

Research Article

Lactobacillus with probiotic potential from homemade cheese in Azerbaijan

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Abstract

Introduction: *Lactobacillus* is believed to be beneficial in human health, thus the search for isolation and identification of friendly human bacteria from traditional fermented foods is important in medicine. One of the dairy products, traditional cheese as a highly-consumed dairy product could be a valuable source of these friendly edible germs.

Methods: In this research, home-made cheese from Lankaran, Jalil Abad and Salian regions in Azerbaijan was characterized for the presence of Lactobacilli with probiotic potential. The bacterial suspension was enriched and screened for acid and bile resistances. Then, the isolates were subjected to antibiotic resistance and antibacterial effects against convenient pathogenic bacteria. The isolates were identified by 16s rDNA sequencing.

Results: The results clearly revealed two probiotics with higher homology to *Lactobacillus planetarum* and *Lactobacillus fermentum*.

Conclusion: No antibiotic resistance was detected in any of the potentially probiotic lactobacilli isolates in these regions, where people continue to follow a life-style that is largely traditional, with traditional medications.

Introduction

Probiotics are defined as live microorganisms or their components which when administered in adequate amounts could confer health benefits such as the improvement of human immune system, rearrangement of intestinal microflora, and establishment of antagonistic effect on the growth of harmful bacteria.¹

Lactic acid bacteria (LAB) especially *Lactobacillus* are the most common types of microbes defined as probiotics.² *Lactobacilli* are commonly used as starter culture of dairy products such as yogurt and traditional cheese.² Consumption of native foods containing such indigenous microorganisms possesses many advantages;³ therefore, the study of probiotics is ubiquitous and many countries have made a great endeavor to isolate probiotic bacteria from natural sea salt,⁴ plants,⁵ and so from the human gastrointestinal tract,⁶ particularly with many endeavors from traditional fermented foods such as cheese.⁷

It should be noted that the complex microflora intensity of traditional cheese is due to so many processing and fermentation steps such as clarification of raw milk, heating, cooling, incubation, salting, and usage of homemade cheese starter.⁸ Although there are many of commercial probiotic supplements and dairy products, there is an emphasis on the isolation of probiotics with respect to the native microbiome.^{9,10} Hence, in this research we aimed to

study the rural regions of Azarbyjan including Lankaran, Jalil Abad and Salian that their homemade cheese could be a valuable source of probiotic microorganism.

Materials and methods

Sampling and isolation of bacteria

Thirty cheese samples were collected from Lankaran, Jalil Abad and Salian regions, and then 1 g of each sample was homogenized into 10 ml sodium citrate. Then, 1 ml was inoculated with MRS broth (Fluka, Buchs, Switzerland) and incubated in aerobic condition for 48 h at 37 °C. For screening the tolerance of lactobacilli to acidic condition (harsh condition of gastrointestinal tract), 1 ml of each enriched culture was inoculated in 10 ml PBS buffer (pH = 2.5)¹¹ and incubated for 3 h. After centrifugation, survived organisms were resuscitated by addition to 10 ml MRS broth and incubation for 24 h at 37 °C. Additionally, the modified method was used for LAB screening against bile salt.¹² Briefly, the overnight cultures of LAB were inoculated in MRS broth containing 0.3% (w/v) oxgall (Sigma, Louis, USA) and incubated for 4 h at 37 °C. Serial dilutions were prepared from acid and bile resistant cultures, then 0.01 ml of 10⁻⁵ dilution were spread onto MRS-agar plates and incubated for 24-48 h at 37 °C. Several single colonies were randomly picked up and incubated in 10 ml MRS broth. Preliminary screening of isolates was

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performed by morphological evaluation (gram staining, cell morphology) of the single clones. The isolates were subcultured in MRS broth and then conserved in MRS broth with skim milk (10%) and glycerol (25%) at -70°C .

Antibiotic susceptibility of potentially probiotic isolates

The resistance of the isolates were determined using the NCCLS modified Kirby–Bauer disc diffusion method¹³ for the following clinically important antibiotics: chloramphenicol (30 μg), vancomycin (30 μg), tetracycline (30 μg), erythromycin (15 μg), Ampicillin (10 μg), and methicillin (10 μg). All antibiotic discs were purchased from Padtan Teb Co (Tehran, Iran). Antibiotic susceptibility assays were performed according to the producer's guideline and the isolates were classified into mediate and sensitive. Then the sensitive isolates were subjected to further characterization.

Assay of antimicrobial activity of isolates

The antibacterial activity of antibiotic sensitive isolates was assayed by the disc diffusion method.¹³ The overnight cultures of isolated lactobacilli in MRS broth were centrifuged at 12000 rpm for 5 min, then filter sterilized (0.2 μm filter). The blank discs of 6 mm diameter were immersed in bacterial free supernatant, then the discs were placed on inoculated nutrient agar (Sigma, Louis, USA) with approximately 10^5 - 10^7 CFU/ml of the following indicator bacteria: *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923) and native isolate of *Escherichia coli* O157 H7. Positive results were identified as clear zones around the discs after overnight incubation at 37°C . Similarly, the discs containing pronase (Sigma Deisenhofen, Germany) treated supernatant were recruited for distinction of non-bacteriocin and bacteriocin inhibitory effect on bacterial growth and the isolates with positive inhibition zone were expunged from the experiment.¹⁴

Amplification of 16S rDNA

Total chromosomal DNA was extracted from overnight broth cultures of the strains according to the method reported by Atashpaz *et al.*¹⁵ Amplification of the 16s rDNA was carried out using the primer pair reported previously:¹⁶ 16lacF 5'-AGAGTTTGATCMTGGCTCAG-3' 16lacR 5'-TACCTTGTTAGGACTTCACC-3'. PCR amplification was performed using master mix (cinacon, Iran), 0.5 μM primer, 50 ng DNA, and the final volume was reached to 25 μl . The cycling program was as follows: denaturation at 94°C for 4 min, 32 cycles of: 94°C for 40 sec; 59°C for 40 sec; 72°C for 80 sec and a final extension was performed for 5 min at 72°C .

Sequencing and analysis of PCR-amplified 16s rDNA

PCR products were cloned in pGEM vector (promega, US) according to the manufacturer's instructions and transformed in *E. coli* DH5 α according to the vibration method.¹⁷ The inserts of purified plasmids were sequenced (Macrogen, Korea). Having used BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), the determined sequences were compared with the sequences deposited in NCBI GenBank as 16s rDNA gene of different *Lactobacillus* species.

Results

Screening of acid and bile resistant isolates

The screening of isolates in simulated condition of human gastrointestinal system (i.e., pH=3 for 2.5 h and 0.3% bile salts for 4 h) led to the attainment of acid and bile resistant rod-shaped isolates.

Antibiotic susceptibility of potentially probiotic isolates

As shown in Fig. 1, approximately 100% of the selected isolates were sensitive or semi-sensitive to the entire routinely used antibiotics in the inhibition zone evaluation.¹⁸

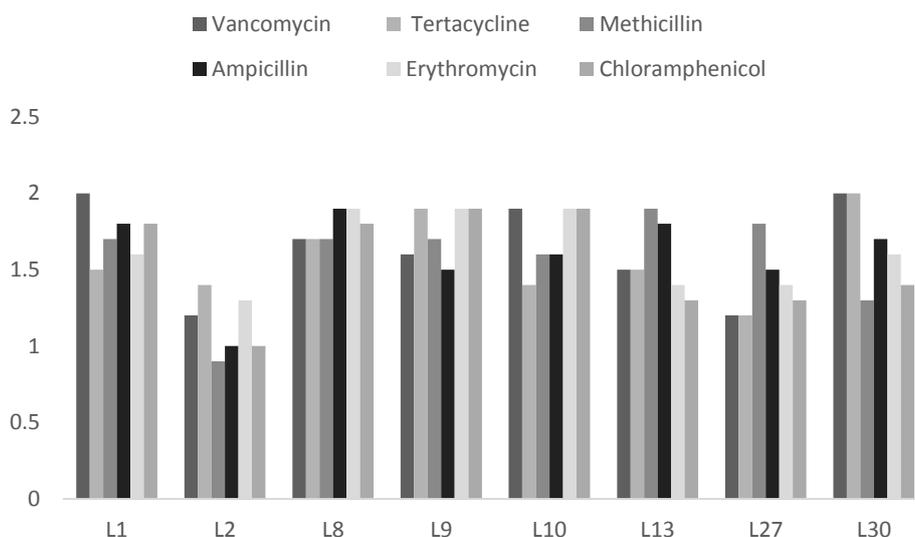


Fig. 1. The inhibition zone of routinely used antibiotics (Vancomycin, Tetracycline, Methicillin, Ampicillin, Erythromycin, Chloramphenicol) against selective isolates

Analysis of antimicrobial activity

The results showed that only two isolates (i.e., L1, L13) were able to inhibit the indicator bacteria through the bacteriocin effect, while other isolates displayed inhibitory effect by the non-bacteriocin effect(s), as shown in Fig. 2. Based on our findings, *S. aureus* strains were the most tolerant bacteria to Lactobacillus inhibition as compared to the other pathogenic bacteria (Fig. 2).

Identification of Lactoacilli by 16s-rDNA pattern analysis

Identification of new strains of *Lactobacillus* have been frequently described in the literature using amplification of 16s rDNA gene which is highly conserved in some part, however it has very variable regions that can provide strain specific signature.¹⁹ Therefore, to confirm the *Lactobacillus* in the genus level, PCR amplification of 16S rDNA gene and ARDRA was performed. Fig. 3 represents the electrophoresis of PCR and ARDRA. As shown in Fig. 3, the fragments (~1500 bp) corresponding to the full length of 16S rDNA in different *Lactobacillus* species were obtained. The result of ARDRA showed two distinct isolates and both of isolates were sequenced to determine the species.

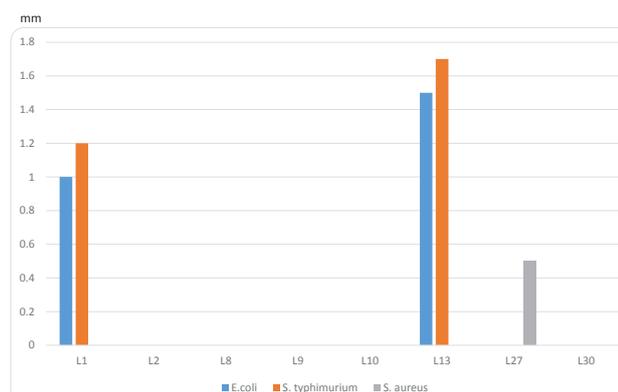


Fig. 2. The inhibitory effect of acid-bile resistant isolates against pathogenic bacteria including native *S. aureus* (ATCC 25923), *S. typhimurium* (ATCC 14028), and *E. coli*

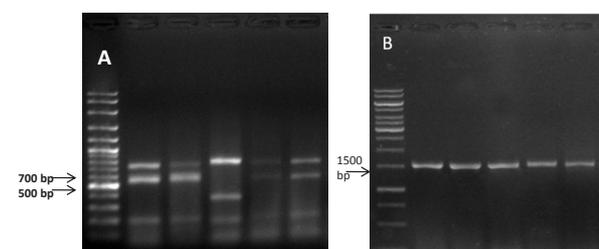


Fig. 3. A; ARDRA pattern of PCR products, digested with *TaqI* enzyme. 100 bp DNA ladder has been used for the size of cleavages. B; 1% Agarose gel electrophoresis of 16S rDNA gene (~1500 bps), shown with 1kb DNA ladder

Discussion

It is recommended that human friendly bacteria be isolated with respect to native foods⁹ due to their efficacy in the same population.¹⁰ In this study, we found out that in the rural regions of Lankaran, Jalil Abad and Salian in Azarbyjan, homemade cheese could be a valuable source to get the probiotics. It could be applied in designing starter culture for industrial dairy products to save the natives' health and prevent modern diseases that the world suffers from as a result of industrial lifestyles. Also, according to the WHO guideline, probiotic bacteria such as *Lactobacillus* are expected to display high sensitivity to conventional antibiotics. This implies that use/abuse of antibiotics can change the bacterial resistance patterns in different regions. In this region due to large traditional medications, no antibiotic resistance was detected in any of the isolates. Another feature of health beneficial probiotic LAB in WHO guideline is its inhibitory effect on the growth of pathogenic bacteria. The health beneficial impacts from probiotics can be merely stemmed from the effect of bacteriocin secretion.²⁰ Therefore in this study, pronase treatment was applied for the degradation of bacteriocin and discrimination of bacteriocin and non-bacteriocin effects.

Conclusion

No antibiotic resistance was detected in any of the isolates in Lankaran, Jalil Abad and Salian regions in Azerbaijan, where people have a traditional life-style and continue to follow largely the traditional medications. Hence, traditional cheese in these areas has been identified as a good candidate for the isolation of new probiotic strains.

Ethical issues

There is none to be declared.

Competing interests

There is none to be disclosed.

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