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Isolation and identification of potential probiotic *Lactobacillus* species from feces of infants in southwest Iran

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ABSTRACT

Objectives: To evaluate the potential probiotic properties of *Lactobacillus* strains isolated from feces of infants and also to determine their antimicrobial activity against some enteropathogenic bacteria.
Methods: The Fecal samples were prepared from 120 infants aged less than 24 months. In total, 105 *Lactobacillus* strains were identified by phenotypic tests. Thirty isolates were randomly selected to study their potential probiotic properties. These isolates were examined for resistance to acid (pH: 2.5, 2 h) and bile (oxgall 0.3%, 8 h), adhesion to HT-29 cells, antibiotic susceptibility, and antimicrobial activities.
Results: On basis of *16S rRNA* sequencing, 30 isolates identified as *Lactobacillus fermentum* (n = 11; 36.7%), *Lactobacillus plantarum* (n = 9; 30%), *Lactobacillus rhamnosus* (n = 6; 20%), and *Lactobacillus paracasei* (n = 4; 13.3%). All tested strains survived at acid and bile conditions. Six *Lactobacillus* strains revealed high adherence to HT-29 cells. Three strains including the *L. fermentum* (N2, N7), and the *L. plantarum* (N20) showed good probiotic potential and inhibited the growth of *Yersinia enterocolitica* ATCC 23715, *Shigella flexneri* ATCC 12022, *Salmonella enterica* ATCC 9270, and enteropathogenic *Escherichia coli* (EPEC) ATCC 43887. The antibiotic resistance test showed that all the isolates were susceptible to tetracycline, and chloramphenicol.
Conclusions: Lactobacillus strains like *L. fermentum* (N2, N7), and the *L. plantarum* (N20), could be potential probiotic, but further *in vitro* and *in vivo* studies on these probiotic strains are still required.

Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of gram-positive bacteria which are natural inhabitants of the human gastrointestinal tract. They have very important role in maintaining the microbial ecosystem of the colon (Kirtzalidou et al., 2011). Since they are assumed to be extremely advantageous nonpathogenic species for the human population, currently being assessed as possibly probiotic microorganisms (Fijan, 2014). Probiotics are live microorganisms that, when ingested in adequate amounts confer a health benefit on the host and may be used as a possible alternative to conventional treatments for many intestinal diseases (Nagpal et al., 2012). Lactobacilli are also generally associated with infantile intestinal microbiota and play important roles in nutrition, metabolism, immunity, and defense against pathogens (Kerry et al., 2018). Many of microflora that colonize the neonatal gut, have different origins and may be affected by various factors such as the type of delivery, feeding pattern, antibiotic treatment, contact with parents, and hospital staff (Savino et al., 2011). Diarrhea is one of the prime reasons for morbidity and mortality among children in developing countries (Lanata et al., 2013). It has also been reported that diarrhea

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caused by enteric pathogens such as *Shigella, Salmonella,* pathogenic *Escherichia coli, Campylobacter, Vibrio cholera*, and *Rotavirus* are the main cause of infants and children mortality in developing countries (Lanata et al., 2013). There are several shreds of evidence that many strains of probiotic microorganisms can hinder the growth and activities of some of enteropathogenic bacteria (Fijan et al., 2018; Fooladi et al., 2014). Probiotics, such as various strains of *Lactobacillus*, are now being examined for their probiotic properties, and have been proposed as preventive/adjunctive therapy for acute infectious diarrhea in the pediatric population (Delcaru et al., 2016). Since the research of new probiotic strains is essential to meet the increasing needs of the market, this study aimed to evaluate the potential probiotic properties of the *Lactobacillus* strains isolated from feces of infants and also to determine their antimicrobial activity against some enteropathogenic bacteria.

Material and methods

Ethics statement

This study was approved by the Ethical Clearance Committee of the Abadan School of Medical Sciences, Abadan, Iran (IR.ABADANUMS.REC.1394.12). Written informed consent was obtained from all the infants' parents prior to enrollment.

Phenotypic diagnosis of Lactobacillus *strains*

This study was performed from April 2016 to March 2017. Overall 120 fecal samples were collected from infants under 2 years of age. All samples were rapidly transferred to the laboratory and cultured anaerobically in Man Rogosa Sharp (MRS) broth (Merck, Darmstadt, Germany) at 37 °C for 48-72 h. Then, the samples were subcultured on MRS agar (Merck, Darmstadt, Germany) and incubated anaerobically at 37 °C for 48 h. The suspected bacterial colonies were first analyzed based on their Gram reaction, morphology, catalase activity, and gas production from glucose in MRS broth, and then characterized by their growth at different temperatures (15 °C, 45 °C) (Kılıç and Karahan, 2010; Davoodabadi et al., 2015a). The suspected lactobacilli isolates were stored in MRS broth containing 20% glycerol at −80 for long preservation and further analysis.

Molecular characterization of lactobacilli by 16S rRNA *gene sequencing*

The identification of the *Lactobacillus* strains was confirmed by *16S rRNA* sequence analysis. Chromosomal DNA from each isolate was extracted according to a previously described procedure (Heilig et al., 2002). In the present study, the polymerase chain reaction (PCR) primer sequences were as previously described: forward, 5 -AGAGTTTGATCCTGGCTCAG-3 and reverse, 5 -CCGTCAATTCCTTTGAGTTT-3 (Sinaclon, Tehran, Iran) (Tulumoglu et al., 2013). The PCR assay was performed in an Eppendorf thermal cycler PCR system (Roche, Mannheim, Germany) in a final volume of 25 μl which consisted of 5 μl template DNA, 0.4 pmol/μl of each primer, 10 μl master mix 2 × (Ampliqon, Denmark) and sterile distilled water up to 25 μl. The PCR was carried out with the following program: 94 °C/ 5 min, 35 cycles of 94 °C /30 s, 55 °C /30 s and 72 °C /30 s; and final extension 72 °C/7 min. Then 10 μl of PCR products were separated by electrophoresis in a 1.5% (w/v) agarose gel and visualized by staining with ethidium bromide (Sigma, USA). The 900 bp amplified fragments were purified using a gel extraction kit (Qiagen, Germany) and sequenced by Bioneer Company (Bioneer, Korea) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the standard protocol of the supplier. Finally, the sequencing results were compared with the known sequences in the database of the GeneBank using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Acid and bile tolerance

The acid and the bile tolerance of isolates were examined according to the previously described methods with minor modification (Klingberg et al., 2005; Kaboré et al., 2012). The acid resistance was examined in MRS broth adjusted with hydrochloric acid (HCl) 1N to obtain a final pH of 2.5. Briefly, 0.1 ml of an overnight culture of *Lactobacillus* strains (10^6 CFU/ml) was inoculated into 10 ml of MRS broth previously adjusted to pH 2.5 with HCl 1N and incubated anaerobically at 37 °C for 2 h. After incubation, the number of viable bacteria was measured by plating serial dilutions on MRS agar. Acid tolerance was estimated by comparing the viable *Lactobacillus* strains counts on MRS agar for surviving cells after incubation at pH 2.5 for 2 h. The plate count method was used to assess the survival rate (%) on MRS agar, after 0 and 2 h of incubation.

Lactobacillus strains that survived in the acid tolerance assay (pH 2.5, 2 h) were further analyzed for bile resistance property. A 100-μl volume (10^6CFU/ml) of an overnight culture of each isolate was inoculated on 10 ml of MRS broth with and without 0.3% (w/v) oxgall bile salt (Sigma, USA) and incubated at 37 °C for 8 h. Bile tolerance was recorded by comparing the viable *Lactobacillus* strains counts on MRS agar for surviving cells after incubation at 0.3% (w/v) oxgall bile for 8 h. The plate count method was used to assess the survival rate (%) on MRS agar, after 0 and 8 h of incubation.

The survival rate (%) was defined as comparison of the number of viable cells after incubation (N) to the initial number of viable bacteria (N_0) according to the formula (N/N_0) × 100%.

Adhesion assay

The ability of the *Lactobacillus* isolates to adhere to human epithelial cells was studied according to the procedure described by Verdenelli et al. (2009). The HT-29 cell lines (Pasteur Institute, Tehran, Iran) were grown routinely in a controlled atmosphere of 5 % CO2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 2 mM L-glutamine, 10% (v/v) fetal bovine serum (FBS) (Sigma), and 50 unit/ml penicillin-streptomycin (Sigma). For the bacterial adhesion assay, monolayers of HT-29 cells were seeded on glass coverslips that were placed in 6-well tissue culture plates (Sarstedt, Nümbrecht, Germany). After 24 h of incubation at 37 °C in 5% CO2-95% air atmosphere the monolayers were washed twice with sterile PBS (pH 7.4) and 10 ml of bacterial suspension (1×10^7 CFU/ml) was added to each plate. The inoculated plates were incubated at 37 °C (5 % CO2 and 95 % air) for 2 h followed by washing 3 times with PBS (pH 7.4) to remove non-adherent bacteria. The adherent bacteria were detached from polystyrene wells by utilizing a solution of trypsin-EDTA (0.05%) and resuspended in 10 ml of saline solution. Then, serial dilutions of bacteria were cultured on MRS agar and incubated in anaerobic condition at 37 °C for 24–48 h. The percentage of adhesion was determined by comparison of the number of adhered cells to the total cells of the examined bacterial suspension. *Lactobacillus rhamnosus GG* (ATCC 53103) used as the control. Each adherence assay was performed in duplicate for all strains, and the values were expressed as Mean value ± Stand Deviation (SD).

Antibacterial activity

The antimicrobial activity of the *Lactobacillus* strains against some enteropathogenic bacteria was assessed using the well-diffusion method as described previously (Davoodabadi et al., 2015b). Pathogenic strains, including *Yersinia enterocolitica* ATCC

23715, *Shigella flexneri* ATCC 12022, *Salmonella enterica* ATCC 9270, and enteropathogenic *Escherichia coli* (EPEC) ATCC 43887 were inoculated in nutrient broth (Merck, Germany). After overnight incubation at 37 °C, the microbial density was adjusted to 10^7 CFU/ml and subcultured on Muller Hinton agar (Merck, Germany). Cell-free culture supernatants (CFCS) were prepared by centrifugation (10.000 *g*, 10 min) of LAB cultures grown in MRS broth at 37 °C for 24 h. Then, 100 μL of the CFCS was placed into 8 mm diameter wells which were punched in the Muller Hinton agar plates, followed by incubation at 37 °C for 18-24 h. The antimicrobial activity was then measured as growth-free inhibition zone surrounding the well. The diameter of the clear zones was scored as follows: less and equal 11 mm (negative, -), 12-16 mm (weak, +), 17-22 mm (strong, ++), and more than 23 mm (very strong, +++). The *L. rhamnosus GG* and non-cultured MRS broth were used as positive and negative controls.

Characterization of antimicrobial substances

The nature of the antimicrobial substances (organic acids, hydrogen peroxide, and bacteriocin) was investigated by the previously described method (Shokryazdan et al., 2014). An overnight culture of strains in 25 mL of MRS was centrifuged at 4000 × *g* for 10 min at 4 °C. The supernatant of each strain was used for different assays. The bacteriocin assay was performed by treatment of the supernatant (5 ml) with 1 mg/ml trypsin (Sigma, USA). For organic acids assay, the supernatant (5 ml) was adjusted to pH 6.5 using 1 N NaOH, and for hydrogen peroxide assay, the supernatant (5 ml) was treated with 0.5 mg/ml catalase (Sigma, USA). Treated supernatants were filter sterilized through 0.22 μm pore-size filters (Sartorius, Germany), and 100 μL was placed into wells (7 mm diameter) of MRS agar plates, overlaid with 10 mL of soft nutrient agar (Merck, Germany), and inoculated with 1% (v/v) of an overnight culture of test pathogen. The plates were incubated for 48 h at 37 °C and diameters of inhibition zones (including the 7 mm well diameter) were measured.

Antibiotic susceptibility assay

Antibiotic susceptibility assay was performed by the disc diffusion method using commercially available antibiotic discs (MAST, Berkshire, UK) including penicillin (10 μg), vancomycin (30 μg), cephalothin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), and gentamicin (10 μg). *Lactobacillus* strains were inoculated in MRS broth. When broth cultures reached the turbidity equal to the 0.5 McFarland standards at 37 °C, the suspensions were streaked onto previously prepared MRS agar plates with a cotton swab, and then antibiotic disks were seeded onto the agar. After 24-48 h of incubation at 37 °C, the inhibition zone diameters were measured, and results were expressed in terms of resistance (≤15 mm), moderate susceptibility (16–20 mm), or susceptibility (≥21 mm) (Hashemi et al., 2014).

Statistical analysis

All Statistical calculations were performed with SPSS™ software version 22.0 (IBM Corporation, Armonk, NY, USA). A P value of < 0.05 was considered statistically significant.

Results

Preliminary identification of Lactobacillus *strains*

By Gram staining and other phenotypic tests, a total of 105 strains were identified as *Lactobacillus* species. All isolates showed Gram positive and catalase negative properties. Thirty isolates were randomly selected to study their potential probiotic properties.

Molecular identification by 16S rRNA *sequencing*

All 30 isolates were positive for the desired 900 bp *16S rRNA* fragment by PCR. The identification of the isolates was confirmed by sequencing of *16S rRNA* gene. According to Sanger sequencing of the *16S rRNA* gene, the following *Lactobacillus* strains were identified: *Lactobacillus fermentum* (n = 11; 36.7%) (N1 to N11), *Lactobacillus plantarum* (n = 9; 30%) (N12 to N20), *Lactobacillus rhamnosus* (n = 6; 20%) (N21 to N26), and *Lactobacillus paracasei* (n = 4; 13.3%) (N27 to N30). *Lactobacillus fermentum* was the most prevalent isolate.

Acid and bile resistance

The acid and bile tolerance profile of *Lactobacillus* strains are summarized in Table 1. The results revealed that all tested strains tolerated acid conditions and the residual cells were more than 50 % of the initial cells even after 2 h of incubation at the pH 2.5. Despite the variations in the degree of survival rate, there was a reduction in the viable count (log CFU/ml) after the prescribed time. In acid tolerance assay, *L. fermentum* (N2, N3), *L. plantarum* (N15), *L. rhamnosus* (N22, N24), and *L. paracasei* (N29) showed a little reduction in viable count compared to the initial number of viable bacterial cells after 2 h. Also, these strains showed more than 90% of the survival rate after 2 h in comparison with the reference strains *L. rhamnosus GG* (ATCC 53103) that showed a survival rate of 58% in acid tolerance test.

In bile resistance assay, while all strains involved in this study could tolerate 0.3 % oxgall, *L. fermentum* (N2, N7, N11), *L. plantarum* (N14, N18), *L. rhamnosus* (N26), and *L. paracasei* (N27) were the most resistant strains of all and showed more than 90% of the survival rate after 8 h in comparison to the reference strains *L. rhamnosus GG* (ATCC 53103) that showed a survival rate of 66% in bile tolerance test.

Adhesion assay

The results of HT-29 epithelial cells adherence indicated that the isolates possessed variable adhesion ability compared to the reference strain after 2 h incubation (Table 2). Among the tested isolates, 6 strains had higher adhesive properties in comparison with the positive control *L. rhamnosus GG*. These 6 strains were *L. fermentum* (N2, N7, N11), *L. plantarum* (N17, N20), and *L. paracasei* (N27). The N7 strain exhibited the highest adhesion (14.46 ± 0.93) and other 25 strains displayed moderate-to-low adhesive properties.

Antibacterial activity

As mentioned in Table 3, all 30 strains except to *L. plantarum* N14, showed inhibitory effects against at least two enteropathogens. All 30 strains exhibited the antimicrobial activity against *S. flexneri* ATCC 12022. In addition, three strains of *L. fermentum* (N2, N7, and N8), three strains of *L. plantarum* (N12, N16 and N20), *L. rhamnosus* (N25), and *L. paracasei* (N30), like the *L. rhamnosus GG* indicated inhibitory activity against all examined pathogens used in this study. *Lactobacillus* strains N3, N5, N13, N21, N23, and N27 inhibited the growth of all pathogens other than *S. enterica* ATCC 9270; the strains N15, N17, N19, N22 and N28 inhibited all tested pathogens except *EPEC* ATCC 43887; and *L. fermentum* N1 inhibited all tested pathogens other than *Y. enterocolitica* ATCC 23715. Further, among all *Lactobacillus* strains, *L. plantarum* N17 and *L. paracasei* N30 had the strongest inhibitory activity against *S. flexneri* ATCC 12022.

Table 1
Acid and bile survival rate and adhesion of 30 tested Lactobacillus isolates.

Isolate identity	Isolate name	Acid resistance (pH:2.5)			Oxgall bile tolerance (0.3%)		Adhesion (Mean ± SD)
		0h	2h	Survival rate[a] (%)	8h	Survival rate[b] (%)	2h
Control	*L. rhamnosus GG*	7.85	4.56	58	5.20	66	8.14 ± 1.12
N1	*L. fermentum*	8.46	7.55	89	6.54	77	5.14 ± 0.14
N2	*L. fermentum*	8.36	8.10	97	7.65	91	12.40 ± 0.19
N3	*L. fermentum*	7.59	7.12	94	5.14	68	1.55 ± 0.25
N4	*L. fermentum*	8.17	7.25	89	6.34	78	7.42 ± 0.30
N5	*L. fermentum*	9.10	7.98	88	7.20	79	0.50 ± 0.10
N6	*L. fermentum*	8.47	7.10	84	5.54	65	5.7 ± 2.20
N7	*L. fermentum*	8.45	7.46	88	7.88	93	14.46 ± 0.93
N8	*L. fermentum*	7.66	6.14	80	5.76	75	4.77 ± 1.20
N9	*L. fermentum*	7.55	5.11	68	6.55	87	7.70 ± 2.01
N10	*L. fermentum*	7.98	5.98	75	7.12	89	0.91 ± 0.14
N11	*L. fermentum*	8.50	6.24	73	7.77	91	11.5 ± 1.80
N12	*L. plantarum*	8.45	7.45	88	4.98	69	3.24 ± 0.02
N13	*L. plantarum*	8.46	6.98	82	7.54	89	6.55 ± 1.71
N14	*L. plantarum*	8.75	7.12	81	8.10	92	1.24 ± 0.50
N15	*L. plantarum*	8.46	8.10	96	4.33	51	5.52 ± 1.12
N16	*L. plantarum*	8.35	6.55	78	5.45	65	0.81 ± 0.03
N17	*L. plantarum*	9.10	7.99	88	5.14	56	12.18 ± 2.55
N18	*L. plantarum*	8.15	6.44	79	7.49	92	6.73 ± 2.53
N19	*L. plantarum*	8.45	5.10	60	7.12	84	4.68 ± 1.11
N20	*L. plantarum*	7.55	6.45	85	3.78	50	12.0 ± 3.05
N21	*L. rhamnosus*	9.10	5.95	65	8.14	89	2.24 ± 1.04
N22	*L. rhamnosus*	8.36	8.12	97	6.24	75	7.87 ± 1.95
N23	*L. rhamnosus*	7.65	5.35	70	3.98	52	5.84 ± 0.98
N24	*L. rhamnosus*	8.46	8.14	96	7.15	84	0.54 ± 0.20
N25	*L. rhamnosus*	8.88	5.18	58	6.19	70	4.90 ± 1.14
N26	*L. rhamnosus*	8.40	6.40	76	7.87	94	5.12 ± 1.66
N27	*L. paracasei*	8.98	5.45	61	8.17	91	11.12 ± 2.21
N28	*L. paracasei*	7.90	7.12	90	7.10	90	7.65 ± 1.98
N29	*L. paracasei*	9.15	8.40	92	7.44	81	5.92 ± 2.11
N30	*L. paracasei*	7.55	4.50	60	4.87	64	0.77 ± 0.21

[a] 2 h/0 h × 100.
[b] 8 h/0 h × 100.

Table 2
Antimicrobial activity of lactobacilli strains against the enteropathogenic bacteria.

Isolate name	Species	*Y. enterocolitica* ATCC 23715	*S. flexneri* ATCC 12022	*S. enterica* ATCC 9270	*EPEC* ATCC 43887
	L. rhamnosus GG	++[a]	++	++	++
N1	*L. fermentum*	-	+	+	+
N2	*L. fermentum*	+	++	+	+
N3	*L. fermentum*	+	+	-	+
N4	*L. fermentum*	-	+	+	-
N5	*L. fermentum*	++	++	-	+
N6	*L. fermentum*	-	++	++	-
N7	*L. fermentum*	+	++	+	+
N8	*L. fermentum*	++	+	++	++
N9	*L. fermentum*	-	++	+	-
N10	*L. fermentum*	-	++	-	++
N11	*L. fermentum*	-	+	+	-
N12	*L. plantarum*	+	+	+	+
N13	*L. plantarum*	+	++	-	+
N14	*L. plantarum*	-	+	-	-
N15	*L. plantarum*	+	++	+	-
N16	*L. plantarum*	++	+	++	++
N17	*L. plantarum*	++	+++	+	-
N18	*L. plantarum*	-	+	-	+
N19	*L. plantarum*	+	++	++	-
N20	*L. plantarum*	+	++	+	+
N21	*L. rhamnosus*	+	++	-	+
N22	*L. rhamnosus*	++	+	+	-
N23	*L. rhamnosus*	+	++	-	+
N24	*L. rhamnosus*	-	+	+	-
N25	*L. rhamnosus*	++	++	+	+
N26	*L. rhamnosus*	-	+	++	-
N27	*L. paracasei*	+	++	-	+
N28	*L. paracasei*	+	++	+	-
N29	*L. paracasei*	-	++	+	-
N30	*L. paracasei*	++	+++	++	+

[a] Interpretation of zone inhibition diameter: ≤ 11 mm (-), 12-16 mm (+), 17-22 mm (++), and more than 23 mm (+++).

Table 3
Antibiotic susceptibility test of *Lactobacillus* isolates from infant feces.

Isolate identity	Isolate name	P	V	CF	TE	C	GM
N1	*L. fermentum*	S	R	I	S	S	I
N2	*L. fermentum*	S	R	I	S	S	S
N3	*L. fermentum*	S	R	S	S	S	R
N4	*L. fermentum*	S	R	S	S	S	R
N5	*L. fermentum*	S	R	S	S	S	S
N6	*L. fermentum*	R	R	S	S	S	R
N7	*L. fermentum*	S	R	S	S	S	S
N8	*L. fermentum*	S	R	S	S	S	R
N9	*L. fermentum*	S	R	S	S	S	R
N10	*L. fermentum*	S	R	S	S	S	S
N11	*L. fermentum*	S	R	S	S	S	R
N12	*L. plantarum*	S	R	S	S	S	R
N13	*L. plantarum*	S	R	S	S	S	S
N14	*L. plantarum*	S	R	S	S	S	R
N15	*L. plantarum*	S	R	S	S	S	R
N16	*L. plantarum*	S	R	S	S	S	R
N17	*L. plantarum*	S	R	S	S	S	R
N18	*L. plantarum*	S	R	S	S	S	R
N19	*L. plantarum*	S	R	S	S	S	S
N20	*L. plantarum*	S	R	S	S	S	R
N21	*L. rhamnosus*	S	R	S	S	S	S
N22	*L. rhamnosus*	S	R	S	S	S	S
N23	*L. rhamnosus*	S	R	S	S	S	S
N24	*L. rhamnosus*	S	R	S	S	S	R
N25	*L. rhamnosus*	S	R	S	S	S	S
N26	*L. rhamnosus*	S	R	S	S	S	R
N27	*L. paracasei*	S	R	R	S	S	R
N28	*L. paracasei*	S	R	R	S	S	S
N29	*L. paracasei*	S	R	S	S	S	R
N30	*L. paracasei*	S	R	S	S	S	R

S: sensitive, I: intermediate, R: resistant.
P: Penicillin (10 μg), V: Vancomycin (30 μg), CF: Cephalothin (30 μg), TE: Tetracycline (30 μg), C: Chloramphenicol (30 μg), GM: Gentamicin (10 μg).

Characterization of antimicrobial substances

The results showed that the trypsin treated supernatants of all *Lactobacillus* strains did not affect their inhibitory activities against the enteropathogens. This indicated that inhibitory effects of the *Lactobacillus* strains were not due to bacteriocin production. Also, culture supernatants treated with catalase did not affect the inhibitory activities of the *Lactobacillus* strains, confirming that inhibition by the *Lactobacillus* strains was not due to hydrogen peroxide production. However, neutralized supernatants (pH 6.5) of all *Lactobacillus* strains did not have any inhibitory activity against the tested strain, which indicated that the repressive properties of the *Lactobacillus* strains were owing to their organic acid productions.

Antibiotic susceptibility assay

In order to investigate probiotic properties, the antibiotic susceptibility of 30 *Lactobacillus* strains with acid and bile tolerance was performed. The susceptibility patterns of 30 lactobacilli isolates against six antibiotics are shown in Table 3. All the isolates were susceptible to tetracycline, chloramphenicol, and penicillin (except for *L. fermentum* N6), and were vancomycin resistant. Two strains (*L. paracasei* N27 and N28) were cephalothin resistant and two (*L. fermentum* N1 and N2) were intermediate. Also, eleven isolates (N2, N5, N7, N10, N13, N19, N21, N22, N23, N25, and N28) were susceptible to gentamicin.

Discussion

LAB strains such as lactobacilli are the most common microorganisms which are considered as probiotic. Lactobacilli, which inhabit naturally in the human GI-tract, play a prominent role in maintaining the microbial ecosystem of the colon. Lactobacilli with human origin have more beneficial effects than LAB from other sources (Davoodabadi et al., 2015b). The main features for selecting highly potent probiotic species are their tolerance to acidic conditions and bile salts, ability to adhere to the intestinal cells, antibiotic resistance, survival in the GI tract, and especially antimicrobial activity against pathogens (Davoodabadi et al., 2015; Plessas et al., 2017).

In the present study, one hundred and twenty fecal samples were collected from infants from which 105 strains were identified as *Lactobacillus* species. Due to financial constraints, only thirty *Lactobacillus* isolates selected for further evaluation and screening of their probiotic potential that was one of limitations of current study. Similar to our current work, a previous study found that *L. fermentum* and *L. plantarum* were the most common strains identified in fecal samples taken from Iranian infants (Davoodabadi et al., 2015a).

In this study, we tested 30 *lactobacillus* isolates for their tolerance to acid (pH 2.5), resistance to 0.3% bile salts, adherence to HT-29 cells, and antagonism against some enteric pathogens. Our observations indicated that 30 isolated lactobacilli were resistant to pH 2.5 and 0.3% bile salt, and also exhibit good adhesion to HT-29 cells, which was in agreement with previous studies that reported by Lee et al. (2011) and Mandal et al. (2015). This study revealed that most isolates were more resistant to acidic condition than the control strain, which was in agreement with the previous report from Malaysia (Shokryazdan et al., 2014). Also, our results confirmed the claim of the previous study that the ability to tolerate acid by the *Lactobacillus* strains is not related to their source of isolation, since the level of acid tolerance in lactobacilli was very different in this study (Shokryazdan et al., 2014).

According to our statistical analysis, we found that most tested *Lactobacillus* strains had viability percentages higher than 70% against bile and were more tolerance to bile than control strain, which may be due to the use of bile-stress mechanisms such as active efflux of bile, bile salt hydrolysis and changes in the composition of the cell wall (Ruiz et al., 2013). In the current study, we used only single control strain *L. rhamnosus GG* for comparative analysis that could be considered as a limitation.

The ability of probiotic bacteria to adhere to human intestinal epithelial cells is another characteristic for the selection of probiotics. In agreement with previous studies by Davoodabadi et al. (2015b), and Ren et al. (2014), we have observed that six strains include *L. fermentum* (N2,N7,N11), *L. plantarum* (N17,N20), and *L. paracasei* N27 exhibited stronger adhesion as compared to *L. rhamnosus GG*. In this study, *L. fermentum* N7 isolate had greater adhesion to HT-29 cells than did other *Lactobacillus* strains. This result is consistent with the finding of Halimi et al., who found that *L. fermentum* isolates (FH5, FH13, and FH18) had more ability to adhere to epithelial cells than did other isolates (Halimi and Mirsalehian, 2016). Furthermore, the adherence level was different among the strains, confirming that it is strain-specific. Similar findings were reported by Shokryazdan et al (2014).

Since probiotic bacteria may serve as host antibiotic resistance genes, which can transfer them to pathogenic bacteria, the antibiotic susceptibility of lactobacilli is therefore considered as an important criterion from the safety standpoint for potential probiotics. Moreover, awareness of antibiotic resistance may possibly be used to eliminate the probiotic once it outlives its efficacy as a delivery carrier (Lee et al., 2011; Sharma et al., 2014). Today, the resistance of lactobacilli to antibiotics is a double-edged sword. The antibiotic resistance of some probiotics can be useful for people with unbalanced intestinal microflora due to the administration of various antibiotics and, on the other hand, may provide a basis for transferring resistance genes to other bacteria (Imperial and Ibana, 2016). Previous studies by Argyri et al. (2013) and Zhang et al. (2016) reported that some species of *Lactobacillus*

spp. had high levels of resistance to vancomycin, while in the current study; all tested lactobacilli were found to be vancomycin resistant. The results were predictable as lactobacilli are known to be naturally resistant toward vancomycin (Verdenelli et al., 2009). Althogh, a research by Klare et al. (2007) showed that *Lactobacillus* species such as *L. rhamnosus*, *L. paracasei*, *L. plantarum*, *L. reuteri* (MICs: $\geq$ 128 mg/L) and *L. fermentum* (MICs: 32– > 256 mg/L) were resistant against vancomycin, in contrast, the tested species of the *L. acidophilus* group were obviously susceptible to vancomycin, exhibiting MICs between 0.25 and 1 mg/L. Given the importance of this paradox issue, and the fact that we have not been able to use more accurate methods such as determining the minimum inhibitory concentration (MIC) of vancomycin due to lack of financial resources, it is recommended to do so in the next studies. However, the vancomycin resistance genes in lactobacilli are chromosomally encoded and, therefore, not inducible or transferable to other species (Morrow et al., 2012).

Several previous studies also confirmed the broad susceptibility of the lactobacilli species investigated here towards chloramphenicol and tetracycline (Maragkoudakis et al., 2006; Wang et al., 2010). The previous report by Karapetkov et al. has been documented that lactobacilli are generally resistant to aminoglycosides (Karapetkov et al., 2011). In agreement to the above report, more than fifty percent of our isolates were resistant to gentamicin. In this study, due to lack of financial resources, the MIC of studied antibiotics were not determined that could considered as another limitation of our research.

Antimicrobial activity is another criterion for selecting probiotic bacteria. Application of Lactic acid bacteria as bio preservatives has been confirmed in various previous studies, due to their antagonistic effects against common foodborne bacteria (Zbrun et al., 2013; Chaillou et al., 2014). In this study, all tested lactobacilli showed antimicrobial properties which is in agreement with previous reports (Kaboré et al., 2012; Shokryazdan et al., 2014; Halimi and Mirsalehian, 2016). Furthermore, in our study, the ability of lactobacilli strains to inhibit the in vitro growth of enteropathogenic bacteria appeared to be owing to the production of organic acids (especially lactic and acetic acids), which was consistent with results of Shokryazdan et al. (2014).

According to our results, the *L. fermentum* (N2, N7), and the *L. plantarum* (N20), possessed a number of interesting properties that could be considered as good potential probiotic candidates. They should be studied further as biotherapeutic agents for treatments of specific pathogens. The strains should also be investigated further for other probiotic bioactivities that have human health benefits.

In conclusion, based on the results of the present study, it can be concluded that *Lactobacillus* strains with good probiotic potential could be isolated from fecal of infants. Some *Lactobacillus* strains like *L. fermentum* (N2, N7), and the *L. plantarum* (N20) have an antibacterial effect against enteropathogenic bacteria. This work represents an advance in biomedical science because it highlights that *Lactobacillus* strains isolated from feces of infants could be potential probiotic, but further *in vitro* and *in vivo* studies on these probiotic strains are still required.

Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

N.J., M.A., H.H., and M.S.M. contributed to the conceptualization, design; acquisition of data; Z.G., M.S., M.T.S.A., and H.J. contributed to interpretation of the results, drafting, and revising the article; M. A., M.S., M.T.S.A, and H.J. contributed to the analysis of data.

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