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MOLECULAR CHARACTERIZATION OF ISOLATED LACTIC ACID BACTERIA FROM DIFFERENT TRADITIONAL DAIRY PRODUCTS OF TRIBES IN THE FARS PROVINCE, IRAN

Maryam Jafari[1], *Nabi Shariatifar*[1], *Gholamreza Jahed Khaniki*[*1], *Mehran sayadi*[**2], *Abbas Abdollahi*[3]

Address(es): Gholamreza Jahed Khaniki, Mehran Sayadi
[1] Department of Food Safety and Hygiene, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
[2] Department of Food Safety and Hygiene, Faculty of Health, Fasa University of Medical Sciences, Fasa, Iran
[3] Department of Microbiology, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran.

*Corresponding author: Ghjahedkh@yahoo.com , Mehransayadi6262@yahoo.com

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ABSTRACT

Various traditional dairy products could be considered as an abundant source for Isolation/collection of new lactic acid bacteria (LAB) with unique characteristics. The present researchaimed to investigate the morphological, biochemical and genotypic characterization of dominant lactic acid bacteria that were isolated from traditional dairy products in Iranian tribes . A total of 75 samples of traditional yogurt, doogh from dairy units in Fars province were randomly collected. Isolation of lactic acid bacteria, biochemical and genotypic identification were conducted. Totally, 157 LAB isolates were selected. Cocci and rod shape LAB were 53.50% and 46.49%, respectively. Biochemical tests showed the occurrence of 40.6% of the strains were *Streptococcus*, 2.25% of the strains were enterococci and 20.3% of the strains were lactococci. Most isolated lactobacilli were related to *Lactobacillus plantarum* and *Lactobacillus bulgaricus*. Genetically the presence of the following species was verified: *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Lactobacillus fermentum*, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus casei*. The current study showed that thestrains which were isolated from traditional dairy products were not only appropriatefor use as starter adjuncts or cultures, but also they may provide a valuable gene pool for research and production of commercial starters with specific traits.

Keywords: Biochemical Identification; Genotypic Identification; Lactic acid bacteria; Strain selection; Traditional dairy products

INTRODUCTION

Fermentation is the most economical old methods for food preservation and storage that maintaince quality for long period **(Mashak, Sodagari, Mashak, & Niknafs, 2014)**. Fermentation of lactic acid is a known method for producingseveral dairy products, such as yogurt, doogh (savory yogurt-based beverage), kashk (drained yogurt product), gharaghooroot (a nonfat-diary product after boiling Doogh and yogurt), cheese, etc **(Abd El Gawad, Abd El Fatah, & Al Rubayyi, 2010; Azadnia & Khan Nazer, 2009)**. Traditionally, doogh is referred to a drinking product which is produced from yogurt dilution following a strong agitation stage in special waterproof sacs made from sheep or goat skin, called 'Mashk'.**(Kirdar, 2012; Noori, Keshavarzian, Mahmoudi, Yousefi, & Nateghi, 2013)** Produced centuries ago in Bulgaria, yogurt is a semisolid fermented product whichis currently being consumed in different countries. Yogurt is a dairy product which is generated by*Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), two bacteria which convert lactose into lactic acid **(Çelik, 2007; Tamime & Marshall, 1997)**. The existence of living organisms in fermented food is well known and many scientific research were done regarding the traditional fermented products. The microorganisms present in food mostly specify the propertiesof the fermented food(flavor, texture, and acidity)andprovide several nutritional benefits. These organisms exist as food natural microflora or resultfrom adding starter cultures in food fermentation **(Abdi, Sheikh-Zeinoddin, & Soleimanian-Zad, 2006)**.Dairy starter cultures are vital to the production ofhigh quality and safeproducts in the modern dairy industry.Starter cultures are utilized as single strain, mixed strain, or multiple strains depending on the product type **(El Soda, Ahmed, Omran, Osman, & Morsi, 2003)**. Lactic acid bacteria (LAB) are frequently known as dairy starter culture which are used in various fermented milk products **(Moulay *et al*., 2006)**. LABs widely exist as indigenous raw milk microflora in nature. These microorganisms are Gram-positive, catalase-negative andhaveanimportant part in food fermentation **(Maqsood, Hasan, & Masud, 2013)**. The objective of the current research was to studythe phenotypic and genotypic diversity of dominant LABs that were isolated from traditional dairy products in some tribes in Fars province, Iran.

MATERIALS AND METHODS

Sample collection

75 traditional doogh and yogurt sampleswere gleaned randomly from local areas of Fars in Iran and were sampled by transferring 10 ml into in sterile plastic bags maintained at 4°C and analyzed within 24 h. After being transferredunder refrigerated conditions (4°C) to the laboratory, the collected samples underwent microbiological analysis during the next 24 h.

Lactic Acid Bacteria Isolation

In completely sterile conditions, the collected samples were homogenized andserially diluted in Ringer's solution and the aliquots (100 µl) of each dilution were spread-plated for the isolation of LAB. Plate Count Agar (PCA) (Merck, Germany) was employed to improve the isolation of LAB by incubating perti dishes at a temperature of 30°C for 72h. De Man Rogosa Sharpe (MRS) agar (pH 5.7,Merck, Germany) was utilized to isolatelactobacilli, while GM17 agar(pH 7.15,Himedia, Hindi) was used for the isolation of streptococci, lactococci, and enterococci. The culture media were supplemented with 50 mg L^{-1} of natamycin to stop themold and yeasts from growing**(Botes, Todorov, Von Mollendorff, Botha, & Dicks, 2007)**. Through the use of the gas pack system, MRS plates were incubated in aerobical and anaerobical conditions (Merck Anaerocult Type A) at 37°C for 48-72 h, and GM17 plates were incubated under the sameconditions at 30°C and 42°C for 24-48h **(Jokovic *et al.*, 2008)**. Morphologically distinct colonies from PCA, GM17 and MRS agar plates were randomly-picked, sub-cultured and purified by streak plating via the same medium and were incubated at 37℃ for 72 h;subsequently, the strains were maintained at 4°C and refreshed by streaked every 2 weeks. Pure strains were further tested forcatalase production, gram staining, spore formation, cell

morphology, and oxidase activity. Catalase negative and Gram-positive, non-spore forming, oxidase negative, cocci or rods isolates were selected as presumptive LAB and stored in growth (MRS or GM17 or Tryptic soy broth (TSB)) medium containing 15% (vv^{-1}) glycerol as stocks frozen at -80°C.

Biochemical identification

Biochemical identification of the isolated/selected bacteria was carried out following Bergey's manual of determinative bacteriology.(Breed, Murray, & Smith, 1957) For biochemical identification, every single isolate was activated in 5 ml GM17 or MRS or TSB broth medium for 24 h at 37°C prior to use. The tests used for preliminary characterization of isolates included Gram staining, catalase test (H2O2, 3%), oxidase test and endospore formation(spore staining). Isolates with Gram-positive, catalase, and oxidase-negative, non spore forming characteristics were selected for further identification **(Nikita & Hemangi, 2012)**.

Identification of cocci

Inoculated bacterial isolates in GM17 broth medium were incubated at 10°C, 40°C and 45°C. At different NaCl concentrations (2%, 4% and 6.5%), the growth ability was evaluated in the modified GM17 broth media. The change of the color as the evidence for cell growth **(Nikita & Hemangi, 2012)**. Growth at pH 9.6, was detected by cultivating of isolates in the GM17 broth media with pH 9.6 (by pH adjustment with NaOH).

Arginine hydrolysis and gas production from citrate and Reduction of Methylene Blue

Inverted Durham tubes and Reddy broth were applied in this test. The color of cultures usingargininechanged from yellow to violet. To reduce Methylene Blue, overnight cultures were transferred into GM17 broth tubes with Methylene Blue.

Carbohydrate fermentations

The properties of the isolates were further determinedaccording to the profiles related to their sugar fermentation. Sixteen different sugars were utilized. In each test, the strains were inoculated in 3 ml of Phenol red base broth media containing 150 µl of filter sterilized (0,22 µm, Merck Millipore) 10% sugar solutions (0.5%). The acidification as a measure of fermentation ability is reflected in color change from red to yellow.

Identification of lactobacilli

Inverted Durham tubes and MRS broths were usedto identify lactobacilli. The accumulation of gas in Durham tubes indicatedthe production of CO_2 from glucose. Lactobacilli isolates were screened to determine if they were ableto fermentD (-) Raffinose, D Mannitol, D (+) Galactose, D (-) Ribose, Lactose, Maltose, , glucose, L (+) Arabinose, D (-) Salicin, D (+) Xylose, D (+) Mannose, Fructose sucrose, Sorbitol, Trehalose, and Rhamnose as described above.

Genotypic identification

DNA extraction

To extract the DNA, the cultures of isolates grown in MRS and GM17 broth for 18h were employed. Genomic DNA extraction was donethrough phenol-chloroform extraction, which is a modified bacterial DNA extraction protocol depicted by Federici et al.,and the samples of DNA were maintained at -20°C **(Federici *et al*., 2014)**. A single colony of each isolate was inoculated into 10 ml of the appropriate medium broth and incubated overnight at 37°C. Using centrifugation at 7500-8000 rpm for 5 min, the cells were harvested, and genomic DNA was isolated based on a modified genomic DNA isolation protocol **(Ashmaig, Hasan, & El Gaali, 2009)**. The collected bacterial pellet was resuspended in 2.5 ml of TE buffer (1M Tris-HCl, 0.5 mM EDTA pH 8.0) and washed two times with the same buffer. Once washed,the pellet was re-suspended into 250 µl of lyses solution (1% (w/v) SDS, 0.2 mol NaOH). Through gentle shaking, the cell suspension was incubated for 1 h in a water bath at 65°C . The solution was gently emulsified by an equal amount of TE buffer saturated phenolchloroform (1:1); it was thencentrifuged for 5 min at 7500-8000 rpm, and the aqueous phase was transferred intonew tubes. Next, the aqueous solution waswashed two times with an equal amount of chloroform: isoamylalcohol, 24:1, and it was centrifugated for 5 min at 7500-8000 rpm. Oncethe aqueous phase was centrifuged, it was transferred to Eppendorf tube (400 µl/ tube) to which1/10 volume of three molar sodium acetate (pH 2.5) was added. Via adding 2 volumes of ice cooled absolute ethanol, the nucleic acids were precipitated. Subsequently, the mixture underwent centrifugationat 14000 rpm for 10 min; after that,the supernatant was discarded and the created pellet was washed two times by 70% ethanol. The remaining ethanol was eliminated through drying the pelletat room temperature. It was then dissolved in TE buffer and kept at -20°C.

Amplification of 16S rRNA genes and specific PCR conditions

The mixtures of the PCR reaction were prepared in 25 µl volumes containing 2.5 µl of 10X Taq buffer, 1.5 µl MgCl2 (50 mM), 2.5 µl dNTPs (2 mM/µl), 2 µl random primer (16S-FA 5′-AGAGTTTGATCCTGGCTCAG-3′and 16S-RA 5′-AGGAGGTGZTCCAGCCGC-3′), 0.5 µl Taq-DNA polymerase (5 U/µl). Through adding sterilized distilled water, the mixture was diluted up to 25 µl . The amplifications were carried out via an Applied Biometra thermalcycler programmed to repeat the thermal profile.The PCR program included 5 min at 96°C; 30 cycles of 96°C for 30 s, 55°C for 30 s and 72°C for 30 s;another extension step at 72°C for 5 min was also performed in a mastercycler. Using electrophoresis, theamplification products were assessed. Afterwards, the standard marker and the DNA fragments wereseparated in 1.5% ethidium bromide-stained agarose gels. Then, the separated fragments(1200-1500 bp) and their patterns were visualized and photographed with gel documentation system **(Terzic-Vidojevic *et al*., 2014)**.

Multiple sequence alignment

Ultimately, the amplified genes were obtained from the gels by use of a QIA quick gel extraction kit (Bioneer, USA) according to the the manufacturer's instructions. Buffer 1 (gel binding buffer) was added to the cutted piece of gel, incubated for 10 min at 60°C to completely desolve the gel. The solution was transferred to the DNA binding column tube, centrifuged for 1 min at 13000 rpm and poured in the flow-through, re-assembling the DNA binding filter column with 2.0 ml collection tube. 500 µl of buffer 2 (cell lysis buffer) was added to the DNA binding column tube, centrifuged for 1 min at 13000 rpm., poured in thethrough, re-assembling the DNA binding filter column with the 2.0 ml collection tube and dried via another centrifugation for 1 min at 13000 rpm. DNA was eluted from column by adding 30 µl buffer 3 (EB) to the center of the DNA binding filter column and remaining for at least 1 min at room temperature and centrifugation 1 min at 13000 rpm. The purified DNA fragments of amplified genes for 16S RNA were finally subjected to sequencing service (Macrogen, South Korea) for standard sequencing **(Poormontaseri, Hosseinzadeh, & Shekarforoush, 2014)**.

DNA sequence Analysis

DNA sequences were obtained by use of forward and reverse primers assembled and edited through the use of BioEdit sequence alignment editor version 5.0.9. Using BLAST, the homology was searched in the Genbank DNA database, and the sequence similarity was estimated.

RESULTS AND DISCUSSION

Lactic Acid Bacteria Isolation

From the collected samples, approximately 280 colonies were picked at random from selective media for each group of LAB.

Phenotypic Identification

Selected colonies from the fermented food samples showed comparable differences in their colony morphology (size, shape, shine and color). Among the 280 Gram-positive isolates that were extractedfrom the dairy products, 117 (all belonging to the LAB family) were negative for catalase and oxidase activity andnon-spore forming bacteria . A large number ofisolates (60 isolates) had a rod shape, therebypossibly related tothe genus *Lactobacillus*; the rest were cocci (57 isolates).

Physiological and Biochemical Identification

The results of identification of coccoid shaped isolates are shown in Table 1. According to obtained results, the strains were *Streptococcus* (29 isolates), enterococci (12 isolates) and lactococci (16 isolates). The ability of lactobacilli isolates to ferment different types of carbohydrate is shown in Table 2.

Table 1 Technological properties of cocci shaped of cocci LAB that were isolated from dairy products and the fermentation profile of sugars.

Characteristics	*Streptococcus thermophiles*	*Entrococcus faecium*	*Enterococcus fecalis*	*Entrococcus durans*	*Lactococcus lactis* subsp. *lactis*	*Lactococcus lactis* subsp. *cremoris*
Gram staining	+	+	+	+	+	+
catalase production	-	-	-	-	-	-
oxidase activity	-	-	-	-	-	-
spore formation	Non - spore	Non-spore	Non-spore	Non-spore	Non-spore	Non-spore
Growth at pH 9.6	-	+	+	+	-	-
Reduction Methylene Blue	-	+	+	+	±	-
Growth at 10 °C	-	+	+	+	+	+
Growth at 40 °C	+	+	+	+	+	-
Growth at 45 °C	+	+	+	+	-	-
Growth in 2% NaCl	±	+	+	+	+	+
Growth in 4% NaCl	-	+	+	+	+	-
Growth in 6.5% NaCl	-	+	+	+	-	-
Hydolysis of arginine	-	+	+	+	+	-
CO_2 from citrate	-		-		-	-
Acid formed from						
Mannitol	-	±	+	-	±	-
Raffinose	-	±	-	-	-	-
Ribose	-	+	+	+	+	-
Galactose	-	+	+	+	+	+
Maltose	-	+	+	+	+	-
Lactose	+	+	+	+	+	+

+: Growth / Fermentable carbohydrates - : Non-growth / Non-fermentable carbohydrates

Table 2 Carbohydrates fermentation pattern of LAB isolated from traditional yogurt and doogh

	***Lactobacillus* species**								
Characteristics	***Lb. plantarum***	***Lb. delbruecki***	***Lb. heleviticus***	***Lb. brevis***	***Lb. casei* subsp. *tolerans***	***Lb. casei***	***Lb. acidophilus***	***Lb. bulgaricus***	***Lb. salivarius***
Arabinose	+	-	-	+	-	-	-	-	-
Lactose	+	-	+	-	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+
Glucose (gas)	-	-	-	+	-	-	-	-	-
Xylose	+	-	-	+	-	-	-	-	-
Mannitol	+	-	-	-	-	+	-	-	+
Rhamnose	-	-	-	-	-	+	-	-	+
Salicin	+	-	-	-	+	+	+	-	-
Mannose	+	+	-	-	+	+	+	-	+
Ribose	+	-	-	+	+	+	-	-	-
Galactose	+	-	+	-	+	+	-	+	+
Sucrose	+	+	-	+	-	+	+	-	+
Raffinose	+	-	-	-	-	-	+	-	+
Fructose	+	+	-	+	+	+	+	+	+
Sorbitol	+	-	-	-	+	+	-	-	+
Trehalose	+	-	+	-	-	+	+	-	+

Lb: *Lactobacillus*, +: Growth / Fermentable carbohydrates, - : Non-growth / Non-fermentable carbohydrates

Fig 1 shows identification results of the isolated LAB based on the phenotypic characterization. In addition, the results show that LAB isolates can be considered as the predominant bacteria in collected dairy products. Nonetheless, different dairy samples had various predominant LAB.

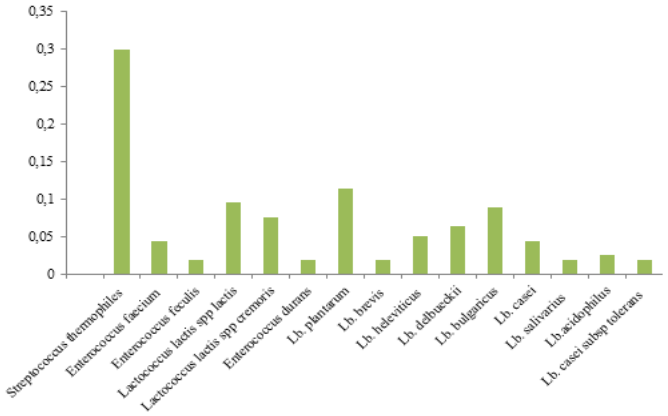

Figure 1 The percentage of LAB species isolated from collected samples

In addition, we found that cocci especially those cultured by pouring plate, have complitely different forms, however with the aid of a microscope, they were seen as spherical or oval, mostly forming short or long chain and in single or pairs. Bacilli were observed to be so differently from each other under a microscope; although, on the plate, they were almost similar except *L. bulgaricus* with a star shape. Diversity of bacterial species can be related to different factors, such asraw milk composition and the breed of the animal,regional influences, the kind of the utilized culture medium, effect of reducing pH environment at different stages of growth, concentration of salt in the environment, and the existenceof calcium**(Kirdar, 2012; Rahimi *et al*., 2012; Wright & Klaenhammer, 1983)**.

In order to classify cocci shaped isolates, growth ability at 10°C, 40°C, and 45°C as well as growth at 2%, 4% and at the consentration of 6.5% NaCl were used. *Streptococcus thermophilus* was not able to show growth at 10°C, but it had a good growth at 40°C and 45°C; 47 isolates showed these characteristics. *Enterococcus* species could grow at 10°C, 40°C and 45°C; 3 isolates showed these characteristics. Unlike other species, *Lactococcus* species indicated growth at 10°C but had no growthat 45°C and only 26isolates possessed such property. *L. lactis* subsp. *lactis* strain could grows poorly at 45°C, while *L. lactis* subsp. *cremoris* grew just at 10°C. *S. thermophilus* and *L. lactis* subsp. *cremoris* did not have the ability to grow at salt concentrations of 4 and 6.5%, while *L. lactis* subsp. *lactis* and *Enterococcus* species could grow at different levels of salt. Reduction methylene blue, growth at a pH of 9.6, arginine hydrolysis, and citrate utilization and carbohydrate fermentations also were examined. Only 11 isolates were able to reduce methylene blue and hydrolyze the arginine. At a pH of 9.6, very few (only 2 isolates) could grow and 5 of them were able to produce gas from citrate. Cocci shape isolates capable of fermenting carbohydrates(raffinose, mannitol, ribose, lactose, maltose, galactose)were also evaluated. *S. thermophilus* and *L. lactis* subsp. *cremoris* bacteria had less ability to ferment carbohydrates, while *Enterococcus* species and some species of *Lactococcus* are able to ferment majority of tested sugars. *S. thermophilus* and *L. lactis* subsp. *cremoris* bacteria differ in the fermentation of galactose; *S. thermophilus* poorly ferments galactose because theits enzyme beta-galactosidase is not able to ferment this sugar. Similarly, most researchers agree on the fermentation of lactose by cocci bacteria **(Begovic *et al*., 2011; Patil, Pal, Anand, & Ramana, 2010)**. On the contrary, atypical characteristic of some *Streptococcus* strains is their galactose fermentation. Almost none of LAB cocci were able to ferment raffinose, however, they are able to break the disaccharide lactose into glucose and galactose that can be used for energy.

For identification of bacilli shaped isolates, carbon dioxide from glucose and carbohydrate fermentation tests were performed by applying 13 different type of carbohydrates. The biochemical tests showed that *Lactobacillus* species could be divided to three groups. Group I comprises obligately homofermentative strains (*L. acidophilus, L. delbruckii, L. helveticus*). The second category includes facultative heterofermentative strains (*L. casei, L. plantarum*), and group III are obligately heterofermentative strains (*L. brevis, L .fermentum*); This means that for the fermentation of sugar, only the 6-PG/PK pathway is accessible. In order to differentiate homofermentative and heterofermentative LAB, the ability of the isolates was studied in the fermentation of pentose sugars such as ribose and xylose. All cocci isolates were able to ferment pentose in order to identify species more accurately. This survey and similar studies indicate the approximate different sugars fermented by *Lactobacillus* species **(Ayad, Nashat, El-Sadek, Metwaly, & El-Soda, 2004; Erkuş, 2007; Khedid, Faid, Mokhtari, Soulaymani, & Zinedine, 2009; Moulay *et al*., 2006; Omafuvbe & Enyioha, 2011)**. Regarding galactose and mannose sugars ability fermentation by *L. bulgaricus*, contradictory findings were reported by researchers. **Asmahan (2011)**, reported that *L. delbrueckii* is not capable of fermenting the mannose sugar, while Kiaei *et al*. in his research reported that mannose was fermented by *L. delbrueckii* **(Ali, 2011)**. Majority of researchers have reported in their studies that *Lactobacillus* strains are not able to ferment galactose (Ashmaig *et al*., 2009), but Maqsood et al. have suggested that *L. bulgaricus* can ferment the galactose. *L. delbueckii, L. bulgaricus* and *L. heleviticus* possess weak fermentation ability and are only able to ferment few suagrs **(Maqsood *et al*., 2013)**. In a previously reported research by **Kirdar (2012)** in meaditerian area, three *Lactobacillus* species were noted. Unlike to other lactobacilli, *L. casei* subsp. *pseudoplantarum* is able to obtain energy and produce acid from most of the carbohydrates **(Abdullah & Osman, 2010)**. The ability to ferment galactose is considered as the main factor for distinction between *L. helveticus* species as galactose positive and *L. bulgaricus* known as galactose negative. *L. lactis* and *L. acidophilus* ferment sugars in the same way, however, the only distinction between them is in the fermentation of raffinose, in which *L. lactis* is not able to use the sugar for its energy supply. Roshanzade *et al*. reported the similar action of these two species in the fermentation of sugars **(RoushanZadeh, Eskandari, Shekarforoush, & Hosseini, 2014)**. Bacterial strains oxidize environmental energy sources by producing different enzymes; however, using energy sources depends on the synthesis of specific enzymes by each bacteria **(Ashmaig *et al*., 2009)**. According to the literature, new bacterial species identified are discovered in almost the same studies carried out previousely, pointing that the main reason for diverzification could be adaptation to the local conditions of the region. Biochemical tests for the representative LAB isolated from traditional yogurts in various parts of Fars province revealed the occurrence of 52 (44.44%) *L. lactis* subsp *cremoris* and 65 (55.56%) *L. mesenteroides* subsp *cremoris* among LAB cocci and regardingLAB bacilli ,*L. helveticus* 85 (15.3%); *L. plantarum* 124 (22.3%); *L. brevis* 117 (21%) ;*L. casei* subsp. *casei* 86 (15.5%) and *L. delbruckii* subsp. *bulgaricus* 144 (25.9%) were detected **(Azadnia & Khan Nazer, 2009)**. To investigate different species isolated from Iran using phenotypical and molecular tests, Tajabadi Ebrahimi *et al*. performed a study on traditional Iranian dairy products. Isolated species belonged to *L. plantarum* (cheese, fermented milk, yogurt, kashk), *L. brevis* (cheese, yogurt), *L. casei* (cheese, yogurt, fermented milk), *L. acidophilus* (yogurt), *L. jensenii* (cheese), *L. salivarius* (yogurt), *L. lactis* (cheese), *L. alimentarium* (yogurt), *L. rhamnosus* (cheese), *L. arieminis* (cheese), *L. agilis* (kashk) **(Ebrahimi, Ouweh, Hejazi, & Jafari, 2011)**.

Acurcio *et al*. (2014), **García-Cano *et al*. (2019)** and **Masalam *et al*. (2018)** noted that strains of *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Streptococcus* and *Weissella* isolated from raw milk and dairy products have *in vitro* probiotic features, including resistance to biliary salts and gastric juiceand antagonism against reference pathogens, including*Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., and *Shigella sonnei* **(Acurcio *et al*., 2014; Bin Masalam *et al*., 2018; García-Cano *et al*., 2019)**. The quality of cheese and other fermented food products depends on the ability of microorganisms to produce flavour and aroma in the starter culture. Various LABs have been assessed to determine if they are ableto degrade amino acids into aroma compounds. *L.lactis* subsp. *lactis* and *L.lactis* subsp. *cremoris*, *L.lactis*, *L. helveticus*, *L. bulgaricus* and *L.casei* have this ability to degrademethionine to methonethiol, dimethyledisulphide and dimethyltrisulphide **(Abiona & Adegoke, 2017)**. These strains, which have high lipolytic and proteolytic activities were genetically identified and proved to playa crucial role in cheese ripening **(Abosereh, Abd El Ghani, Gomaa, & Fouad, 2016; García-Cano *et al*., 2019)**.

Genotypic identification

Since 1990, some alternatives have emerged for the classical phenotypic and biochemical identification of LAB . These methods might not be sufficient to definitively attribute a strain to a certain species. To thoroughly identify and classify the species in bacterial systematics, polyphasic approaches **(Coeuret, Dubernet, Bernardeau, Gueguen, & Vernoux, 2003)**, such asphenotypic, chemotaxonomic, and genotyping methods are still recommended.

To identify the LAB species more accurately, PCR assays were used with primers that target 16S rRNA gene (Table 3). To confirm the belonging to certain species, the nucleotide sequences of the 16S rRNA gene related to all the isolates were examined and specified using the BLAST program on NCBI. The results were categorized based onthe maximum identity andrecorded in terms of coverage. The sequence similarity with 90% or higher cut-off was regarded as significant.The optimum hit was considered as the sequence with the most maximum similarity to the query sequence.

Table 3 Genotypes of the selected isolated LAB as 16S rDNA gene sequence alignments submitted to the NCBI GeneBank database.

Species	Similarities (%)	Source of Isolation
Entrococcus faecium	99%	**Doogh**
Streptococcus termophilus	100%	**Yogurt**
Lactococcus lactis subsp. *lactis*	99%	**Doogh**
Entrococcus durans	100%	**Yogurt**
Lactococcus salivarius	95%	**Doogh**
Lactobacillus heleviticus	98%	**Yogurt**
Lactobacillus delbrueckii subsp. Bulgaricus	100%	**Yogurt**
Lactobacillus plantarum	97%	**Yogurt**
Lactobacillus casei	98%	**Yogurt**
Lactobacillus acidophilus	95%	**Doogh**

With the current rapid growth in biotechnology, more and more molecular techniques are utilizedin the genetic diversity studies on LAB. Typing methods based onmajor advantages because of their highly discriminatory power, being applicable universally and having independent culture. Methods like pulsed field gele electrophoresis (PFGE), DNA/DNA hybridization, ribotyping, polymerase chain reaction (PCR), real-time PCR hybridization and sequencing of rRNA are able to distinguish even the close related strains **(Miljkovic et al. 2019; Axelsson, 2004; Furet, Quénée, & Tailliez, 2004)**.

In categorizing LABs, a large number of studies have compared the foregoing genetic methods. A highly comprehensive study in this regardis still the collaborative work carried out by M. Sta°hl, M.-L. Johansson andcolleagues. They made use of a set of *Lb. reuteri* and *Lb. plantarum* strains. These strains weresystematically characterized via phenotypic tests, DNA-DNA homology, REA, automated PCR sequencing of rRNA, ribotyping, and RAPD. Their work clearly elucidates that every method has its own upsides and downsides,and that one technique cannot bethe answer to all problems, but rather different methods complement one another.

CONCLUSION

In the present study, in-vitro approaches were chosen to examine the diversity of the LAB in dairy products from Iran. Previous studies have been conducted on LAB isolates from the Iranian traditional cheeses and yogurts, with less attention to others traditional and industrial dairy products. This work showed that other fermented dairy products could be significant sources of LAB. Some studies reflected the fact that LAB strains detected from dairy products, produce more pleasant flavor and volatile compounds than industrial strains. Local LABs are highly potential as starters or adjuncts. To optimally utilize novel strains, the specific conditions of use have to be definedand more detailed research must be conducted on their technological features.

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Conflict of Interest: None to declare.

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