

Identification and Characterization of Lactic Acid Bacteria Isolated from Iranian Traditional Dairy Products

Galia Amirbozorgi^{1*}, Hamidreza Samadlouie², Seyed Ahmad Shahidi¹

1. Department of Food Science and Technology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran.

2. Department of Food Science and Technology, Faculty of Agriculture, Shahrood University of Technology, Shahrood, Iran.

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The lactic acid bacteria (LAB) are an industrially important group of probiotic organisms that play an important role in human health by inhibiting harmful and pathogen bacteria growth, boosting immune function, and increasing resistance to infection. This study aimed to isolate, identify, and biologically characterize probiotic LAB strains from Iranian traditional dairy products. A total of 20 samples of traditional fermented milk were collected from six villages of Mazandaran province, Iran. LAB strains were identified according to phenotypic characterization and 16S rRNA gene sequence analysis. All isolates belonged to the Lactobacillus strains. According to the 16S rRNA gene sequences the isolated bacteria were divided into two sub-groups Lactococcus lactis helveticus and Lactobacillus brevis. The dominant isolated Bacillus genus was Lactobacillus helveticus (76.92% of lactobacilli population). The results of the present study showed slight diversity of LAB among traditional dairy products in Mazandaran province, Iran. These bacteria can be introduced as valuable sources for further starter selection.

Key words: Probiotic, lactic acid bacteria, 16S rRNA

According to international dairy federation and international organization for standardization, lactic acid bacteria (LAB) are defined as a group of Gram-positive, cocci or rods, non sporing and anaerobic organisms with low G C content, which produce lactic acid as the major end product during the fermentation of carbohydrates. The LAB are an industrially important group of probiotic organisms (1-2). Probiotics are known as live microorganisms that play an important role in our health by inhibiting growth of harmful and pathogen bacteria and also

boosting immune function and increasing resistance to infection (3-5). Moreover, the consumption of milk and its derivatives such as yoghurt, cheese and dough is essential to the diet of several millions of people worldwide because these products are source of vitamins and minerals. Information on microb-iological composition of traditional dairy products is helpful in choosing LAB strains with useful function and stable fermentation properties from the traditional dairy products, and their usage as dairy starter in the

*Correspondence: Department of Food Science and Technology, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran.
E-mail: Galia Amirbozorgi

Table 1. Demographic data and measured values of the elderly people in Amirkola

Sample Type	Location	No. of samples
Cheese	Kheshvash	1
	Yoosh	1
	Chelav	1
Yoghurt	Yoosh	1
	Chelav	1
	Larijan	1
	Baladeh	1
	Sangechal	1
Dough	Kheshvash	1
	Yoosh	1
	Chelav	1
	Larijan	1
	Baladeh	1

manufacture. The aim of the present study was to isolate and identify different genera of LAB in traditional dairy products in order to represent a collection of Iranian province LAB strains using conventional culturing method and also by molecular analysis-based 16S rRNA gene sequence analysis.

Materials and methods

Sample collection

20 samples of traditional fermented milk including dough, yoghurt and cheese were collected from six villages in Amol (Mazandaran province, Iran) during the period of November 2014 to February 2015 (Table 1). The samples were collected in sterile tubes and kept at 4 °C for further use.

Isolation of lactic acid bacteria

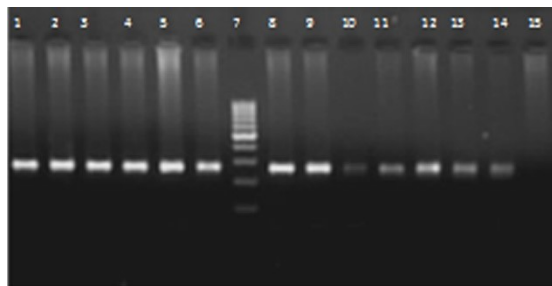


Figure 1. PCR products of purified DNA from isolated strains amplified with primers for 16s rRNA. Lane 7: 100bp ladder; lane 15: no template control.

10 g of each dairy sample was suspended in 90 ml of sterile sodium citrate and homogenized using Stomacher 400 Circulator. Then, 10 mL of each homogenized sample was added to 100 mL Man, Rogosa, Sharpe (MRS) broth and incubated for 24 h in anaerobic conditions (37 °C, 5% CO₂). Colonies with distinct morphologies (color, shape, and size) were sub-cultured on new MRS agar to obtain single pure colonies. Gram staining, catalase test and microscopic observations were performed for all isolates and gram-positive, catalase-negative isolates were stored in 30% (w/v) glycerol at -80 °C for further assessments.

DNA extraction

Bacterial DNA was extracted from all isolated strains using boiling method. Briefly, the single colonies on the growth agar plate were selected and transferred into MRS broth culture medium and incubated for 24 h at 37 °C. After 24 h, 1.5 ml of culture media was centrifuged at 5000 rpm for 5 min at room temperature, then 100 µl of PBS was added to the pellet which was further incubated for 30 min at 100 °C. At the end of the exposure time, 20 µl of TE-buffer was added and the mixture was centrifuged at 10000 rpm for 2 min at room temperature. The supernatant was transferred into a properly labeled 1.5 ml tube for further assessments.

Molecular analyzes of 16S rRNA

Amplification of DNA fragments encoding 16S rRNA was performed using forward (5'-CTCAAACTAAACAAAGTTTC -3') and reverse (5'-CTTGTACACACCGCCCGTCA -3') primers under following conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, extension at 72 °C for 1 min and a final elongation step at 72 °C for 7 min. The PCR products were sequenced by Macrogen Company (Korea). The chromatograms were edited using the Chromas version 3.1 software. Thereafter,



Results

nucleotide sequencing of the *16S rRNA* gene in 13 samples (Figures 1-2) and compared with available sequences in GenBank database using the BLAST program at NCBI. The results confirmed that all strains belonged to *Lactobacillus* genera. *rRNA* gene sequence of isolates showed 94-100% similarity to related bacterial sequences in the GenBank. According to the *16S rRNA* sequences, the isolated bacteria were divided into two sub-groups *Lactococcus lactis helveticus* and *Lactobacillus brevis*. Among 14 *Lactobacillus* strains which were isolated from traditional dairy products, 10 and 3



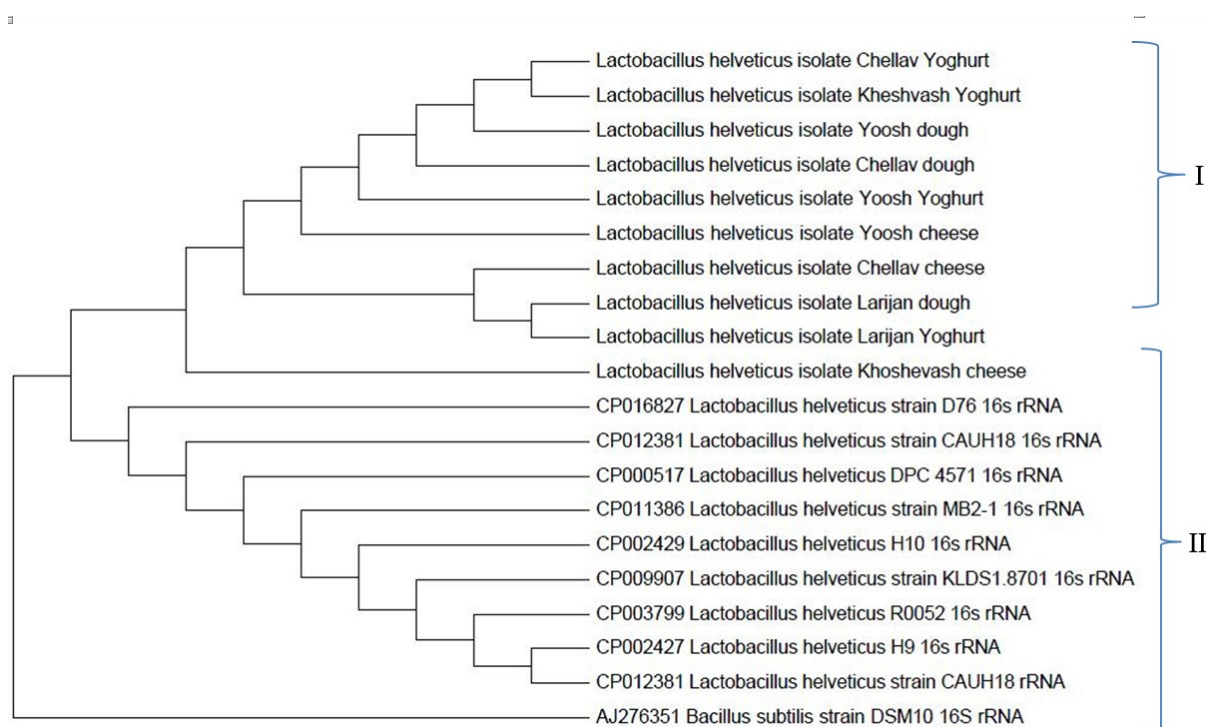


Figure 4. Phylogenetic relationship between the isolated 16s rRNA gene sequence of *Lactobacillus brevis* and various sequences of *Lactobacillus brevis* by maximum parsimony method.

isolates belonged to *Lactobacillus helveticus* and *Lactobacillus brevis*, respectively. The phylogenetic tree diversity between the isolated strains and related reference strains for *Lactobacillus brevis* and *Lactobacillus helveticus* is shown in figures 3 and 4, respectively. The phylogenetic tree showed that the isolates and related reference strains were mainly divided into two clusters. As shown in figures 3 and 4, all *Lactobacillus* samples isolated from traditional dairy products were grouped together in a separate sub-cluster in comparison to related reference strains.

Discussion

In recent years, there has been increasing interest in isolation, identification and classification of LAB in traditional dairy products. LAB are the most proposed probiotics, and the key components of the starters used in dairy products. *Lactobacillus* is one of the most important genera of LAB in dairy products such as cheese, yoghurt and dough. In the present study, we analyzed traditional dairy products

samples for possible presence of potential probiotic bacteria by molecular methods in order to improve our knowledge about specific characteristics of LAB in Iran. *Lactobacillus* is generally predominant in the traditional naturally-fermented milk (6-11). We found two sub-groups: *Lactococcus lactis helveticus* and *Lactobacillus brevis* in traditional dairy products. *Lactococcus lactis helveticus* constituted the majority of isolated lactobacilli (71.5 %). The diversity of LAB species in dairy products is variable and area specific. In similar studies from Iran, *Lactobacillus plantarum* was isolated with high frequency from most traditional koopeh cheese and tarkhineh. While *L. brevis* was present only in 3% koopeh cheese and *L. Helveticus* in 14.8% Tarkhineh isolated lactobacilli (10, 12). In addition, Davati et al. found that isolated strains of camel milk were classified into five groups including *Leuconostoc*, *Lactobacillus*, *Enterococcus*, *Weissella* and *Pediococcus* genus. Lactobacilli were observed in 15% of samples and they were all *Lactobacillus casei* (13). Azadnia et al. showed that among

Lactobacillus strains which were isolated from traditional drinking yoghurt, 25.9, 22.3%, 21% and 15.5% of isolates belonged to *Lactobacillus delbrueckii subsp. Bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus helveticus*, respectively (14). RoushanZadeh et al. reported that *Lactobacillus delbrueckii subsp. Bulgaricus* was the most abundant isolate in traditional drinking yoghurt, while *Lactobacillus helveticus* and *Lactobacillus brevis* were present in 15 and 10% of isolated samples, respectively (15). In traditional Spanish cheese; *L. casei ssp. casei* and *L. brevis* were predominant (16) while *L. paracasei ssp.* and *L. sake* were the dominant species in Greek goat cheese (17). Lollo et al. showed that the predominant species in Brazilian fresh cheese was *L. acidophilus* (18). Sun et al. found that among LAB strains isolated from the kurut samples, 35.1% of isolates belonged to the *Lactobacillus* strains. Moreover, *Lactobacillus delbrueckii subsp* was the predominant species and *L. helveticus* was observed only in 25% of samples (19). Bao et al. showed that *Lactobacillus helveticus* and *Lactobacillus casei* were considered as the predominant populations in the yak milk products (11). Yu et al. showed that *Lactobacillus helveticus* and *Lactobacillus brevis* were present in 27.3% and 0.44% of mongolian dairy products, respectively (20). Also, Yu et al. in another study showed that the predominant LAB isolates were *Lactobacillus helveticus* (176 out of 599 strains) and *Lactobacillus plantarum* (63 out of 599 strains), which represented 39.9% of all isolates in traditional fermented dairy foods of Russia (21).

The present study focused on the utilization of LAB resources and analysis of LAB composition in Iranian traditional dairy products by molecular methods. We confirmed that the major LAB in traditional dairy products are lactobacillii. The obtained results showed slight diversity of LAB among traditional dairy products. *Lactobacillus*

helveticus was the most abundant isolated species. Phylogenetic tree from traditional dairy products suggests that these isolated bacteria can be new subspecies of *Lactobacillus brevis* and *Lactobacillus helveticus*. However, regarding annual economic losses due to the importation of starters, further studies need to be performed on traditional dairy products before their commercial uses.

Conflict of interest

The authors declared no conflict of interest.

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