification step including 35 cycles of denaturation at 93 °C for 35 sec, annealing at 59 °C for 60 sec, and extension at 72 °C for 90 sec, and final extension at 72 °C for 5 min. The PCR products were visualized using 3% agarose gel electrophoresis followed by SYBR green staining (Partovi et al., 2015).

3 Results

3.1 Phenotypic identification of LABs

Morphological assessments showed that all seven LAB isolates were lactobacilli. The results obtained from gram staining and catalase activity revealed that LAB isolates were Gram-positive and catalase negative. They were able to grow at 37 °C on MRS agar medium under micro-aerophilic conditions.

3.2 Carbohydrate fermentation profile of LAB isolates

Fermentation of six sugars maltose, lactose, arabinose, galactose, melezitose and sorbitol by the isolated strains were investigated. The fermentation profile revealed that LAB A was positive, negative, positive, positive, positive and negative for maltose, lactose, arabinose, galactose, melezitoze and sorbitol, respectively, which are shown as + - + + + -. As the same, LAB C and LAB E were + + + + + - and + + + + - -, respectively. LAB B, LAB G, and LAB H were negative for all sugars. LAB D was positive for all sugars (Table 1).

3.3 Antibiotic resistance of LAB isolates

The sensitive strains showed a clear zone around the antibiotic discs (inhibition zone). The results represented as sensitive or resistant according to diameters of the inhibition zone. As presented in Table 2, most of the isolated LABs were resistant to Ampicillin. Five LABs B, C, E, G and H were also resistant to ciprofloxacin while LAB isolates A and D were susceptible to it. Of seven isolated strains, LAB A, B and D showed a zone of 10, 13 and 12 mm around the penicillin disk and the rest of LABs were resistant.

3.4 Probiotic characterization of LAB isolates

Acid tolerance of LAB isolates at pH 2.0

Survival rate of the LABs cultured in acidic conditions of pH 2.0 showed a significant decrease during 1 h. All strains showed a progressive reduction of colony numbers following

Table 1. The results of carbohydrate fermentation test.

	Maltose	Lactose	arabinose	Galactose	Melezitose	Sorbitol
LAB A	+	-	+	+	+	-
LAB B	-	-	-	-	-	-
LAB C	+	+	+	+	+	-
LAB D	+	+	+	+	+	+
LAB E	+	+	+	+	-	-
LAB G	-	-	-	-	-	-
LAB H	-	-	-	-	-	-

Table 2. Antibiotic resistance of LAB isolates.

	Ciprofloxacin	Ampicillin	Penicillin
LAB A	S (~ 17 mm*)	R	S (~ 10 mm)
LAB B	R	R	S (~ 13 mm)
LAB C	R	R	R
LAB D	S (~ 14 mm)	R	S (~ 12 mm)
LAB E	R	R	R
LAB G	R	R	R
LAB H	R	R	R

* represented the diameter of inhibition zone. R: resistance to antibiotic; S: sensitive to antibiotic.

incubation in acidic condition for either 30 or 60 min (Table 3). LAB A was the most sensitive strain to acidic pH, as the number of its colonies reduced from 1.3×10^7 to less than 100 after a 30 min incubation. Whereas LAB E showed the highest resistance to acidic condition. LAB isolates C and D retained a constant level of viability after exposure to pH 2.0 for both 30 and 60 min.

Bile tolerance of LAB isolates

Bile condition showed the most effect on the viability of LABs B and D (Table 4). The number of their colonies was significantly reduced from about 10^7 to 10^2 after 1 hour and to less than 100 after 2 and 4 hours ($p < 0.05$). Other strains also exhibited a reduction of viability but with a slower trend during 4 h exposure to bile condition. LABs C and E maintained their viability during exposure to oxgall. Both isolates did not showed a significant decrease of colony numbers after 1 h compared to 2 h ($p > 0.05$). We can considered them as tolerant strains against 0.30% bile salt.

Resistance to the simulated gastrointestinal conditions

Of all seven isolates, LABs A, C, H and D were sensitive to intestinal conditions throughout the study period (120 min). Of three remained isolates, LABs E and G showed a decreased viability less than 100 after 2 h incubation in intestinal conditions. Whereas LAB B presented relative resistance to intestinal condition. The simulated stomach conditions had less influence on the viability of LAB isolates even 30 min after incubation. As shown in Table 5, the more time passed, the more strains became so sensitive to the simulated stomach conditions that their colony numbers decreased to less than 100 after 120 min, except for LAB E which represented resistance against simulated stomach condition.

Bile Salt Hydrolase (BSH) activity of LAB isolates

The ability to hydrolyze bile salts of glycocholic acid, tarocholic acid, glycocholic acid was investigated. There was no sedimentary halo around the colonies when the isolates were incubated with the mixture of three salts. Exposing to each salt separately, just glycocholic acid prevented the bacterial growth while all strains formed colonies at the presence of tarocholic acid and glycholic acid.

Table 3. Acidic pH tolerance of LAB isolates.

	T_0	T_{30}	T_{60}
LAB A	1.3×10^7	100>	-
LAB B	8×10^7	2×10^6	3.5×10^4
LAB C	1.5×10^7	1×10^3	100>
LAB D	1×10^7	4×10^3	
LAB E	2.3×10^7	1×10^6	5.5×10^4
LAB G	1.1×10^7	5×10^5	1.5×10^4
LAB H	9×10^7	4×10^3	100>

Table 4. Bile tolerance of LAB isolates.

	T_0	T_{60}	T_{120}	T_{240}
LAB A	$1/3\times10^7$	$1/5\times10^4$	9×10^3	1×10^3
LAB B	8×10^7	1×10^2	100>	100>
LAB C	$1/5\times10^7$	5×10^6	1×10^6	5×10^5
LAB D	1×10^7	1×10^2	100>	100>
LAB E	$2/3\times10^7$	4×10^6	$1/5\times10^6$	1×10^5
LAB G	$1/1\times10^7$	8×10^3	2×10^3	1×10^2
LAB H	9×10^7	$1/3\times10^4$	7×10^3	4×10^2

Antimicrobial and antifungal properties of LAB isolates

None of strains showed antifungal activity against *Aspergillus parasiticus* and *Geotrichum candidum*, whereas all of them showed an antibacterial activity against *Escherichia coli*, producing clear inhibition zone of more than 5 mm. Furthermore, none of LABs had antimicrobial activity against *Staphylococcus aureus* (Table 6).

Cell surface hydrophobicity of LAB isolates

Cell surface hydrophobicity is a considerable property in adhesion capacity of probiotics. Among the isolated LABs, as shown in Table 7, LABs A, B, D and H showed the highest hydrophobicity ability, respectively, suggesting these LABs with higher hydrophobicity capacity can efficiently adhesion to host cells and act therefore as more eligible probiotics. The LAB G had the lowest hydrophobicity percent.

Auto-aggregation and Co-aggregation features of LAB isolates

Auto-aggregation and co-aggregation are necessary abilities of probiotics for adhesion to host epithelial cells and colony

Table 5. Evaluation of bacterial resistance to simulated intestine and stomach conditions.

	Intestinal condition				Stomach condition			
	T_0	T_{30}	T_{60}	T_{120}	T_0	T_{30}	T_{60}	T_{120}
LAB A	5×10^7	100 >	100 >	100 >	5×10^7	-	-	-
LAB B	9×10^7	^{4} 10×2.1	^{4} 10×1	^{3} 10×3	9×10^7	1.1×10^7	2×10^5	-
LAB C	4×10^7	100 >	100 >	100 >	4×10^7	3.1×10^7	100 >	100 >
LAB D	1.5×10^7	100 >	100 >	100 >	1.5×10^7	2×10^3	100 >	100 >
LAB E	4×10^7	^{3} 10×1	100 >	100 >	4×10^7	2×10^6	3×10^5	3×10^3
LAB G	1×10^7	^{3} 10×1.1	10	100 >	1×10^7	2×10^6	4×10^4	25
LAB H	1.1×10^7	100 >	100 >	100 >	1.1×10^7	1.1×10^4	100 >	100 >

formation. The obtained data for auto-aggregation revealed that LAB A had the highest auto-aggregation capacity in all studied times compared to other LABs (Table 8). Over time, it was also found that the auto-aggregation ability increased. Moreover, co-aggregation abilities of LABs with *E.coli*, *Streptococcus agalactiae, Staphylococcus aureus*, and *Salmonella enteritidis* have been shown in Tables 9, 10, 11, and 12, respectively. Over time, three LABs A, G, and H showed a higher co-aggregation feature with *E.coli*, *Streptococcus, Staphylococcus, and Salmonella* in comparison to other five LABs.

Hemolytic activity

All isolated LABs were positive for hemolytic activity (Table 13). LABs A and C showed beta hemolysis, hemolyzing the blood completely and producing a clear zone around the colonies on the blood plates. While other isolates exhibited alpha hemolysis and were able to partially hemolyzed the blood, producing a greenish zone.

Indole production capability of LAB isolates

As the produced indole in combination with Kovac's reagent lead to a color change of the solution from yellow to cherry red and our results showed no color change, it is concluded that all LABs were negative for indole.

Solubility activity P

According to the resulting data, no solubility activity P was observed for all LAB isolates.

EPS production capability of LAB isolates

The total saccharide content of LAB isolates was measured according to McCready et al. (1950). According to Table 14, all LABs were capable of EPS production.

Lactic acid production of LAB isolates

Acid production is another beneficial feature for bacteria to be used as probiotics in food industry and therapeutic strategies. The ability of lactic acid production was evaluated in LAB isolates based on the NaOH volume used for acid titration. As shown in Table 15, the LABs A and H demonstrated the highest and lowest levels of lactic acid production, respectively.

Table 6. Evaluation of antifungal and antimicrobial properties of lactic isolates based on the formation of growth inhibition zone.

	a: Antimicrobial activity		a :Antifungal activity	
	Escherichia coli	*Staphylococcus aureus*	*Aspergillus parasiticus*	*Geotrichum candidum*
LAB A	+	-	-	-
LAB B	+	-	-	-
LAB C	+	-	-	-
LAB D	+	-	-	-
LAB E	+	-	-	-
LAB G	+	-	-	-
LAB H	+	-	-	-

a: (-) no inhibition; (+) inhibition zone.

Table 7. Percentage of surface hydrophobicity.

	Surface hydrophobicity
LAB A	94.2029
LAB B	91.9355
LAB C	68.1818
LAB D	84.6154
LAB E	50.7692
LAB G	22.3880
LAB H	82.2580

Table 8. Percentage of Auto-aggregation ability of LAB isolates.

	0	1h	2h	3h	4h	5h
LAB A	1	7.25	15.94	27.54	65.22	56.52
LAB B	1	3.23	4.83	6.45	11.29	17.74
LAB C	1	3.03	9.09	10.60	16.67	24.24
LAB D	1	0	6.15	23.08	10.77	20
LAB E	1	6.15	10.77	18.46	24.62	30.77
LAB G	1	5.97	10.44	14.93	22.39	37.31
LAB H	1	3.23	4.84	11.29	24.20	38.71

Bacteriocin production ability of LAB isolates

The ability of LAB isolates to produce bacteriocin was evaluated by three spot plate, disk and well diffusion methods. The results obtained from well diffusion assay showed that the LAB isolates produce bacteriocin against *E.coli* and then inhibit the *E.coli* growth, indicated by a clear zone around the disks coated with cell-free LAB secretions.

Table 9. Percentage of co-aggregation mixture with *E.coli*.

Time	A/E.coli	B/E.coli	C/E.coli	D/E.coli	E/E.coli	G/E.coli	H/E.coli
0	0	-6.59	0	1.62	0.54	2.67	-6.59
1h	0.15	-6.67	0.010	1.69	0.55	1.14	-2.22
2h	0.53	-5.88	1.14	2.70	1.081	1.64	-0.59
3h	1.29	-5.14	2.92	2.98	1.29	2.59	1.71
4h	2.46	0	3.64	3.17	2.30	6.79	3.82
5h	15	1.24	4.72	4.32	4.24	13.81	10.13

Table 10. Percentage of co-aggregation mixture with *Streptococcus agalactiae*.

Time	A/Sterp	B/Sterp	C/Sterp	D/Sterp	E/Sterp	G/Sterp	H/Sterp
0	4.83	0.5	0.52	-1.67	0.99	3.68	2.5
1h	2.65	1.06	1	0	0.51	3.90	3.061
2h	5	2.04	1.63	1.16	1.09	5.53	5.29
3h	10.56	2.66	2.17	2.49	1.60	6.42	8.09
4h	17.54	7.73	3.53	2.96	2.29	15.17	8.65
5h	20	6.43	4.42	3.80	3.03	16.05	11.39

Table 11. Percentage of co-aggregation mixture with *Staphylococcus aureus*.

Time	A/Staph	B/ Staph	C/ Staph	D/ Staph	E/ Staph	G/ Staph	H/ Staph
0	3.01	-2.60	2.92	2.05	1.25	2.030	0.52
1h	4.17	-3.19	3.049	2.65	1.60	3.66	0.56
2h	6.45	-0.53	3.19	3.45	2.65	5.32	1.06
3h	8.05	1.10	3.57	3.63	2.82	8.29	5.88
4h	13.04	1.75	4.17	5.75	3.23	8.33	7.98
5h	17.36	2.42	4.92	7.83	3.077	12.18	12.50

Table 12. Percentage of co-aggregation mixture with *Salmonella enteritidis*.

Time	A/Sal	B/Sal	C/Sal	D/Sal	E/Sal	G/Sal	H/Sal
0	0.52	-5.38	-0.56	-5.88	0	0/52	1.064
1h	1.12	-4.52	-1.05	-0.57	0.53	1.11	1.68
2h	1.23	-3.91	1.16	0	1.03	1.60	2.26
3h	3.53	-1.12	1.66	0.53	1.12	3.95	3.64
4h	7.36	2.31	2.22	0.55	1.62	7.65	4
5h	10.96	5.39	2.41	1.55	1.86	13.92	12.99

Acidification and coagulation potential of LAB isolates

As shown in Table 16, all LAB isolates exhibited the ability of acidification and coagulation at 37 °C. In contrast, none of LAB isolates presented coagulation potential at 30 °C.

Molecular identification of LAB isolates

The PCR results showed that all seven isolated strains presented the same 16S rDNA as appeared on 3% agarose gel. These results definitely confirmed that the isolated strains belong to lactobacilli (Figure 1). Moreover, the amplified 16S rDNA fragment related to each LAB isolate was sequenced and the resulting data were recorded in NCBI database as following defined accession numbers: MT509522.1, MT509521.1, MT509520.1, MT509525.1, MT509524.1, and MT509523.1.

Table 13. Blood hemolysis capability of LAB isolates.

	Alpha Hemolysis	Beta Hemolysis
LAB A		+
LAB B	+	
LAB C		+
LAB D	+	
LAB E	+	
LAB G	+	
LAB H	+	

4 Discussion

Probiotics are normal flora in human gastrointestinal tract, offering some benefits for human health and can be used in therapeutic, medical and food industry (George Kerry et al., 2018). In this study, probiotic bacteria were isolated from milk

Table 14. The measurement of total saccharide produced by LAB isolates.

	50µl	100µl	ppm	ppm
LAB A	0.283	0.482	238	218.5
LAB B	0.254	0.425	209	190
LAB C	0.231	0.4	186	177.5
LAB D	0.248	0.409	203	182
LAB E	0.308	0.527	263	241
LAB G	0.284	0.47	239	212.5
LAB H	0.395	0.705	350	330
ppm	50	100	200	300
Absorbance	0.106	0.167	0.327	0.42

Table 15. Lactic acid production of LAB isolates.

	Volume of NaOH consumed	Acidity g/l of Lactic acid
LAB A	2.5	2.25
LAB B	1.4	1.26
LAB C	1.3	1.17
LAB D	1.2	1.08
LAB E	1.1	0.99
LAB G	1.5	1.35
LAB H	1	0.9

Table 16. pH measurement and coagulation ability.

	pH	Ability to create clots
LAB A	3.85	+
LAB B	5.30	+
LAB C	6	+
LAB D	4	+
LAB E	5.75	+
LAB G	5.35	+
LAB H	5.5	+

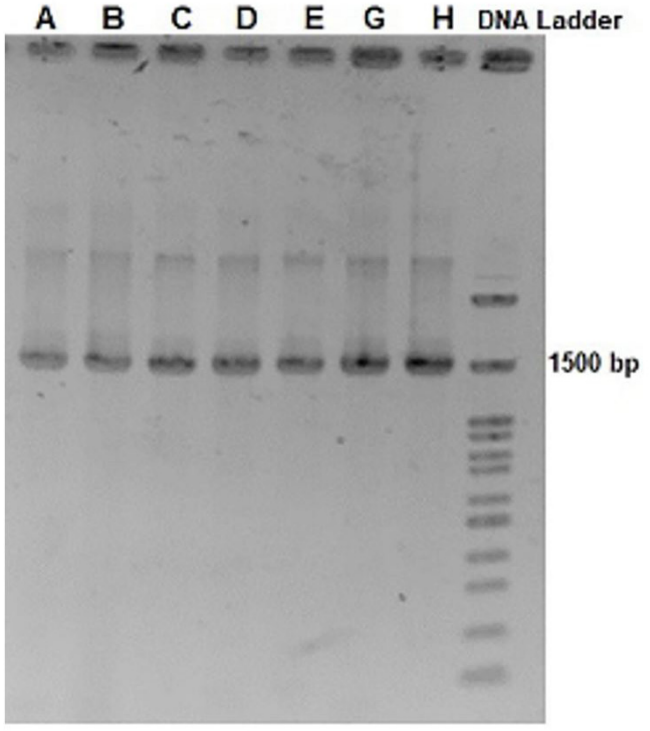

Figure 1. Molecular identification of the isolated LAB strains using 16S DNA-based method. As shown, all seven LABs exhibited the same 16S rDNA fragment with the expected size of 1500 bp, confirming their lactobacillus identity.

enzymatic clots. Our data showed that 7 of 13 isolated bacteria were morphologically bacilli. The results of staining and catalase activity assays revealed that the isolates were Gram-positive and catalase negative. They were able to grow at 37 °C in MRS agar medium under microaerophilic conditions. Fermentation of six sugar was tested for these strains. Except for LABs B, G and H, all of them were able to ferment all or some of the sugars. Also all seven isolated strains presented 16S rDNA which definitely confirmed that the isolated strains belong to lactobacilli. Our results showed that all isolates had antibacterial properties against *E. coli*. These activities may be associated with some factors such as metabolites, organic acids, and bacteriocins released from LABs to their environment (Zuo et al., 2016). No antimicrobial activity was observed in the case of *Staphylococcus aureus*, which these results was in agreement with the results obtained by Braïek Olfa and his coworkers whereas in the case of *Escherichia coli*, their results were opposite of ours (DeLisle & Perl, 2003). This discrepancy maybe attributed to species- and strain-dependency of antimicrobial activity. All our isolates were resistant to Ampicillin, whereas some of them showed susceptibility and sensitivity toward penicillin and some showed resistance toward them. Sensitivity of LABs to antibiotics depends on the specific target sites recognized by an antibiotic, so resistance against an antibiotic would be achieved, when its specific target site is absent (Santos et al., 2016). These results are consistent with those reported by Angmo et al. (2016), however, they have used different antibiotics and showed no data related to penicillin and ciprofloxacin (Angmo et al., 2016). Our data were in accordance with those were shown by Teles Santos and coworker. They reported sensitivity and moderate sensitivity against penicillin, as the susceptibility of some strains of our isolated lactobacilli to penicillin was appeared by creating a growth inhibition zone (Casarotti et al., 2015). In this line, Tanja Zugic Petrović et al. (2021) also reported that their LABs isolated from fermented sausages showed high sensitivity to most antibiotics such as penicillin, amoxicillin, tetracycline, and erythromycin. Their examined isolates showed a significant inhibitory property against a broad range of pathogens and good tolerance at low pH in the simulated stomach and intestine conditions, making them good candidate as probiotic functional food (Petrović et al., 2021). In our study, I was found that of all seven LAB isolates, four LABs A, C, H and D were sensitive to simulated intestinal conditions, two LABs E and G showed a decreased viability less than 100 after 2 hours in simulated intestinal conditions, and finally LAB B presented relative resistance to low pH condition in comparison to other ones. The simulated stomach conditions had low influence on LABs' viability even 30 min after incubation. As shown in result section, as the time was increased, more strains became sensitive to the simulated stomach conditions somehow

colony numbers decreased to less than 100 after 120 min expect for LAB E which represented resistance against this condition. Therefore, LABs B and E seemingly showed good tolerance to low pH conditions, which make them potential probiotics using in food production. In this case, Casarotti et al. (2015) reported that probiotic survival in the simulated gastrointestinal conditions might be depend on the type of matrix. They evaluated the effects of three different matrices including milk, MRS and milk with inulin on the survival of some probiotics (*Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subsp. lactis BB-12) in the simulated gastrointestinal conditions. Their results showed that both probiotic strains had low viability in the gastrointestinal conditions, however, milk and milk with inulin could partly protect the probiotics from harsh gastrointestinal conditions, indicating the significance of the selection of a proper food matrix as probiotic carrier (Jones et al., 2013).

As bile tolerance is considered as a significant factor for employing bacteria in probiotic uses, we examined the effects of three bile salts on survival of the studied strains. Of three bile salts, glycodeoxycholic acid prevented the bacterial growth while it was observed that the bacterial colonies grew at the presence of tarocholic acid and glycolic acid salts, confirming their BSH activity against tarocholic acid and glycolic acid. In human intestine, the ability of LABs to hydrolyze bile salts is correlated to the reduction of cholesterol absorption (Abushelaibi et al., 2017; Miremadi et al., 2014). Our data related to this factor showed this correlation, however, they are not in accordance with those reported by Miremadi et al. (Chalas et al., 2016). The Results of strain cultivation in bile conditions indicated that all strains were susceptible to bile salts, except for LAB C and E which showed relative resistance to oxgall, validating these two strains as good candidates for probiotics uses. LAB E also revealed the most resistance to acidic environment compared to LAB B and G. Nevertheless, these three strains showed more viability in comparison to other LABs A, C, D and H in acidic conditions. The last ones were sensitive to acid and bile simulated gastric and intestinal conditions, respectively. Consequently, tolerance to gastrointestinal conditions is a critical criterion for selecting bacteria as probiotics in food industries (Abushelaibi et al., 2017; Archer & Halami, 2015). In this study, LABs B, E, and G, therefore, seems to be potentially eligible for employing as probiotics.

Finally, all LABs exhibited partially hemolytic activity, except for LABs A and C which exhibited a complete hemolytic activity. In this case, Halder et al. (2017) reported that their lactobacilli isolated from curd samples were non-hemolytic (Halder et al., 2017). As generally many pathogenic bacteria possess hemolytic activity, the eligible bacteria as probiotics not only show no hemolytic activity, but also they exert a protective effect against pathogens responsible for hemolysis (Deidda et al., 2020).

5 Conclusion

In conclusion, this study has shown that LABs B, E, and G isolated from cow milk enzymatic clots presented good features as probiotics and could be used for producing probiotic dairies and functional foods. However, further studies on these potentially probiotic LAB isolates are required.

Conflict of interest

Sanaz Kousha, Hamed Ahari, Guity Karim, and Seyed Amir Ali Anvar declare that they have no conflict of interest.

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Authors' contributions

Sanaz Kousha carried out the experiments and wrote the MS draft. Hamed Ahari supervised the work and revised the manuscript. Guity Karim supervised the work and validated the results. Seyed Amir Ali Anvar contributed to this work as the advisor.

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