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RESEARCH ARTICLE

Evaluation of Probiotic Potentials of the Lactic Acid Bacteria (LAB) Isolated from Raw Buffalo (*Bubalus bubalis*) Milk

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A B S T R A C T

Lactic acid bacteria are widely used as probiotics to produce and preserve foods. This study aimed to isolate and evaluate probiotic potentials of lactic acid bacteria from raw buffalo milk. The bacterial colonies (n=130) were isolated from 30 randomly collected raw buffalo milk samples. Based on morphology and biochemical tests, twenty isolates were identified as lactobacilli and evaluated for tolerance to pH (2, 3, 4.5, 6.5), bile (0.5, 1% w/v) and lysozyme (50 and 100 µg/mL). Five out of twenty lactobacilli isolates showing highest resistance towards acid, bile and lysozyme were selected for further probiotic characterization. Tests for tolerance to gastrointestinal fluids, adhesion characteristics, antibiotic susceptibility, hemolytic activity, antibacterial activity, β-galactosidase activity, exopolysaccharide production and heat resistance were performed. The survival of the isolates in simulated gastric fluid and simulated intestinal fluid ranged from 6.60-7.03 Log CFU/mL and 8.36-8.74 Log CFU/mL respectively. The isolates showed autoaggregation (14-62%), hydrophobicity (11-55%) and adhesion to Caco2 cell lines (21-60%). The lactobacilli isolates were positive for β - galactosidase activity, exopolysaccharide production, heat resistant and negative for hemolysis. Isolates were susceptible to most of the tested antimicrobial agents and showed antimicrobial activity against all tested food borne pathogens. The isolates were identified through 16S rDNA sequencing as *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus fermentum* and *Lactobacillus reuteri*. All isolates produced probiotic based yogurt and survived in cold storage (4°C) after 28 days. The results suggest that isolated probiotics have potential use for the development of fermented food products.

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INTRODUCTION

Food products containing probiotics are considered as functional foods and provide various health benefits (Abushelaibi *et al.*, 2017). Probiotics are non-pathogenic microorganisms that provide positive health benefits to the host when consumed in adequate amount (Morelli and Capurso, 2012). Most of the commercial and widely used probiotic bacteria belong to genera *Lactobacillus* and *Bifidobacterium*. Probiotic bacteria have several desirable characteristics such as resistance against acid and bile salts, tolerance to gastrointestinal tract conditions, antimicrobial activity against pathogens, capability to adhere and colonize intestinal cell wall and absence of unusual antibiotic resistance (Ng *et al.*, 2015). Probiotics maintain microbial balance in intestine, prevent and inhibit the growth of pathogenic bacteria and cure various intestinal diseases. These effects occur due to production of organic acids, hydrogen peroxides (H_2O_2), bacteriocins or other inhibitory substances and by competing with pathogenic bacteria for nutrients (Anal and Singh, 2007).

Lactobacilli, a group of lactic acid bacteria (LAB), are found in human and animal body fluids, plants, raw food materials and fermented foods (Rivera-Espinoza and Gallardo-Navarro, 2010). The LAB are generally regarded as safe (GRAS) by United States Food and Drug Administration (FDA) and are widely used as probiotics to produce and preserve foods (Wang *et al.*, 2016). Native

LAB isolated from foods are more suitable for the development of fermented products. These LAB have potential genetic makeup that make them resistant to the manufacturing conditions and antagonistic activities that allow their dominance in environment (Sáez *et al.*, 2018). The different antimicrobial compounds produced by LAB can inhibit the growth of both Gram-positive and Gram-negative food borne pathogens including *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* (Akbar and Anal, 2014; Akbar *et al.*, 2016). Although there are a number of commercially available LAB strains, isolation and characterization of novel probiotics strains from unexplored materials is still of great importance (Vinderola *et al.*, 2008). Probiotics have received increasing attention in recent years due to their long safe use and therapeutic benefits to human health (Chen *et al.*, 2018).

Buffalo milk is an important commodity and plays a major role in human nutrition especially in developing countries. It is richer in almost all main milk nutrients when compared to cow milk (El-Salam and El-Shibiny, 2011). None of the study has yet been reported for the detail characterization of LAB from buffalo milk. This study focuses on isolation, identification and probiotic characterization of LAB from buffalo milk including milk fermentation to produce probiotic enriched yogurt.

MATERIALS AND METHODS

Sample collection: Buffalo milk samples (individual, n=30) were randomly collected under hygienic conditions in sterilized plastic bottles from a dairy buffalo farm, Chachoengsao province, Thailand and transported in icebox to the Bioprocess Technology Laboratory, Asian Institute of Technology, Pathumthani, Thailand.

Isolation of lactic acid bacteria: Aliquots of raw buffalo milk (0.1 mL) along with serial dilutions were plated (spread plate method) on de Man, Rogosa and Sharpe (MRS, Himedia, India) agar and incubated at 37°C for 48 h. One hundred and thirty bacterial colonies were subjected to Gram staining, cell morphology, motility, catalase test and cytochrome oxidase activities, thus twenty lactobacilli isolates were identified.

Acid, bile and lysozyme tolerances: The lactobacilli isolates (n=20) were evaluated for the acid, bile and lysozyme tolerances by following the method as described by Rajoka *et al.* (2018). For acid tolerance, the cell culture of LAB isolates was mixed with MRS broth (Himedia, India) adjusted with pH 2, 3, 4.5 and 6.5 (control). For bile tolerance, the LAB culture was mixed with MRS broth containing 0.5 and 1% bile (w/v) (Sigma-Aldrich, USA). MRS broth without bile salt was used as control. Similarly, for tolerance to lysozyme, the cell culture of LAB was mixed with lysozyme (Sigma-Aldrich, USA) at final concentrations (50 µg/mL and 100 µg/mL). Culture without lysozyme was used as control. For each experiment, samples were incubated for 3 h at 37°C and viability was determined through total plate count method on MRS agar.

Evaluation in gastrointestinal fluids: Five out of twenty lactobacilli isolates showing highest resistance towards acid, bile and lysozyme were selected for further probiotic characterization. The behavior of LAB cells in gastrointestinal fluids was evaluated following the method described by Ng *et al.* (2015). Resistance in terms of total viable count was determined after 3 h in simulated gastric fluid (SGF) and 4 h in simulated intestinal fluid (SIF).

Auto aggregation, hydrophobicity and adhesion to Caco-2 cell line: Auto aggregation activity and hydrophobicity were evaluated as described by Feng *et al.* (2017). Adhesion to human colon carcinoma cell-lines (Caco-2 cells) was performed following the method described by Manini *et al.* (2016). The adhesion percentage was calculated as

$$\text{Adhesion } (\%) = (N_t/N_o) \times 100$$

N_t = Viable count of adhered bacteria, N_0 = Viable count of total bacteria added

Antibiotic susceptibility, hemolytic and antibacterial activity: Susceptibility towards antibiotics, hemolytic and antibacterial activities against food borne pathogens were evaluated as described by Kumar *et al.* (2017). Antibiotic discs (Himedia, India) used in this study were ampicillin (10 µg), clindamycin (2 µg), erythromycin (15 µg), streptomycin (10 µg), tetracycline (10 µg), trimethoprim (5 µg), penicillin (5 µg) and vancomycin (30 µg). For hemolytic activity, lactobacilli isolates were streaked on blood agar (Himedia, India) plates supplemented with 5% (v/v) sheep blood. Plates were incubated at 37°C for 48 h and observed for clear zones (β - hemolysis), greenish halos (α - hemolysis), no reaction (γ - hemolysis). For antibacterial activity Tryptone Soya Agar (20 mL, TSA, Himedia) was mixed with overnight culture (500 µL) of indicator pathogens at 45°C, vortexed and poured into petri plates. Wells were made into agar surface using sterile cork borer. The cell free supernatant (CFS) of LAB strains was obtained through centrifugation (10,000 rpm for 10 min at 4°C) of overnight culture and sterilized through 0.2 µm membrane filter (Sartorius, Minisart, Germany). The CFS of each strain was placed in each well. Plates were incubated 37°C for 24 h and diameter of inhibition zone was measured.

β - Galactosidase activity, exopolysaccharide production and heat tolerance: β - Galactosidase activity and exopolysaccharide (EPS) production were analyzed using the method described by Angmo *et al.* (2016). Tolerance to heat was evaluated following the method described by Abushelaibi *et al.* (2017). Samples were heated in water bath at 60°C for 5 min and cooled down on ice. Cell viability was determined through total plate count after incubation at 37°C for 48 h.

Molecular Identification of LAB isolates: Selected isolates were identified by 16S rDNA gene sequencing as described by Akbar and Anal (2015). PCR primers 518F (5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') were used. Sequence alignments and phylogenetic tree were constructed using MEGA software (ver. 7.02).

Production of probiotic based yogurt: Probiotic based yogurt was produced as described by Guo *et al.* (2009).

Samples were taken every 7th day and bacterial viability through total plate count and changes in pH were recorded.

Statistical analysis: Results are expressed as Mean±SD of three independent replicates. Statistical significance (P<0.05) of results was determined through one way ANOVA and Tukey's test using SPSS 23.0 program (SPSS Inc., Chicago, IL).

RESULTS

Isolation of lactic acid bacteria: One hundred and thirty bacterial colonies, obtained from thirty buffalo milk samples were evaluated to identify LAB isolates. Twenty isolates were Grams positive, rod shaped, non-motile, catalase and oxidase negative and showed typical characteristics of LAB.

Acid, bile and lysozyme tolerances of isolated LAB: The twenty lactobacilli isolates from buffalo milk were screened for acid, bile and lysozyme tolerances (Table 1). All isolates showed high survival of 8-9 Log CFU/mL in pH 4.5 and 6-8 Log CFU/mL in pH 3. However, the isolates showed reduction in survival i.e. 3-7 Log CFU/mL after 3 h incubation (P<0.05) at pH 2. Five isolates (19, 42, 60, 93, 112) survived 6-7 Log CFU/mL after 3 h incubation. The survival of LAB in bile ranged from 5 - 8 Log CFU/mL (P<0.05) in 0.5% and 4-8 Log CFU/mL (P<0.05) in 1% bile. Tolerance of LAB to 50 µg/mL lysozyme ranged from 7-8 Log CFU/mL, while for 100 µg/mL it was 5-7 Log CFU/mL. Isolate 96 showed lowest tolerance (60%) and isolate-19 showed highest (87.78%) tolerance towards 100 µg/mL lysozyme. Overall isolates (19, 42, 60, 93, 112) showed highest resistance towards acid, bile and lysozyme and were selected for further probiotic characterization.

Evaluation in simulated gastrointestinal fluids: Survival in SGF of selected isolates ranged from 6.60 - 7.03 Log CFU/mL after 3 h, while survival in SIF ranged between 8.36-8.74 Log CFU/mL after 4 h incubation. Isolate-93 demonstrated highest survival rate (77.76%) in SGF and SIF (96.04%) (Table 2).

Auto aggregation, hydrophobicity and adhesion to Caco-2 cell lines: Results of auto aggregation activity and hydrophobicity are shown in Fig. 1. Auto aggregation of isolates ranging from 14 to 62% after 24 h incubation. Isolate 93 exhibited highest auto aggregation activity (62.74±0.7%) (P<0.05), while least aggregation activity was shown by isolate 112 (14.54±1.3%) (P<0.05). Hydrophobicity test revealed variable results ranged from 11 to 55% in xylene. Highest hydrophobicity (P<0.05) was exhibited by isolate 93 (55.26±1.8%) followed by isolates 42, 60, 19 and 112. Adhesion of LAB to Caco-2 is shown in Table 3. All strains adhered to Caco-2 cells, highest adhesion percentage (P<0.05) was observed in isolate 93 (60.99%).

Antibiotic susceptibility, hemolytic and antibacterial activity: Three out of five isolates (19, 42 and 93) were susceptible towards all antibiotics tested viz ampicillin, clindamycin, erythromycin, streptomycin, tetracycline, trimethoprim, penicillin and vancomycin. One isolate (60) was resistant towards ampicillin, penicillin and vancomycin, whereas one isolate (112) was resistant towards vancomycin. None of the tested isolates produced hemolytic activity. Antibacterial activity against five food borne pathogens is shown in Table 4. Antibacterial activity ranged from 12.6 to 18.6 mm against *L. monocytogenes*, *S. aureus*, *S. typhimurium*, *E. coli* and *Listeria innocua*. Highest inhibitory activity was exhibited by isolate 93 against *L. monocytogenes* (18.6 mm).

β-Galactosidase activity, exopolysaccharide production and heat tolerance: All tested isolates (19, 42, 60, 93, 112) were positive for β - galactosidase and EPS production. Tolerance of LAB isolates to heat stress is shown in Table 5. Cell viability ranged from 7.65 - 8.12 Log CFU/mL at 60°C after 5 min exposure. Highest tolerance to heat was shown by isolate 42 (P<0.05).

Table 1: Effect of low pH, bile and lysozyme in terms of log CFU/mL on the viability of LAB isolates after 3 h incubation

Isolate	pH tolerance				Bile tolerance			Lysozyme tolerance		
	pH 6.5	pH 4.5	pH 3	pH 2	Control	0.5%	1%	Control	50 µg/mL	100 µg/mL
7	9.09±0.04^{d}	8.65±0.05^{c}	6.98±0.04^{b}	3.65±0.09^{a}	9.11±0.04^{c}	7.02±0.05^{b}	4.85±0.08^{a}	9.07±0.04^{c}	7.91±0.05^{b}	6.66±0.08^{a}
16	9.00±0.05^{d}	8.72±0.06^{c}	7.28±0.03^{b}	3.86±0.07^{a}	9.08±0.04^{c}	7.25±0.03^{b}	5.05±0.04^{a}	9.12±0.04^{c}	7.89±0.04^{b}	6.60±0.07^{a}
19	8.99±0.03^{c}	8.90±0.05^{c}	8.28±0.04^{b}	6.97±0.04^{a}	9.05±0.06^{b}	8.10±0.04^{a}	7.97±0.06^{a}	9.09±0.05^{c}	8.60±0.07^{b}	7.98±0.05^{a}
21	9.08±0.04^{d}	8.61±0.07^{c}	7.86±0.08^{b}	4.20±0.04^{a}	9.07±0.05^{c}	6.87±0.07^{b}	4.98±0.04^{a}	9.00±0.04^{c}	7.63±0.07^{b}	6.31±0.03^{a}
33	8.99±0.04^{c}	8.87±0.06^{c}	8.10±0.05^{b}	5.73±0.07^{a}	8.95±0.08^{c}	5.98±0.07^{b}	4.73±0.06^{a}	9.05±0.04^{c}	7.95±0.04^{b}	6.71±0.07^{a}
39	9.07±0.03^{d}	8.68±0.06^{c}	7.95±0.09^{b}	3.74±0.10^{a}	9.03±0.06^{c}	6.36±0.02^{b}	4.66±0.11^{a}	8.95±0.06^{c}	7.38±0.03^{b}	6.20±0.04^{a}
41	8.93±0.10^{c}	8.85±0.04^{c}	8.04±0.03^{b}	4.92±0.05^{a}	8.94±0.06^{c}	6.91±0.04^{b}	5.94±0.08^{a}	8.98±0.05^{c}	7.71±0.07^{b}	6.32±0.03^{a}
42	9.10±0.02^{c}	9.04±0.03^{c}	8.65±0.11^{b}	7.14±0.06^{a}	9.14±0.04^{c}	8.64±0.08^{b}	8.12±0.04^{a}	9.11±0.03^{c}	8.40±0.03^{b}	7.62±0.08^{a}
48	9.04±0.06^{d}	8.74±0.07^{c}	8.17±0.04^{b}	3.99±0.04^{a}	9.02±0.08^{c}	6.72±0.05^{b}	4.21±0.03^{a}	8.96±0.05^{c}	7.39±0.03^{b}	5.95±0.05^{a}
54	8.98±0.04^{d}	8.80±0.05^{c}	8.27±0.03^{b}	5.68±0.07^{a}	9.04±0.05^{c}	7.08±0.03^{b}	5.18±0.02^{a}	9.07±0.04^{c}	8.00±0.04^{b}	6.73±0.08^{a}
56	9.01±0.04^{c}	8.84±0.06^{bc}	8.66±0.09^{b}	5.83±0.09^{a}	8.99±0.08^{c}	6.82±0.05^{b}	5.84±0.06^{a}	9.10±0.03^{c}	8.13±0.04^{b}	6.95±0.05^{a}
60	9.09±0.03^{c}	9.0±0.04^{c}	8.40±0.02^{b}	7.07±0.05^{a}	9.11±0.04^{c}	8.37±0.03^{b}	7.99±0.05^{a}	9.18±0.03^{c}	7.84±0.05^{b}	7.18±0.03^{a}
66	8.96±0.06^{d}	8.62±0.07^{c}	7.62±0.06^{b}	4.02±0.04^{a}	9.05±0.03^{c}	6.67±0.08^{b}	4.15±0.03^{a}	8.94±0.04^{c}	7.31±0.03^{b}	5.60±0.07^{a}
68	8.99±0.08^{d}	8.71±0.06^{c}	6.94±0.04^{b}	3.90±0.05^{a}	9.09±0.07^{c}	7.22±0.04^{b}	5.12±0.05^{a}	9.15±0.03^{c}	7.70±0.08^{b}	6.20±0.04^{a}
79	8.91± 0.09^{c}	8.83±0.05^{c}	8.18±0.04^{b}	5.73±0.09^{a}	8.93±0.06^{c}	6.79±0.07^{b}	5.97±0.05^{a}	9.07±0.04^{c}	8.23±0.03^{b}	6.87±0.05^{a}
84	9.06±0.03^{d}	8.69±0.08^{c}	8.21±0.04^{b}	4.12±0.03^{a}	9.10±0.05^{c}	6.34±0.04^{b}	4.89±0.04^{a}	8.96±0.06^{c}	7.37±0.03^{b}	5.85±0.05^{a}
93	9.13±0.05^{c}	8.98±0.04^{c}	8.74±0.08^{b}	7.25±0.04^{a}	9.14±0.04^{b}	8.30±0.04^{a}	8.21±0.03^{a}	9.17±0.03^{c}	8.33±0.03^{b}	7.25±0.03^{a}
96	9.07±0.06^{d}	8.81±0.05^{c}	7.73±0.10^{b}	3.83±0.09^{a}	9.08±0.03^{c}	6.90±0.04^{b}	4.64±0.09^{a}	8.94±0.04^{c}	7.17±0.03^{b}	5.37±0.03^{a}
112	9.06±0.05^{d}	8.88±0.03^{c}	8.37±0.02^{b}	6.89±0.05^{a}	9.09±0.05^{c}	8.20±0.03^{b}	7.75±0.06^{a}	9.01±0.04^{c}	8.35±0.03^{b}	7.77±0.07^{a}
117	8.98±0.08^{d}	8.76±0.06^{c}	7.95±0.07^{b}	5.70±0.06^{a}	9.04±0.05^{c}	6.98±0.05^{b}	6.06±0.07^{a}	9.13±0.04^{c}	7.80±0.06^{b}	6.75±0.06^{a}
GG	9.04±0.04^{c}	8.93±0.06^{c}	8.34±0.03^{b}	6.96±0.05^{a}	9.02±0.06^{c}	8.62±0.06^{b}	8.02±0.05^{a}	9.08±0.04^{c}	8.29±0.03^{b}	7.33±0.03^{a}

Each value represents Mean±SD of three independent readings. For each row at each parameter, different subscripts indicate significant difference at P<0.05. *L. rhamnosus* GG was used as reference.

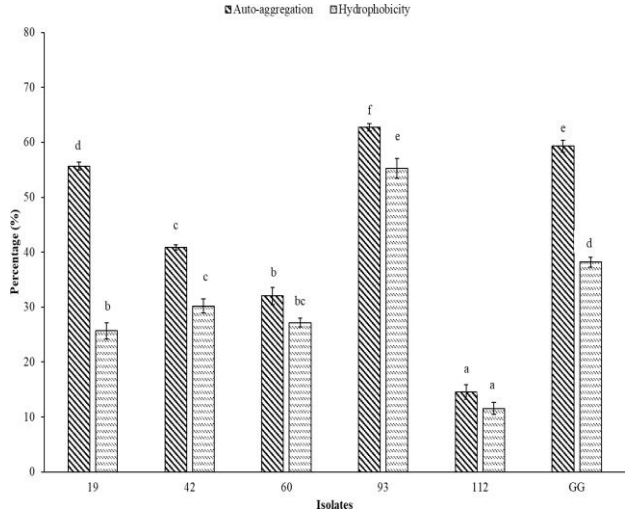

Fig. 1: Auto aggregation activity and hydrophobicity of *Lactobacillus* isolates. Each value represents Mean±SD of three independent readings *Lactobacillus rhamnosus* GG was used as reference. Different subscripts indicate significant difference at P<0.05.

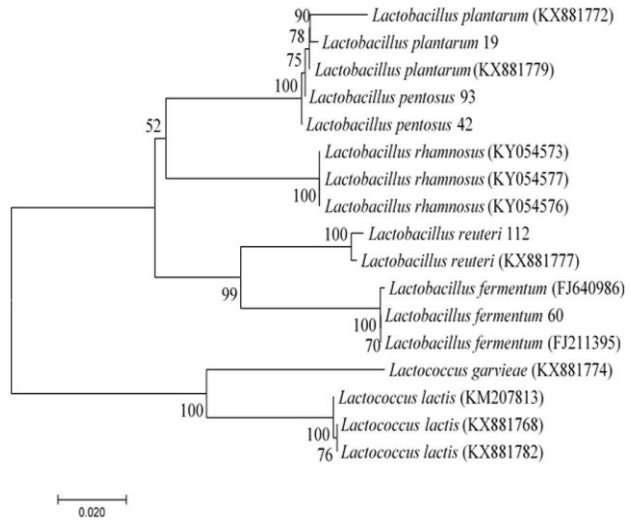

Fig. 2: Phylogenetic tree derived from 16S rDNA gene sequence of LAB isolates from buffalo milk.

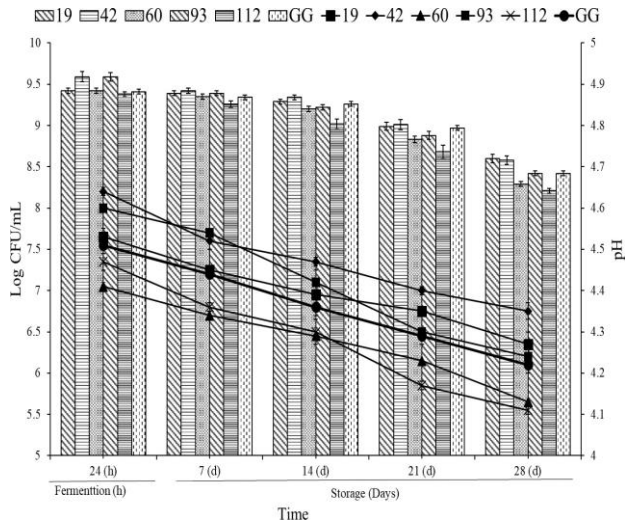

Fig. 3: Survival of LAB isolates in terms of Log CFU/mL and pH values after fermentation and storage at 4 °C for 28 days in yogurt. Each value represents Mean±SD of three independent readings. Bar graph indicates Log CFU/mL survival of each isolate while pH is indicated by line graph.

Table 2: Effect of Gastrointestinal fluids on the viability in terms of log CFU/mL of LAB isolates

Isolate	SGF		SIF	
	0 h	3 h	0 h	4 h
19	8.89±0.05^{a}	6.60±0.08^{a}	8.93±0.05^{a}	8.40±0.03^{a}
42	9.02±0.04^{a}	6.95±0.04^{d}	9.06±0.04^{b}	8.61±0.07^{b}
60	8.97±0.07^{a}	6.88±0.05^{cd}	9.03±0.04^{ab}	8.65±0.09^{b}
93	9.04±0.03^{a}	7.03±0.04^{d}	9.10±0.03^{b}	8.74±0.07^{b}
112	8.90±0.07^{a}	6.63±0.08^{ab}	8.97±0.06^{ab}	8.36±0.03^{a}
GG	8.93±0.04^{a}	6.78±0.05^{bc}	9.01±0.03^{ab}	8.39±0.03^{a}

Each value represents Mean±SD of three independent readings. For each column different subscripts indicate significant difference at P<0.05; SGF= Simulated gastric fluid, SIF= Simulated intestinal fluid *L. rhamnosus* GG was used as reference.

Table 3: Adhesion percentage of LAB strains to Caco-2 cells

Bacteria	Caco-2 adherence (%)
Isolate 19	34.13±1.2^{b}
Isolate 42	46.77±1.4^{d}
Isolate 60	37.03±1.3^{b}
Isolate 93	60.99±1.1^{e}
Isolate 112	21.83±1.6^{a}
L. rhamnosus GG	42.27±1.5^{c}

Mean±SD of results from three replicates, Different subscripts indicate significant different at P<0.05. *L. rhamnosus* GG was used as reference.

Table 4: Antibacterial activity of *Lactobacillus* isolates against food borne pathogens

LAB isolate	Bacterial pathogens				
	L. monocytogenes	*S. aureus*	*S. typhimurium*	*L. innocua*	*E. coli*
	Diameter of inhibition zone (mm)				
19	15.3±0.5	16.3±0.5	13.0±0.0	13.3±0.5	12.6±0.5
42	17.0±1.0	17.6±0.5	16.6±0.5	15.3±0.5	15.6±0.5
60	16.6±0.5	13.0±1.0	15.3±0.5	14.0±0.0	16.0±0.5
93	18.6±0.5	17.3±0.5	15.6±0.5	17.0±0.0	15.3±0.5
112	13.6±0.5	15.0±1.0	14.6±0.5	13.6±0.5	14.3±0.5
GG	15.6±0.5	16.3±0.5	16.6±0.5	15.3±0.5	16.0±0.0

Mean±SD of results from three replicates; *L. rhamnosus* GG was used as reference.

Table 5 Heat resistance of LAB isolates

Isolate	Before treatment	After treatment	Survival percentage (%)
19	9.07±0.04^{ab}	7.86±0.05^{c}	86.65
42	9.12±0.03^{b}	8.12±0.03^{d}	89.03
60	9.14±0.04^{b}	7.65±0.06^{a}	83.69
93	9.08±0.03^{ab}	7.82±0.05^{bc}	86.12
112	8.98±0.05^{a}	7.68±0.07^{ab}	85.52
GG	9.05±0.04^{ab}	7.90±0.04^{c}	87.29

Data represented in log CFU/mL, Mean±SD of results from three replicates; For each column, different subscripts indicate significant difference at P<0.05; *L. rhamnosus* GG was used as reference.

Molecular identification of LAB: The LAB was identified through 16S rDNA gene sequencing, which showed the similarity with known *Lactobacillus* species. Isolates 19, 42, 60, 93 and 112 were identified as *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus fermentum*, *Lactobacillus pentosus* and *Lactobacillus reuteri*. The phylogenetic tree indicated the genetic relationship between the isolated strains and other published isolates sequence from milk as illustrated in Fig. 2.

Production of probiotic based yogurt: Fermentation profiles of LAB in yogurt and changes in pH during 28 days at 4°C are shown in Fig. 3. All isolates produced yogurt and grew well in fermented milk, viable count ranged from 8.21-8.60 Log CFU/mL after 28 days at 4°C while all lactobacilli isolates exhibited ability to acidify milk resulting in lower pH (4.11-4.35). *L. plantarum* exhibited highest survival of 8.60 Log CFU/mL with pH 4.27.

DISCUSSION

In the present study LAB isolated from buffalo milk were evaluated for their probiotic potential including milk fermentation to produce probiotic enriched yogurt. Twenty out of 130 isolates showed typical characteristics of LAB out of which five showed very promising probiotic potential. The survival of probiotic strains in stomach is an important property of any potential probiotic bacteria (Noureen *et al.*, 2018). The LAB strains *L. plantarum* (isolate 19), *L. pentosus* (isolate 42), *L. fermentum* (isolate 60), *L. pentosus* (isolate 93) and *L. reuteri* (isolate 112) showed good resistance to acid (pH 2) and survived more than 6 Log CFU/mL after 3 h incubation, which indicated that they could survive in the low acidic pH of stomach and reach the intestine and colon to produce beneficial effects. Abushelaibi *et al.* (2017) also reported high survival (5-9 Log CFU/mL) of selected LAB isolated from camel milk in pH 2 after 2 h incubation. Certain LAB strains resist the harmful effect of bile by releasing bile salt hydrolase enzyme, which acts as catalyst for the hydrolysis of bile salts into amino acid residues and free bile salts (Guo *et al.*, 2009). The selected isolated LAB tolerated bile (0.5 and 1%) and lysozyme (50 µg/mL and 100 µg/mL) after 3 h incubation. These results are in agreement with Angmo *et al.* (2016) who reported similar survival of LAB strains in bile and lysozyme in 1% bile and 100 µg/mL lysozyme after 3 h incubation.

All five isolates demonstrated survival in SGF and SIF after 3 and 4 h incubation. The survival of strains was more in SIF than GIF. Feng *et al.* (2017) also reported good survival of LAB strains in GIF and SIF. Similarly, LAB isolates showed high auto aggregation and hydrophobicity characteristics. Hydrophobicity was correlated with aggregation activity; strains with high hydrophobicity had high auto aggregation activity. Abushelaibi *et al.* (2017) also reported high auto aggregation and hydrophobicity of LAB strains from camel milk. Adhesion to Caco-2 cells leads to several health benefits such as immune system modulation and pathogen exclusion. The LAB isolates showed adhesion (21-60%) to the Caco2 cell line. Our findings are consistent with Argyri *et al.* (2013) who reported high adhesion to Caco2 cells (30-74%) for lactobacilli strains isolated from fermented olives.

The LAB isolates were susceptible to most of antibiotics, however vancomycin resistance was found in two isolates. Similar findings about vancomycin resistance in LAB strains were also reported by Plessas *et al.* (2017). Absence of hemolysis is important characteristic and prerequisite for the selection of any probiotic bacteria. All LAB isolates in this study were found non-hemolytic. Antimicrobial activity of LAB improves the safety and shelf life of food products when used for bio preservation (Akbar and Anal, 2014). Selected LAB isolates inhibited both Gram-positive and Gram-negative pathogens. Many studies have confirmed the antimicrobial activity of LAB against the tested pathogens (Abushelaibi *et al.*, 2017; Feng *et al.*, 2017).

β-galactosidase and EPS production are essential characteristics of probiotic bacteria (Kumar *et al.*, 2017). The LAB in this study were positive for both β-galactosidase and EPS production. Our results are consistent with Kumar *et al.* (2017) and Angmo *et al.* (2016) who reported β - galactosidase and EPS production by LAB using same tests. Similarly, the results for the heat tolerance assay indicate that LAB isolates can tolerate heat shock and remain viable without significant loss in cells. Shafakatullah and Chandra (2014) reported the survival of LAB strains isolated from buffalo milk at 50°C but strains lost viability with increased temperature. Production of fermented milk and survival in acidic environment are characteristic properties of probiotic bacteria (Abushelaibi *et al.*, 2017). The isolated probiotic strains produced probiotic based yogurt and survived up to 4 weeks in cold storage. The pH was lowered due to breakdown of hexoses to lactic acid. The viability of the isolates was higher than reported by Mortazavian *et al.* (2007) for the survival of LAB in yogurt.

Conclusions: The present study reveals that buffalo milk is a potential source of probiotic bacteria. *L. plantarum* (isolate 19), *L. pentosus* (isolate 42), *L. fermentum* (isolate 60), *L. pentosus* (isolate 93) and *L. reuteri* (isolate 112) obtained from raw buffalo milk exhibited promising probiotic characteristics as they possessed resistance to pH 2, bile (1%), lysozyme (100 µg/mL) and gastrointestinal fluids. The probiotic strains showed high auto aggregation, hydrophobicity, adhesion to Caco-2 cell line, heat resistance and inhibition of food borne pathogens. Furthermore, all five isolates were able to ferment milk and survive in cold storage conditions up to 28 days. Thus, these probiotic cultures can be used as starter culture for the development of functional food due to their ability to survive in environmental stress conditions and inhibition of food borne pathogens.

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