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SCREENING, ISOLATION AND IDENTIFICATION LACTIC ACID BACTERIA WITH PROBIOTIC POTENTIAL FROM TRADITIONAL DAIRY PRODUCTS OF AZERBAIJAN

Abstract Probiotics are defined as live bacterial preparation with clinical documented health effects in human. Human health is deemed to be maintained by the crosstalk among the body and probiotic bacteria. Thus the search for isolation and identification of friendly human bacteria from traditional fermented foods is important in medicine. Lactic acid bacteria (LAB) are a major group of probiotics. In this research, as five traditional dairy product (home-made cheese, suzme) from Dashkasan, Ismailli and khachmaz regions in Republic of Azerbaijan was characterized for the isolation 15 species of Lactic acid bacteria with probiotic potentiality. Afterwards, the selected strains were examined for their tolerance to acidic pH=3 and 0.3% bile salt. Finally, the isolates were identified by 16s rDNA sequencing. The results clearly revealed two species with higher homology to the L.brevis and L.plantarum with high probiotic potentiality were isolated. This study showed that the Traditional Dairy Products of Azerbaijan contained probiotic bacteria, hence, 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, which are capable of fighting against pathogenic bacteria and living in the digestive tract.

Key words: Probiotic, lactic acid bacteria, acid and bile resistant bacteria, 16s rDNA sequencing

Introduction. Probiotic terms derived from Greek words Pro (favor) and bios (life) [1]. Probiotics are a subgroup of microorganisms with positive effects such as the improvement of human immune system, rearrangement of intestinal microflora, and establishment of antagonistic effect on the growth of harmful bacteria [2,3]. On the host health through improving the gut bacterial balance. These bacteria were first discovered by Metchnikoff in 1907 [4]. Lactic acid bacteria (LAB) are the most common types of probiotics. These bacteria have a long-term survival in fermented products [5]. Lactobacillus is a Gram-positive, non-sporeforming, rarely motile bacteria, while Lactococcus is a Gram-positive, spherical and rarely motile bacteria, both of which are present in considerable amounts in dairy products [6]. LAB make an acidic condition and prevent the growth of pathogens by converting the milk sugar (lactose) into lactic acid [7]. In the food industry, LAB is widely employed as starter cultures and has been indexed as part of human microbiota. Yogurt, cheese and fermented

milk products are mentioned as the main food sources of probiotics. The use of Lactic Acid Bacteria (LAB) in foods and food supplements has a long history and most strains are considered commensal microorganisms with no pathogenic potential lactic acid bacteria (LAB) are widely used in fermented food production and are considered as generally recognized as safe (GRAS) organisms which is safely applied in medical and veterinary functions. Today, the probiotic human-friendly bacteria are isolated from foods, cheese yogurt [8] as well as human himself, human milk [9] infant feces [10] women vagina [11] 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. To analyses and rapidly identify bacteria from microbial communities, classical physiological and biochemical tests are not adequately efficient, since bacterial population involved often has similar nutritional requirements and grows under similar

environmental conditions. Therefore, a clear identification within the species by simple phenotypic tests may sometimes be difficult. The development of molecular techniques has opened up new perspectives for characterizing strains from fermented dairy foods [12].

Objective: isolation and identification lactic acid bacteria with probiotic potential from traditional dairy products of Azerbaijan.

Materials and methods. Sampling and isolation of bacteria. 5 cheese and suzme (curds) samples were collected from Dashkasan, Ismailli and khachmaz 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) [12] 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 [13]. Briefly, the overnight cultures of LAB were inoculated in MRS broth containing 0.3% (w/v) oxgall (Sigma, Louis, USA) and incubated for 4h 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 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 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 method [14] for the following clinically important antibiotics: chloramphenicol ($30 \mu g$), vancomycin ($30 \mu g$), tetracycline ($30 \mu g$), erythromycin ($15 \mu g$), Ampicillin ($10 \mu g$), and methicillin ($10 \mu g$). 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 andsensitive. Then the sensitive isolates were subjected to further characterization.

DNA extraction and molecular identification of probiotic bacteria. The bacterial genomic DNA was extracted according to a previously published method [15].

Amplification of the 16s rDNA was carried out using the primer pair reported previously as: 16 16lacF5'- AGAGTTTGATCMTGGCTCAG-3' 1 6 lacR5' TACCTTGTTAGGACTTCACC-3'[16]. _ 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 [17]. The plasmids were then sent to a commercial sequencing facility (Macrogene, 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.

Results and discussion. The screening of isolates (strains) 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 fig.1 and bile fig. 2 resistant rod-shaped isolates.

Antibiotic susceptibility of potentially probiotic isolates. As shown in fig. 3, approximately 100% of the selected strains were sensitive or semisensitive to the entire routinely used antibiotics in the inhibition zone evaluation [18].

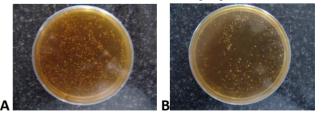


Fig. 1. Screening of lactobacilli tolerance to acidic condition in simulated condition of human gastrointestinal system at pH=3 for 2.5 h. A: the colonies are mix of resistant and unresistant bacteria in acidic condition, that many of them should be removed in screening process.B: the colonies are resistant bacteria in acidic condition.

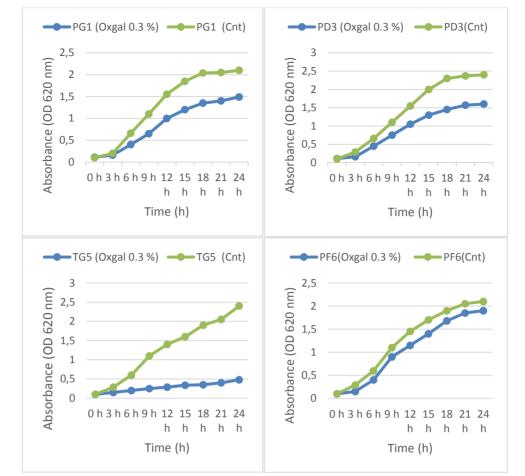


Fig. 2. The growth inhibitory of oxgal (0.3%) for candidate probiotics at overnight incubation. Strain TG5 was very sensitive to bile as compared with strains PF6, PG1, and PD3.

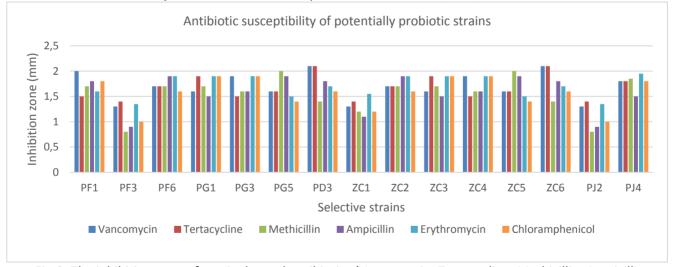


Fig.3. The inhibition zone of routinely used antibiotics (Vancomycin, Tertacycline, Methicillin, Ampicillin, Erythromycin, Chloramphenicol) against selective strains.

Identification of Lactoacilli by 16s rDNA pattern. The 16s rDNA PCR product of the isolates with high probiotic potential and physiological characterization of selected *Lactobacillus*, namely, PF6 and PD3 were cloned in plasmids and sequenced fig. 4, 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.brevis* and *L.plantarum*, with high probiotic potentiality were isolated.

It is recommended that human friendly bacteria be isolated with respect to native foods

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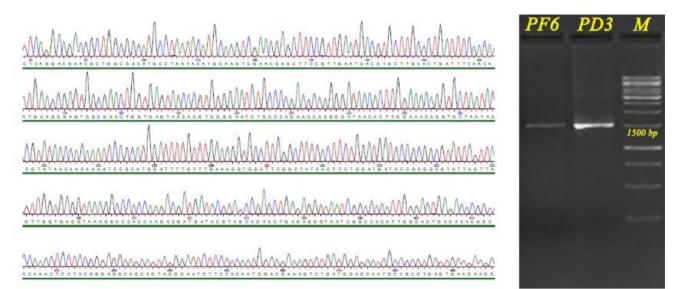


Fig. 4. PCR electrophoresis of 16s rDNA. After amplification the PCR product was inserted on pGEM vector and sequenced. The sequence of our strains was blasted on NCBI for identification.

[19] due to their efficacy in the same population [20] in this study, we found out that in the rural regions of Dashkasan, Ismailli and khachmaz in Republic of Azerbaijan, 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 sensitivitv 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. Therefore in this study, pronase treatment was applied for the degradation of bacteriocin and discrimination of bacteriocin and non-bacteriocin effects.

Conclusion. The acid- and bile-resistant lactobacilli strains from traditional dairy product (home-made cheese, suzme) from Dashkasan, Ismailli and khachmaz regions in Republic of Azerbaijan, where people have a traditional life-style and continue to follow largely the traditional medications, were identified by 16s rDNA as *L.brevis* and *L.plantarum*. These bacteria were

preserved in a biobank for future studies for medicinal applications and food industry.

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