



Antagonistic activity of probiotic lactobacilli against human enteropathogenic bacteria in homemade tvorog curd cheese from Azerbaijan

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Abstract

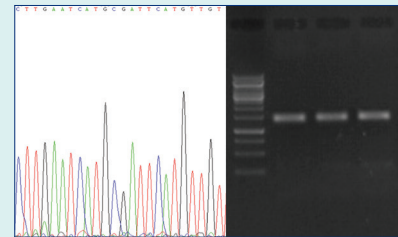
Introduction: Human health is deemed to be maintained by the crosstalk among the body and probiotic bacteria. Disruptions in this composition are associated with the pathogenesis of numerous diseases. Through modernization, traditional foods consumption has been abandoned and native food starters have been substituted with industrial products. Hence, we aimed to isolate and evaluate probiotic bacteria from traditional fermented foods which can

be used as probiotics as well as starter cultures in food industry and in medicine against antibiotic resistant pathogens. To this end, an intact village was recognized in the Republic of Azerbaijan with traditional dairy products, yielding a variety of potential probiotics.

Methods: In this study, tvorog as a traditional dairy product from Dashkasan, Ismaili and khachmaz regions in Azerbaijan was characterized for the isolation of Lactobacilli with probiotic potentiality. The bacteria were tested for the resistance to the acid, bile, and the antagonistic effect of human pathogenic bacteria. The isolates were identified by 16s rDNA sequencing with a blast to the databank.

Results: Three species with higher homology to the *L. planetarium*, *L. rhamnosus*, and *L. casei* with high probiotic potentiality and antibacterial effects were isolated.

Conclusion: Homemade tvorog curd cheese in Azerbaijan harbor a variety of probiotics with industrial applications as well as potentiality to be preserved in a biobank for the future medicinal applications especially against antibiotic resistant pathogens.



Introduction

According to a definition by the World Health Organization (WHO), probiotics are distinguished as microorganisms with beneficial effects for human health.¹ It is around millions years that the indigenous microflora has co-evolved with humans and they have preserved the inherited microbiomes via consumption of fermented foods and interactions with environmental microbes.² Through modernization, traditional foods consumption has gradually been abandoned and native food starters have been substituted with industrial products. This gradual change has reduced human contact and exposure to microbial symbionts and led to the shrinkage of core microbiome.³ Besides, the openings of large numbers of antibiotics during the last three decades have caused anxiety about the threat of bacterial resistance. The widespread use of antibiotics by the population and in hospitals has inflamed this crisis. Mechanisms such as

antibiotic control programs, better hygiene, and synthesis of agents with improved antimicrobial activity seem necessary to be adopted in order to restrict the bacterial resistance.⁴ In this regard, many research have been conducted on the isolation and evaluation of probiotic potential bacteria and in turn establishment of a biobank to save them from destruction, for future applications in medicine and food industry. Today, the probiotic human-friendly bacteria are isolated from foods, cheese,⁵ yogurt,^{6,7} fermented olives,⁸ and wine,⁹ as well as human himself, human milk,^{10,11} infant feces,¹² women vagina,¹³ etc. According to WHO guidelines for evaluation of probiotics, putative strains should be screened for resistance to gastric acidity and bile salts, antimicrobial compound production and safety properties such as antibiotic resistance. Other recommendations are for the isolation with respect to the natives' microbiome, indigenous acid- and bile-resistant bacteria¹⁴ and the traditional native food and environment.



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On the whole according to the literature, it has been emphasized that the isolation of probiotics should be done from native's.¹⁵ Azerbaijan has many villages with traditional lifestyles that could be superb sources of super potential probiotics.¹⁶ In this research, we aimed to isolate lactobacilli from traditional dairy products named tvorog curd cheese, produced in the rural regions; Dashkasan, Ismailli, and khachmaz of Azerbaijan.

Materials and methods

Sampling and isolation of acid-bile resistant bacteria

We screened bacterial species isolated from six samples of tvorog collected from Dashkasan, Ismailli, and khachmaz regions of Azerbaijan for potential beneficial features. We focused in our search on lactobacilli and evaluated their ability for or against human enteropathogenic bacteria. Seventeen tvorog samples were collected from Dashkasan, Ismailli, and khachmaz regions and then 1.0 g of each sample was suspended in 10 ml PBS. Then, 500 μ L of suspension was added to MRS broth (Fluka, Buchs, Switzerland) and incubated at 37°C for 48 h. For screening the tolerance of lactobacilli to harsh condition of stomach, 1.0 mL of each enriched culture was incubated in 10.0 mL PBS buffer (pH = 3) for 3 h. After centrifugation, pH resistant bacteria were resuscitated by addition of 10.0 mL MRS broth and incubation at 37°C for 4 h. Additionally, the second test was used for screening the bacteria against bile salt. Briefly, the acid resistant bacteria were inoculated in MRS broth containing 0.3% (w/v) oxgall (Sigma, Louis, USA) and incubated at 37°C for 4 h. The dilutions were prepared from acid and bile resistant enriched bacterial culture, then 100 μ L of 10⁻⁵ dilution were spread onto MRS-agar plates and incubated at 37°C for 24-48 h. Different clones were picked up and cultured in 10 mL MRS broth. First, the isolates were evaluated by gram staining and cell morphology. Then, the isolates were conserved in MRS broth with skim milk (10%) and glycerol (25%) at -70°C.

Screening for antagonistic activity

Antagonistic activity of isolates was evaluated as described in literature.¹⁷ Overnight cultures of the isolates were centrifuged. Then the supernatant was filtered by 0.25 μ m microfilter. Two-hundred microliters of the supernatant were spotted onto the blank disk (Pad Tan Teb, Tehran, Iran) that was created on Muler Hinton Agar (MHA) plate with enteropathogenic bacteria spread and incubated at 37°C for 24 h. The plates were checked for the inhibition zones. Inhibition was scored positive if the width of the clear zone around the clones of the producer strain was 7 mm or larger. The following enteropathogenic bacteria were used as indicator strains: *Escherichia coli* PTCC 1399, *Yersinia enterocolitica* PTCC 1151, *Shigella flexneri* PTCC 1234, *Klebsiella pneumoniae* PTCC 1290.

DNA extraction and molecular identification

The bacterial genomic DNA was extracted according to a previously published method.¹⁸ Amplification of the 16s rDNA was carried out using the

primer pair reported previously as: 16lacF5'-AGAGTTTGATCMTGGCTCAG-3' 16lacR5'-TACCTTGTTAGGACTTCACC-3'.¹⁹ Reactions were performed in an automatic thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at 94°C for 4 min; 32 cycles of 94°C for 50 s, 59°C for 50 s and 72°C for 90 s and final extension at 72°C for 10 min and holding at 4°C. PCR products were ligated to the pGEM T/A cloning vector (Promega, Madison, WI, USA) according to the manufacturing instruction. Then, they were transformed to the *E. coli* DH5 α according to the literature.²⁰

The plasmids were then sent to a commercial sequencing facility (Macrogen, Seoul, Korea). The sequences were compared to those reported in GenBank, using Basic Local Alignment Search Tool (BLAST) algorithm. The isolates were identified by similarity with standard strains in GenBank.

Result

Screening of acid and bile resistant isolates

The isolates were screened against simulated harsh condition of human gastrointestinal system (i.e., pH=3 for 3 h and 0.3% bile salts for 4 h) as shown in Fig. 1.

Analysis of antimicrobial activity

The antagonistic activity against human enteropathogenic bacteria was tested. The results are shown in Fig. 2. These

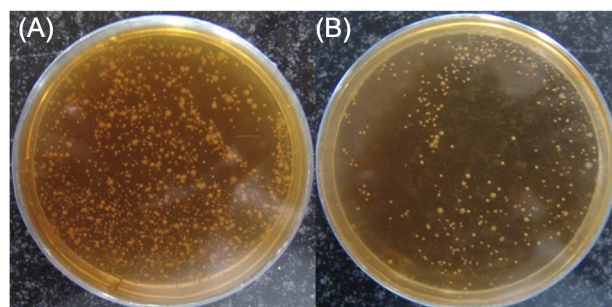


Fig. 1. Clony of bacteria before and after enrichment in 3% bile and low pH. A: the clones are mix of resistant and unresistant bacteria that many of them should be removed in screening process.

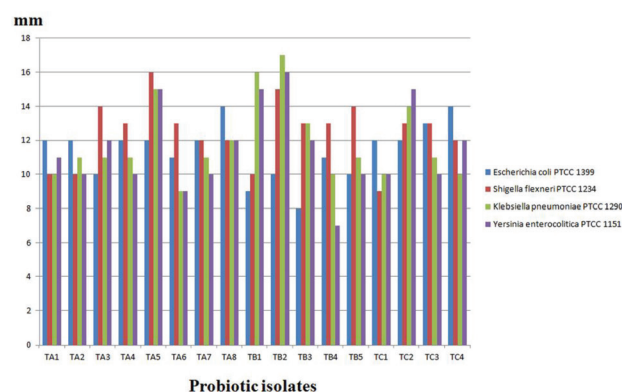


Fig. 2. Antagonistic effect of selected isolates for human enteropathogenic bacteria.

17 isolates were able to inhibit the indicator bacteria, but the strains TA5 (*L. casei*) and TB2 (*L. rhamnosus*) showed antibacterial effects for all of indicators with high efficiency (Fig. 3). The probiotic potential and physiological characterization of selected *Lactobacillus* is shown in Table 1. Overnight culturing of selected isolates in oxgal (0.3%) is shown in Fig. 4.

Identification of Lactoacilli by 16s rDNA pattern

The 16s rDNA PCR product of the isolates with high antibacterial activity, namely, TA5, TB2, and TC2 were cloned in plasmids and sequenced (Fig. 5). Then the sequencing results were aligned using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and compared with the sequences deposited in NCBI GenBank for different *lactobacillus* species. The isolates had 100% similarity with *L. Casei*, *L. plantarum*, and *L. rhominos*. All these species represented antibacterial effects on the indicator bacteria.

Discussion

In recent researches, resistance to antibiotics in the treatment of bacterial infections has been increasingly observed, creating serious problems in treatment of infectious diseases. The resistance has been reported against almost any antibiotic discovered, and hence this issue has turned into one of the most urgent public health concerns and future medicinal challenges. In this regard, probiotics could be considered as living drugs that reduce antibiotic consumption. The aim of this study was the

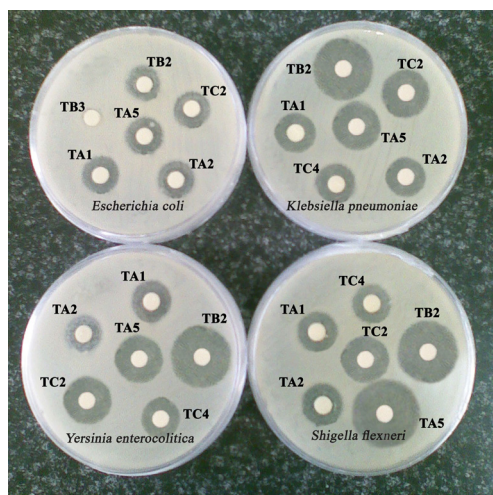


Fig. 3. The inhibition zone surrounding the *Lactobacillus* isolated against human enteropathogenic bacteria.

Table 1. The probiotic potential and physiological characterization of selected *Lactobacillus*

Isolates	Growth	pH resistancy	Bile resistancy	Antibiotic sensitivity	Antagonistic activity
TA5	Fast	%80<	+	+	+
TB2	Slow	%60<	+	+	+
TC2	Medium	%60<	+	+	+

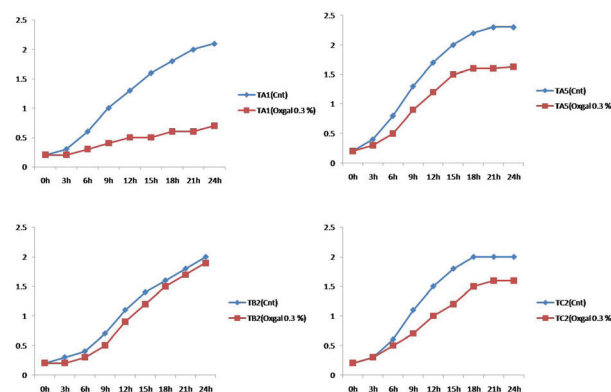


Fig. 4. The growth inhibitory of oxgal (0.3%) for candidate probiotics at overnight incubation. TA1 isolate was very sensitive to bile compared with TB2, TC2, and TA5.

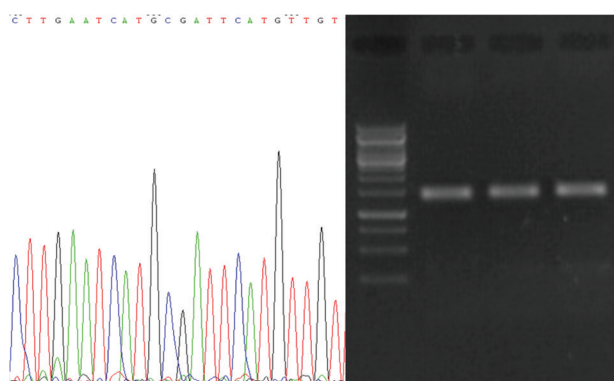


Fig. 5. PCR electrophoresis of 16s rDNA. After amplification the PCR product was inserted on pGEM vector and sequenced (Supplementary files 1-3). The sequence of our strains was blasted on NCBI for identification.

isolation of lactobacilli against human enteropathogenic bacteria for future designing of starter cultures, its application for medical purposes and perhaps the extraction of bacteriocin that is a safe natural antimicrobial for food preservation²¹ and an alternative substance to antibiotics.^{22,23} Bacteriocins, which are antimicrobial peptides produced by probiotic bacteria, might seriously be considered as alternatives to antibiotics. These proteins have significant exhibit potency against other human enteropathogenic bacteria as well as antibiotic-resistant strains that are stable and manipulate the future medicine.²³ Homemade tvorog curd cheese in Azerbaijan that is consumed as a health promoting food, could also be a natural resource for other probiotic organisms such as yeasts.

Conclusion

In conclusion, screening of the acid- and bile-resistant lactobacilli strains from tvorog curd cheese resulted in the isolation of antibacterial samples which were identified by 16s rDNA as *L. plantarum*, *L. casei* and *L. rhominos*. These bacteria that were isolated from traditional regions as native microbiome, were preserved in a biobank for future studies for medicinal applications and food industry,

Research Highlights

What is current knowledge?

- ✓ Probiotic candidate bacteria were isolated from traditionally fermented foods.
- ✓ Antibiotic resistance has turned into one of the most urgent public health issues.
- ✓ Probiotic therapy could be used instead of antibiotic therapy.

What is new here?

- ✓ Homemade tvorog curd cheese in Azerbaijan harbor a variety of probiotics.
- ✓ The isolated probiotics have antagonistic activity.
- ✓ The isolated probiotics could be conserved in a biobank for medical applications.

especially in culture starter designing.

Acknowledgments

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Ethical issues

There is none to be declared.

Competing interests

There is none to be disclosed.

Supplementary materials

Supplementary files 1-3 consist of sequenced data.

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