Probiotic Potential of Five *Lactobacillus* Strains Isolated from Traditional Persian Yoghurt in Fars province, Iran: Viewing Through the Window of Phylogenetics

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Probiotics are dietary supplements of live microorganisms which presence of them in gastrointestinal tract in sufficient quantities can be beneficial to host health. Amongst various bacterial strains, lactic acid bacteria (LAB) are the most common probiotic bacteria. The aims of this study were to isolate and identify potentially LAB probiotics from the fermented yoghurt products of townships and villages in Fars province, Iran. Thirty samples of traditional dairy products were collected from different regions of Fars province. Laboratory methods such as gram-staining, motility; and biochemical approaches including carbohydrate utilization and catalase activity were employed to isolate LAB and assess their relevant properties. In five final isolates, 16S rDNA genes were extracted, amplified through PCR and electrophoresed. These genes were then sequenced and compared with the library of the sequences of the NCBI databases. Five different isolates of *Lactobacillus* were identified and reported as the normal flora in traditional dairy products. These included *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. jensseni*, *L. crispatus* and *L. gasseri*. Phylogenetic analysis revealed a genetic relationship between the five studied strains together and with some previously reported probiotic strains. The obtained strains are potentially probiotics and these isolates can be used in dairy product industries to achieve beneficial effects of probiotics.

**Key words:** Probiotic properties, lactic acid bacteria, traditional dairy products, functional food, phylogenetics.
Lactobacilli are important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Poorbaghi et al., 2014). They produce various compounds such as organic acids, diacetyl, hydrogen peroxide, bacteriocin and other bactericidal proteins during lactic fermentations (Ravi et al., 2011). Furthermore, Lactobacilli have been associated with numerous other health benefits such as the reduction of lactose intolerance (Fuller, 1991; KíHôvá Sepová et al., 2008), increased immune response (Turroni et al., 2014; Villena et al., 2014), and anticancer properties (Viaud et al., 2014). In the food industry, LAB is widely used as starter cultures and has been cited to be part of human microbiota (Holzapfel et al., 2001). In raw milk and dairy products such as cheeses, yoghurts and fermented milks, Lactobacilli are naturally present or added intentionally, for technological reasons or to generate a functional food with health benefit for the consumers (Hassan and Frank, 2001).

Bacterial identification, including LAB, was performed by isolation and phenotypic study of the microorganism in the traditional microbiology laboratory. However, these methods have two major drawbacks: First, they require special equipment and expertise, second, some microorganisms exhibit biochemical characteristics that do not match the patterns for any known genus and species (Woo et al., 2002). Therefore, identification of Lactobacillus isolates by phenotypic methods is difficult, not reliable and some species are not readily distinguishable in terms of phenotypic characteristics (Afaf, 2012). According to above mentioned, derivation of simple and rapid identification methods is required in order to deal with the large numbers of Lactobacillus isolates obtained during microbial-ecological studies of ecosystems and also in food and health studies. Nucleotide base sequences of Lactobacillus 16S ribosomal DNA (16S rDNA) provide an accurate basis for phylogenetic analysis and identification (Amann et al., 1995; Nikolova et al., 2009). The sequence obtained from an isolate can be compared to those of Lactobacillus species held in data banks. On the other hand, the development of molecular techniques has opened up new perspectives for characterizing strains from fermented dairy foods (Tannock et al., 1999; Delfederico et al., 2006). Among PCR-based methods, 16S rDNA PCR-Restriction fragment length polymorphism (RFLP) analysis is easy, rapid and inexpensive way to identify microbial species such as yeast, acetic acid bacteria, and also few Gram positive bacteria (Claisse et al., 2007).

Iranian people in villages and nomad tribes make various fermented dairy products using cow and sheep milks. The transformation of surplus cow and sheep milk into traditional yoghurt is achieved through fermentation. The aims of the present study were to isolate and identify Lactobacillus existed in cow and sheep milk derived traditional yoghurt from the villages and nomad tribes of Fars province located in south of Iran. In this study, physiological, phenotypic and genotypic methods were used for accurate, valid and exact strain detection.

**MATERIALS AND METHODS**

**Isolation and culturing of microorganisms**

Total 30 yoghurt samples were collected randomly from nomad tribes of the different regions of Fars province, Iran and were used for the isolation of Lactobacillus strains. Sample collection was performed aseptically in sterile bottles kept in an ice-box, and transported immediately to the laboratory. One mL of each milk sample was homogenized with 9 mL of distilled water and mixed thoroughly for 60 S. Serial dilutions were made and aliquots (100 µL) of each dilution were streaked on MRS agar contained (g/L) 1% polypeptone, 1% meat extract, 0.5% yeast extract, 2% glucose, 0.5% sodium acetate, 0.2% ammonium citrate, 0.2% K2HPO4, 0.02% MgSO4.7H2O, 0.005% MnSO4.4H2O and 0.0108% Tween 80. It was incubated under anaerobic conditions for 48 hours at 37 °C.

**Biochemical and probiotic characterization of the isolates**

The morphological and physiobiochemical identification of bacteria were done according to Bergey’s Manual of Determinative Bacteriology (Holt, 1994). Colonies from highest dilution plates were randomly selected and purified by subculturing. Gram-positive, catalase negative and the most unique cultures were stored at -80 °C in MRS.
supplemented with 20% glycerol. Isolates were phenotypically assigned to the genus level on the basis of cell morphology, Gram staining, catalase-negative, indole negative and non-motility, according to the methods described previously (Gusils et al., 2004). Moreover, the acid production from carbohydrates was evaluated by using a miniaturized assay with glucose, D-galactose, D-mannitol, D-salicin, cellobiose, D-lactose, D-mannose, D-melibiose, maltose and D-lactose in microplates. After performing of all above mentioned tests, final and the most unique isolates were selected and genotypic methods of identification were carried out for them.

**Molecular identification of the strains**

DNA extraction and 16S rDNA gene sequencing were performed according to our previously published methods (Ghasemi et al., 2008; Morowvat et al., 2010; Rasoul-Amini et al., 2009; Yazdi et al., 2005). Briefly, 1mL of cultured cell was harvested by centrifugation (13000 g, 3 min at room temperature). Cells were resuspended in 0.5 mL of PBS buffer and the mixture was shaken slowly. The genomic DNA was extracted by heat shock method and used for PCR amplification of 16S rDNA gene. The 16S rDNA of the isolates was amplified using universal 16S ribosomal DNA primers: forward 5’-CAGCCGCGGTAAATAC-3’ and reverse 5’-ACGGGCGGTGTGTAC-3’ which amplifies 800 bp region of the 16S rDNA gene. PCR reaction was performed in a total volume of 25 µL containing 200 µM dNTPs, 0.5 U Taq polymerase, 0.5 µM from each primers, 1 µL template, 50 mM MgCl₂, 2.5 µL PCR buffer 10x and 19 µL DDW. Amplified DNA samples and a 100 bp ladder as molecular marker were electrophoresed in a 1% (w/v) agarose gel using tris borate EDTA (TBE) electrophoresis buffer containing 1µg/mL ethidium bromide. PCR products were purified and then sequenced by CinnaGen Company (Tehran, Iran).

**Phylogenetics analysis of the amplified sequences**

The resulting 16S rDNA gene sequences were aligned and compared to the nucleotide sequences of some known microorganisms in Gene Bank database of the National Center for Biotechnology Information (NCBI) by using Basic Local Alignment Tool (BLAST)(http://blast.ncbi.nlm.nih.gov/Blast.cgi). To create a multiple alignment, MAFFT multiple sequence alignment software version 7 (Katoh and Standley, 2013) was used. It uses a fast Fourier transform approach to align medium to large nucleotide sequences. The phylogenetic studies was conducted using MEGA software version 6 (Tamura et al., 2013). It was based on the 16S rDNA sequence of 800 bp drawn using the neighbor-joining method. *Staphylococcus aurous* was used as out-group strain. The CLC sequence viewer software (Qiagen, Aarhus, Denmark) version 7.5, was used to identify the conserved domains among these studied sequences.

**RESULTS**

Collected yoghurt samples were combination of cow and sheep milk derived in approximately ratio of 2:1 (19 cow milk and 11 sheep milk derived yoghurt). From these 30 samples, Gram positive bacilli were detected only in 20 samples and among them 12 long, five mid to high and three short bacilli were existed. Results of biochemical and morphological test of 20 selected isolates are presented in Table 1. As demonstrated, all strains could utilize glucose, but 90% of them fermented maltose and mannose. Also, 80% of them fermented cellobiose, sucrose and galactose. The fermentative ability of these isolates was 75, 70, 50 and 15 percent for salicin, lactose, melibiose and mannitol, respectively. Results of catalase test of these isolates were 10%, 10% and 80% positive, negative and positive/negative, respectively. All isolates were indole negative and non-motile. From these isolates, totally five isolates were selected according to the most diversity of morphology and biochemical variables (isolates A, C, G, M, Q). All other molecular tests were performed only for these selected isolates. A band of 800 bp which represents 16S rDNA amplified sequence are shown in Figure 1. This band is specific for *Lactobacillus* genus and confirmed that these five selected isolates are belonged to *Lactobacillus* genus. Sequence analysis of this amplified bands and comparing them with NCBI database demonstrated a 98-99% of homology for each isolate with five distinct strains included *L. acidophilus* (isolate A, 98%), *L. delbrueckii*...
### Table 1. Biochemical and morphological tests on 20 selected Lactobacilli isolates

| Characteristics | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T |
| Glucose         | ++ | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Cellobiose      | +  | -  | -  | +  | +  | +  | +  | +  | -  | +  | +  | +  | +  | +  | +  | -  | +  | +  | +  | +  |
| Galactose       | +  | +  | +  | +  | +  | -  | +  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Lactose         | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  |
| Mannose         | +  | -  | -  | +  | -  | +  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  | +  | -  |
| Mannitol        | -  | -  | +  | +  | +  | +  | +  | -  | -  | -  | -  | -  | +  | +  | +  | +  | +  | +  | -  | +  |
| Maltose         | +  | +  | +  | +  | +  | +  | +  | -  | -  | +  | +  | -  | +  | +  | -  | +  | +  | +  | +  | +  |
| Salicin         | +  | +  | +  | +  | +  | +  | +  | -  | -  | +  | +  | -  | +  | +  | -  | +  | +  | +  | +  | +  |
| Melibiose       | +  | +  | +  | +  | +  | +  | +  | -  | -  | +  | +  | -  | +  | +  | -  | +  | +  | +  | +  | +  |
| Sucrose         | +  | +  | +  | +  | +  | +  | +  | -  | -  | +  | +  | -  | +  | +  | -  | +  | +  | +  | +  | +  |
| Catalase        | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Indole          | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Motility        | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

**subsp. bulgaricus** (isolate C, 99%), **L. jensseni** (isolate G, 98%), **L. cripatus** (isolate M, 98%) and **L. gasseri** (isolate Q, 98%).

The multiple sequence alignments, the similarity between the studied sequences and the conserved domains among the studied sequences were shown with a color scale (Figure 2). The green residues are the least conserved and white residues are the most conserved. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The consensus sequence is also shown in the below of ten studied sequences. Besides a yellow colored plot in percent scale, shows the conservation extent of each domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

The constructed phylogenetic tree is depicted in Figure 3. Molecular phylogenetic analysis of five isolated *Lactobacillus* strains with some related strains. The evolution history was inferred by using the Maximum Likelihood method. The bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 11 nucleotide sequences (accession numbers in parenthesis). All positions containing gaps and missing data were eliminated. Evolutionary analysis were conducted in MEGA6. *Escherichia coli* was used as out-group strain. It was revealed that there is a close genetic relationship between *L. acidophilus* and *L. cripatus*. This clade has a near relation with *L. gasseri* on its own. Whilst, *L. delbrueckii* showed just a long distant relationship with all other isolates.

**DISCUSSION**

This study isolated and identified five strains of *Lactobacillus* from 30 different cow
and sheep milk derived traditional yoghurt of villages and nomad population in Fars province, the biggest state of southern part of Iran. Our isolates were *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. jensseni*, *L. crispatus* and *L. gasseri*. All of these strains potentially can be probiotic and help to health of the digestive tract and whole body of the host.
Fig. 3. Molecular phylogenetic analysis of five isolated Lactobacillus strains with some related strains. The evolution history was inferred by using the Maximum Likelihood method. The bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 11 nucleotide sequences (accession numbers in parenthesis). All positions containing gaps and missing data were eliminated. Evolutionary analysis were conducted in MEGA6. Escherichia coli was used as out-group strain.

It must be remembered that conventional biochemical and physiological tests clearly have some limitations in discriminating between large numbers of isolates showing similar physiological characteristics (Zamfir et al., 2006; Mathara et al., 2008). The strategy described in this study is an effective means of identifying unknown strains of the Lactobacillus complex, which is a critical initial step in the selection and development of novel probiotic strains. Similar strategy was used in different previous studies worldwide (Kullen et al., 2000; Lee et al., 2008; Macías-Rodríguez et al., 2008). Also, in Iran, there are several reports about using this methods for identifying and selecting appropriate probiotic strains. In a study conducted by Latifi et al, LAB were isolated and characterized using phenotypic methods (Gram staining, physiological and biochemical tests) from traditional cheese and yoghurt of Heris and Sarab regions. Then their acid and bile tolerance, as the primary probiotic characterizations, were investigated. 16S rDNA gene of Lactobacilli and Enterococci was amplified for identification of bacterial strains. Totally, 15 Lactobacillus spp. and 16 Enterococcus spp. were isolated from traditional dairy products of these regions that could be potentially used in the industrial dairy products (Latifi et al., 2010). In another published study, 17 strains of Lactobacillus were isolated from different Iranian dairy products. Their study showed the highest genetic diversity in yoghurt population. Also, all strains had 99% genetic similarity with L. casei (Tafvizi and Tajabadi Ebrahimi, 2012). Isolation and identification of Lactobacilli in traditional fermented milk from two different provinces in the west of Iran were carried out by Dana et al. Lactobacillus bacteria were isolated, and characterized phenotypically. The 16S rDNA genes from these two strains were amplified and sequenced and a phylogenetic tree was constructed. The sequencing results in combination with phenotypic and biochemical properties showed that both strains were similar to L. crusterum (Dana et al., 2013).

Some differences were seen between biochemical tests (especially carbohydrate fermentation tests) in this study and registered properties for detected strains. These differences may be due to some mutations in the genes that responsible for metabolic enzymes. Further studies are needed for finding these interfering mutations and evaluation of their relationship with probiotic beneficial properties.

CONCLUSION

According to our findings, phylogenetic analysis using 16S rDNA sequences appeared to be a very practical method and highly sensitive in the discrimination of the Lactobacillus species. Also, this study demonstrated that LAB (especially Lactobacillus genus) are the most frequently probiotics which are found in traditional Persian yoghurt.
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