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16S-Amplified Ribosomal DNA Restriction Analysis Assay for Discriminating Potentially Probiotic Lactobacillus Species Isolated from Traditional Dairy Products

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Abstract

Background: Traditional dairy products are the main sources for probiotic bacteria. This study aimed to isolate and characterize the potentially probiotic Lactobacillus strains isolated from traditional dairy products in Iran.

Methods: Microbial population of each dairy product was enriched and screened for acid- and bile-resistant strains. The isolates were primarily characterized by conventional phenotypic and biochemical tests. The species were finally identified using restriction analysis and 16S rDNA sequencing. In addition, the antibacterial activity of the four most common species was evaluated against seven gastrointestinal pathogens.

Results: From a total of nine yogurt and cheese samples, 17 acid-resistant Lactobacillus strains were isolated (survival>50% in PBS buffer, pH 2.5 for 3 h). Eight of them also showed high tolerance to bile. Also, the superiority of molecular techniques over phenotypic tests for effective identification of species was observed. Biochemical tests were useful in combination with 16S-amplified ribosomal DNA restriction analysis (ARDRA) technique to discriminate the isolates at species level.

Conclusion: 16S–ARDRA in combination with biochemical tests is suggested as a potentially useful method for accurate identification of Lactobacillus species where the 16S-rDNA sequencing could not discriminate between two closely related species. Considering the acid and bile resistance capacity of isolated strains, traditional dairy products may be used as important sources of probiotic strains.

developed in the left eye. Two patients had no family history suspicious for keratoconus.

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Introduction

During the past few decades, there has been a growing interest in isolation and characterization of probiotic microorganisms from traditional fermented dairy products (1). Traditional dairy products have a diverse microbial population and are regarded as rich sources of probiotic microorganisms (2). Probiotics as live microorganisms with beneficial health effects (3), mainly belong to lactic acid bacteria (LAB) such as Lactobacillus spp., Bifidobacterium spp. and Enterococcus spp. (4).

The tolerance to acid and bile (5,6) and epithelial cell adhesion are considered as main criteria for in vitro selection of probiotics for human consumption (7). Other criteria for selection of suitable probiotic microorganisms include history of safe use of traditional dairy products (8), assessments of side effects in human studies (9), absences of significant risks with regard to transferable antibiotic resistance (10) and virulence properties (11), availability of data regarding dose, duration and mode of administration, their anti-tumor activity (12-14) and physiological status of the population consuming traditional dairy products (15). However, correct identification of the potentially probiotic isolates at the species level is critical for accurate evaluation of growth conditions, metabolic characteristics and genomic information (16).

Previously, LAB strains were identified based on the morphological, physiological and biochemical characteristics. However, the phenotypic characterization has substantial limitations such as poor reproducibility and discriminatory power and the ambiguity of their results due to the plasticity of bacterial growth and direct relationship between gene expression and environmental conditions (17). Therefore, conventional characterization of bacteria has low accuracy leading to uncertain interpretations. Hence, molecular approaches along with phenotypic methods facilitate detection of bacteria (18,19). Molecular techniques, especially polymerase chain reaction (PCR)-based methods are considered to be specific methods for either characterization or identification of LAB strains.

Recently, molecular techniques, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and amplified ribosomal DNA restriction analysis (ARDRA) have been introduced to distinguish closely-related strains. The latter method is focused on 16S rDNA as a conserved universal marker and appropriate evolutionary clock (17).

The aim of the present study was to assess the probiotic properties (acid and bile salt tolerance, antibacterial activity against seven gastrointestinal pathogens) of Lactobacillus spp. isolated from traditional dairy products and to characterize them using conventional and molecular methods.

Materials and Methods

Isolation of lactic acid bacteria

Totally nine yogurt and cheese samples were collected from different villages in Kaleybar County, East Azerbaijan province, Iran. The yogurt and cheese samples were homogenized by Phosphate-buffered saline (PBS, 0.01 M, PH 7.2) and trisodium citrate 2% (w/v) (Sigma Chemical Co., St. Louis, USA), respectively. One mL of each suspension was inoculated into MRS broth (Merck, Germany) and incubated

at 37°C for 24 h under microaerophilic condition (5% (w/v) CO2).

Rapid screening method was used for isolation of acid-resistant bacteria. Briefly, 10 ml of each enriched bacterial suspension was centrifuged and then dissolved into PBS buffer with pH 3 for 3 hr and pour-plated in MRS agar. Colonies with different morphologies were isolated and examined by light microscopy, Gram staining and catalase reaction. Gram-positive and catalase-negative bacteria were sub-cultured and stored in MRS broth with 25% glycerol at −70°C.

Selection of acid-tolerant isolates

Acid tolerance of individual colonies with different morphologies was determined according to Erkkila & Petaja's method (20). Briefly, each isolate was incubated in PBS buffer with pH 2.5 for 3 hr, and viable counts of Lactobacillus isolates were determined by a pour plate method. Survival rate was calculated according to the following formula:

Survival rate (%) = log cfuN1/ log cfuN0 × 100%

Where N0 and N1 represent the total viable count of isolates before and after treatment by acidic PBS, respectively. Strains with survival rate above 50% were selected for further experiments. The experiments were repeated twice.

Selection of bile-tolerant isolates

The bile tolerance of each acid-resistant isolate was assessed based on the broth assay described by Gilliland et al. (21). For this purpose, MRS broth as a control and MRS broth containing 0.3% (w/v) Oxgall bile (Sigma, Louis, USA) as a test culture, were also used. Growth was evaluated by computing absorbance at 620 nm using Genesys TM 5 spectrophotometer (Spectronic®Genesys TM 5 spectrophotometer, Milton Roy, USA) every 30 min against the corresponding non-inoculated blank samples (MRS broth alone or containing 0.3% Oxgall bile). Growth curves were plotted. The difference (d) between control and test culture was expressed in minutes and considered to be due to the growth prevention by bile salt. The growth delay time (h) between the culture media was regarded as the lag time.

Identification of lactobacillus isolates

Physiological and biochemical characterization

Physiological and biochemical characteristics were identified using Bergey's manual of systematic bacteriology (22). Physiological and biochemical tests used for identification of gram-positive, catalase-negative rod-shaped isolates were as follows:

1. Growth at different temperatures

50 µl of overnight cultures was transferred into the 5-ml MRS broth test media containing phenol red as PH indicator (0.04 g/L). After inoculation, they were incubated for 6 days at 15, 37, or 45°C. Bacterial growth at each temperature was indicated by the change in the color of the cultures, from red to yellow.

2. Growth at different NaCl concentrations

50 µl of overnight cultures was transferred into the 5-ml MRS broth test media containing two NaCl concentrations (4 or 6.5%). They were incubated for 6 days at 37°C. The color changes from red to yellow were considered as an evidence for cell growth.

3. Fermentation of carbohydrates

Fermentation patterns of 20 sugars including Amygdalin, Arabinose, Galactose, D-glucose, D-fructose, D-mannose,

Salicilin, Cellobiose, Maltose, Lactose, Sucrose, Trehalose, Sorbitol, Xylose, raffinose, Rhamnose, Ribose, Melibiose, Melezitose and Mannitol were determined for all the isolates in 5-ml MRS broth medium lacking glucose but containing other filter-sterilized test sugars (1% final sugar concentration) as carbohydrate sources and phenol red as a pH indicator. 50 µl of active overnight cultures were inoculated into each test tube and incubated for 6 days at 37°C. MRS broth containing 2% glucose as a carbohydrate source and without glucose were used as positive and negative controls, respectively. Duplicate reactions were conducted for each sugar fermentation experiment. At the end of incubation, based on the color change from red to yellow and increased turbidity, the results were assessed as strong-positive, weak-positive and negative.

Molecular identification

16S rRNA gene sequencing

Genomic DNA was extracted from overnight Lactobacillus cultures using QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR with primers Hal-6F (5´- AGAGTTTGATCMTGGCTCAG -3´) and Hal-R6 (5´- TACCTTGTTAGGACTTCACC - 3´) (23) in a 25 µl reaction volume containing 1X PCR Master Kit (Ampliqon, Denmark), 50 ng DNA and 0.4 µmol/L of each primer. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 4 min, 32 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 55 sec, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were electrophoresed on1% (W/V) agarose gel and stained with a DNA Safe Stain (CinnaGen, Iran). Images were captured by a gel documentation system (Bio-Rad, Germany). Next, the amplicons (~1500 bp) were purified using High Pure PCR Product Purification Kit (Roche, Indianapolis, IN, USA) and analyzed by restriction enzymes and sent for sequencing (Macrogen, Seoul, Korea).

Amplified ribosomal DNA restriction analysis (ARDRA)

16S rDNA amplification product were digested using restriction enzymes including MspI, BsuRI and Bsp143I (Thermo Fisher, Massachusetts, United States) in separate reactions and then analyzed in 1.5% agarose gel. The digestion reaction was set in a total volume of 15 µL, containing 5 µl PCR product (approximately 0.1 µg of DNA), 1.5 µL 10X enzyme buffer, 1.5 µL enzyme and 7 µL H2O. All three enzymes were incubated for 20 min at 37°C. Restriction patterns of Lactobacillus 16S rDNA as a reference profile generated by the same three enzymes, were also analyzed by GeneDoc software (version 2.7.0).

Determination of antimicrobials activity

The antimicrobial activity of supernatants obtained from four acid- and bile-tolerant isolates (BL1, CL1, GL1 and EL3), was assessed against seven important human pathogenic bacteria including Staphylococcus aureus (ATCC 29213), Yersinia enterocolitica (ATCC 35669), Bacillus cereus (ATCC1431), Listeria innocua (ATCC 33090), Klebsiella pneumonia (PTCC 1290), Shigella flexneri (PTCC 1234), and Escherichia coli (ATCC 1399) using disc diffusion method described by Bhunia et al., (1988) with some modifications (24). Cell-free culture supernatants (CFCS) of isolates were collected through centrifugation (12,000 ×g, 4°C, 10 min) of cultures grown in 15 ml MRS broth at 37°C

for 24 h. After filtration of supernatants through a 0.22 µm filter, they were used at two natural (pH=4) and neutralized pH conditions (pH=7). Indicator bacteria, at concentrations of 10^6 cells/ml, were added to 7 ml BHI soft agar (0, 75% agar), mixed and poured over the surface of 20 ml nutrient agar plates. Subsequently, 30 µl of each filtrate was poured on paper discs (6.5 mm diameter), positioned on the inoculated BHI agar and plates were incubated at 37°C in the atmospheric conditions needed for each test bacteria After 24 hr incubation, antimicrobial activity of the isolates was assessed based on the presence of the clear zone around a disc. The width of the inhibition zone was measured using calipers and expressed in millimeters.

Results

Lactobacillus spp. isolated from yogurt and cheese samples

When the isolated strains were examined for their tolerance to acidic condition (pH=3), 41 isolates including 25 Lactobacillus spp. and 16 Enterococcus species, were selected.

The survival rate of 25 Lactobacillus isolates under acidic condition was as follows: 8 isolates with less than 50%, 4 isolates with 50 - 80% and 13 isolates with a survival rate above 80%.

From 25 Lactobacillus strains, 17 isolates with survival rate of ≥50% were subjected for further experiments. The remaining 8 isolates were considered sensitive and excluded from the study.

The bile tolerance of 17 acid-resistant isolates was tested in MRS broth containing 0.3% Oxgall bile. They were subsequently categorized as resistant, tolerant, weakly-tolerant and sensitive groups.

Among 17 isolates, one (BL1) was not affected by 0.3% bile salts and showed a delayed growth less than 15 min (d<15 min) and classified as resistant isolate (group I). Seven isolates were slightly affected by 0.3% bile salts and showed a delayed growth between 15 and 40 min (15 min<d≤40 min) and were classified as tolerant isolates (group II). Five isolates showed a delayed growth between 40 and 60 min (40 min<d<60 min) and were classified as weakly tolerant isolates (group III). Finally, 4 isolates showed a delayed growth more than 60 min (d>60 min) and were considered as sensitive isolates (group IV). The effect of 0.3% bile salts on the growth curve of Lactobacillus isolates belonged to the four groups including BL1 (resistant), IL1 (tolerant), GL3 (weakly tolerant) and AL3 (sensitive), is shown in Figure 1.

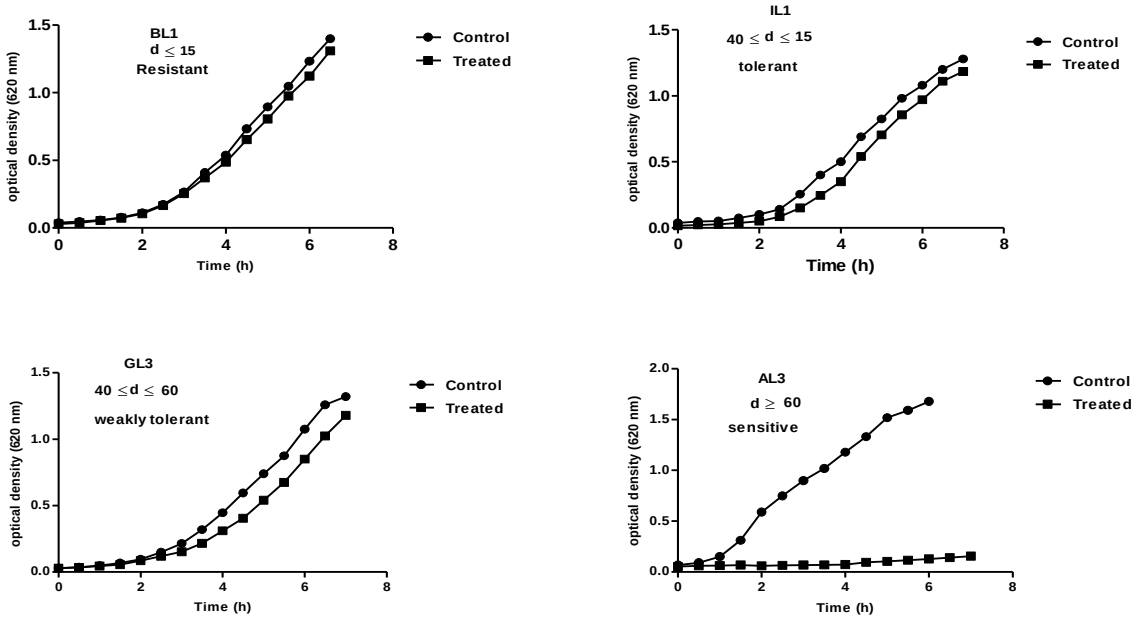

Figure 1. The effect of bile salt (0.3%) on growth of four Lactobacillus strains (BL1, GL3, AL3 and IL1). Letter "d" represents the delayed growth of isolates in MRS broth supplemented with 0.3% bile compared to MRS broth as control. Optical densities of culture media are also shown until 8 hr.

Physiological and biochemical characterization of the isolated Lactobacillus

The 17 acid-resistant rod-shaped isolates were confirmed to be Lactobacillus on the basis of preliminary screening strategies (i.e. Gram positive, rod-shaped, no motile, and catalase-negative). Seven of them were also demonstrated to be bile-tolerant while one of them was bile-resistant. Based on sugar fermentation profile, isolates AL3, BL2, CL2 and EL1 that were isolated from four different types of yogurt showed carbohydrate fermentation profile similar to that of homofermentative L. delbrueckii subsp. bulgaricus. These isolates were able to ferment glucose, fructose, lactose, mannose and salicin to lactic acid. Also, these isolates were able to grow well at 45°C, but not at 15°C and in the presence of two concentration of NaCl (4 and 6.5%). This pattern of growth was similar to that of L. delbrueckii subsp. bulgaricus. The isolates BL2 and EL1 showed identical phenotypic characteristics and carbohydrate fermentation profile. So, the biochemical tests used in this study, could not distinguish between these two isolates. The remaining isolates were classified as heterofermentative Lactobacilli. AL2 was close to L. divergenes while EL3 and GL2 had carbohydrate fermentation profile similar to that of L. casei. Finally, BL1, AL1, EL2, GL3, IL2, CL3, IL1, CL1, IL3 and GL1 were identified as L. brevis and/or L. fermentum and/or L. reuteri based on the growth temperature and carbohydrate fermentation patterns.

Molecular characterization of isolates

Eight isolates comprised of one bile-resistant and seven bile-tolerant were selected for molecular identification. Amplification of the extracted DNA by the above mentioned primers led to the identification of 1500 bp amplicons (Figure 2). Subsequent sequencing of amplicons obtained from 4 acid-

resistant isolates (one bile-resistant and three bile-tolerant) showed that the GL1 and CL1 had 99% sequence similarity with L. brevis (GenBank accession numbers HM8547 and JN368472, respectively) and EL3 had 99% sequence similarity with L. plantarum (GenBank accession number JN368470). Sequence of BL1 showed 97% similarity with L. brevis and it was identified as Lactobacillus sp. (accession number HQ406830). The biochemical tests and 16S–ARDRA analysis both showed that the strain belonged to L. brevis species.

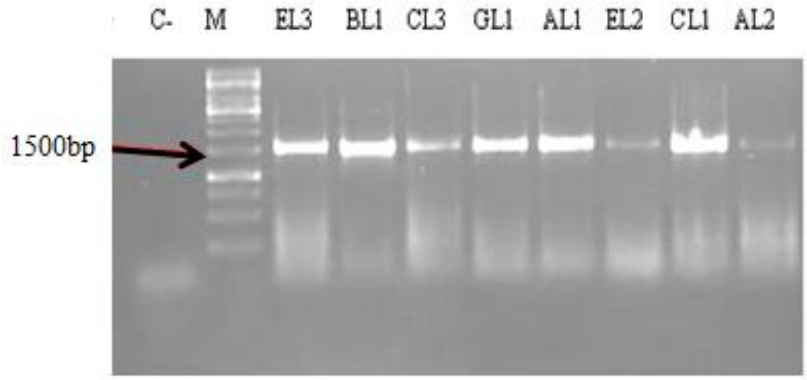

Figure 2. Electrophoresis of 16S rDNA amplicons (1500 bp) in 1% agarose gel. M: 1kb DNA ladder, C-: negative control, EL3-AL2: Lactobacillus spp.

Although, AL1, GL1, BL1, CL3, CL1 and EL2 have been biochemically characterized to belong to one of the L. fermentum, L. brevis or L. reuteri species, but the restriction analysis of these isolates revealed that they belong to L. brevis species.

EL3 and AL2, which were biochemically identified as L. casei and L. divergenes, were identified by molecular analysis as L. plantarum and L. casei, respectively.

Table 1 shows the identification of 8 Lactobacillus isolates using biochemical tests and molecular methods. Restriction analysis (16S-ARDRA) profiles of 8 isolates by restriction enzymes including MspI (A), Bsp143I (B) and BsuRI (C) are also presented in Figure 3.

The combination of two or three restriction profiles obtained from each isolate provided the clearest and discriminating profiles leading to differentiation of the majority of Lactobacillus isolates at the species level.

Table 1. Final identification of the eight acid- and bile-tolerant Lactobacillus isolates and comparison of the classical and molecular methods

Isolates	Biochemical Identification	ARDRA Identification	16S rDNA Sequencing	Sequence Homology in NCBI (%)
AL1	L. brevis / L. fermentum/ L. reuteri	L. brevis	ND*	-
EL3	L. casei	L. plantarum	L. plantarum JN368470	99
GL1	L. brevis / L. fermentum/ L. reuteri	L. brevis	L. brevis HM8547	99
AL2	L. divergenes	L. casei/ L. plantarum	ND	-
BL1	L. brevis / L. fermentum/ L. reuteri	L. brevis	L. spp HQ406830	97
CL3	L. brevis / L. fermentum/ L. reuteri	L. brevis	ND	-
EL2	L. brevis / L. fermentum/ L. reuteri	L. brevis	ND	-
CL1	L. brevis / L. fermentum/ L. reuteri	L. brevis	L. brevis JN368472	99

* Non determined

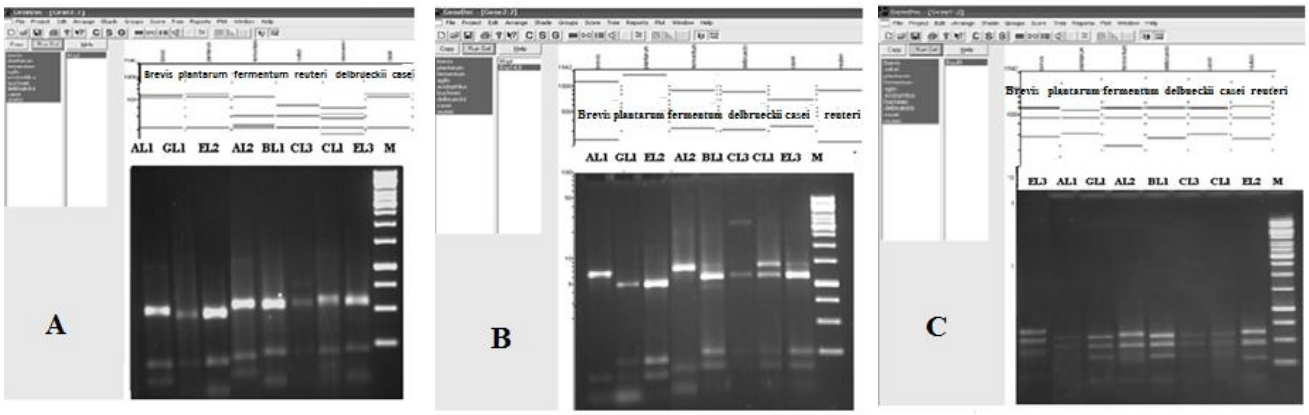

Figure 3. Amplified 16S-rDNA restriction analysis (ARDRA) profiles of eight acid- and bile-tolerant Lactobacillus isolates using MspI (A), Bsp143I (B) and BsuRI (C) restriction enzymes. ARDRA obtained on electrophoresis gel was compared with restriction profiles obtained by the in silico digestion of Lactobacillus species 16S rDNA sequences recorded in NCBI using GeneDoc software. The combination of restriction enzymes profiles was found to give the clearest and discriminating profiles.

Antimicrobial activity of isolates

The antagonistic activity of BL1, CL1, GL1 and EL3 were tested against 7 gastrointestinal pathogens.

The results showed that Yersinia entrocolitica (ATCC 35669) and Listeria innocua (ATCC 33090) were the most resistant strains to CFCS of Lactobacillus isolates. In addition, Bacillus cereus (ATCC1431), Shigella flexneri (PTCC 1234), Klebsiella pneumonia (PTCC 1290), Escherichia coli (ATCC 1399) and Staphylococcus aureus (ATCC 29213) were sensitive to supernatant of L. brevis and L. plantarum cultures. These results are shown in Table 2. Totally, the antimicrobial activity of Lactobacillus strains against pathogenic microorganisms was low to moderate (Table 2).

Table 2. Antimicrobial activity of Lactobacillus strains against pathogenic microorganisms

Isolates	BL1 (L. brevis)		CL1 (L. brevis)		*GL1* (L. brevis)		(EL3) (L. plantarum)	
Cell Free Culture Supernatants	Natural pH	Neutralized pH	Natural pH	Neutralized pH	Natural pH	Neutralized pH	Natural pH	Neutralized pH
E. coli	8.5*±0.62	9.5±0.3	0	0	0	0	8.5±0.55	0
S. aureus	0	0	8.8±0.5	8. 2±.4	9.5±1	8.5±.7	9±1	6.2± 0.46
Y. enterocolitica	0	0	0	0	0	0	5.5±0.4	0
L. innocua	7.5±1	0	0	0	0	0	0	0
B. cereus	9±1	6±0.7	12.5±1.2	9.5±0.6	11.5±1.2	8.6±0.3	10±0.8	7.5±0.6
S. flexneri	8.5±0.45	8.3±0.4	9.5±0.45	0	7.5±0.6	8.5±0.52	7.5±0.4	8.5±0.2
K. pneumoniae	8.5±0.55	9.5±0.21	8.6±0.3	0	0	0	8±0.65	8±1

* Diameter of inhibition zone (mm); Results are expressed as mean±SEM; each data point is the average of duplicate experiments

Discussion

Probiotics have beneficial effects on many aspects of human physiological processes. Notably, they have been shown to exert anti-cancer effects as well (25). Dairy products are regarded as important sources of probiotics (26). Due to specific functions of certain strains, identification of probiotic stains in each product is important. In this study, a conventional and molecular method was used to identify the Lactobacillus species isolated from dairy products. Seventeen acid-resistant Lactobacillus isolates were identified on the basis of their phenotypic properties. Eight isolates comprised of one bile-resistant and seven bile-tolerant were further characterized using molecular methods including 16S-ARDRA and four out of eight isolates were subjected to 16S rDNA sequencing.

In the present study, three groups of acid-sensitive (<50% survival rate), acid-tolerant (50-80%) and acid-resistant (>80%) isolates were obtained from traditional types of yogurt and cheese, and the isolates with a survival rate of >50 % were further assessed for bile tolerance capacity. In bile tolerance tests, the same concentration of bile was used as in the human gastrointestinal tract (0.3% w/v) (27) to find the most clinically relevant results.

Among the 17 isolates, BL1 strain (L. brevis) showed the highest tolerance to bile (classified as resistant strain). Moreover, AL3, BL2, CL2, and EL1 were identified as L. delbrueckii subsp. bulgaricus and were recognized as sensitive strains.

In this study, classical biochemical tests was used for identification of the isolates. The results showed that application of biochemical tests alone frequently led to misidentification of species and molecular techniques are required for correct identification of the isolates. On the other hand, it was demonstrated that biochemical tests could be useful in combination with 16S-ARDRA technique to discriminate isolates at species level. The results also showed that the 16S-ARDRA technique is a simple, quick and highly discriminatory method to identify Lactobacillus species, while the 16S-rDNA sequencing could not discriminate between two closely related species. In silico restriction analysis also

revealed that MspI, Bsp143I and BsuRI restriction enzymes are appropriate for differentiation of Lactobacillus species. Previously, 16S-ARDRA has been successfully applied to identify Lactobacillus spp. in commercial fermented milks (28).

The four potential probiotic isolates showed moderate antagonistic activity against various gastrointestinal pathogens. Although the exact mechanism of this effect was not assessed in this study, but previous studies have suggested the production of organic acid, hydrogen peroxides and bacteriocin as the underlying mechanisms (29,30).

Antimicrobial tests at two natural (pH 4.0 ± 0.2) and neutralized (pH 7.0) conditions revealed that some isolates such as BL1 strain have inhibition zone at both conditions. This may be due to the production of bacteriocin-like compounds, which have antimicrobial activities and are also stable at different temperatures, pHs and enzyme treatments (31).

Conclusion

In the present study, Lactobacillus species were isolated from traditional dairy products in Kaleybar County, East Azerbaijan province, Iran. Based on their acid and bile tolerance capacity and antibacterial effects, the isolates are putative probiotic strains. These dairy products as potential sources for probiotic Lactobacilli. L. plantarum (EL3 strain) isolated in this study showed moderate antimicrobial activity against several gastrointestinal pathogens and can be used in dairy products such as yogurt. Furthermore, 16S–ARDRA in combination with biochemical tests can be potentially useful for accurate identification of Lactobacillus strains where the 16S-rDNA sequencing could not discriminate between two closely related species.

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Conflict of interests

The authors declare that there are no conflict of interests.

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