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Genotypic and phenotypic study of *Lactobacillus* species isolated from traditional yogurt and cheese in southwest Iran

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Abstract

Lactic acid bacteria (LAB) have the property of producing lactic acid from sugars by a process called fermentation. These bacteria are broadly used in the production of fermented food products, such as yogurt, cheeses, sauerkraut and sausage. Among them *Lactobacillus* species are used industrially for the production of yogurt and cheese. The purpose of present study was to identify the genotypic and phenotypic of *Lactobacillus* species in the traditional cheeses and yogurt produced in the Chaharmahal Va Bakhtiari province located in southwest Iran using molecular technique. 18 Dalameh cheeses and 15 traditional yogurt specimens were collected from various groceries and markets in the Chaharmahal Va Bakhtiari province. Genomic DNA was extracted from yogurt and cheese samples, as well as reference strains and PCR reaction was down for amplification of 16S rDNA gene. PCR products were digested by *TaqI* and *HaeIII* restriction enzymes and fragments were analyzed in 2% agarose gel electrophoresis. The results of present study showed that *Lactobacillus delbrueckii* (*Lb. delbrueckii*), *Lb. acidophilus*, *Lb. brevis* and *Lb. lactis* strains from traditional cheese and yogurt is important and more frequent in dairy industry. These findings could be used as starter cultures in manufacturing of cheeses and yogurt to approach better and safety dairy products with high quality.

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Introduction

Cheesemaking began about 8000 years ago and now there are in excess of 1000 cheese varieties worldwide (Sandine and Elliker, 1970). Since the 1960s, the industrial production of fermented milks especially yogurt has increasingly developed worldwide. Dairy yogurt is produced using a culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* bacteria. Several factors account for the success of yogurt: its natural image, its organoleptic characteristics (fresh and acidulated taste and characteristic flavor), nutritional, prophylactic and therapeutic properties, and its moderate cost due to the high productivity of the production lines (Sandine and Elliker, 1970). The ripening of cheese is a very complex process involving a range of biochemical reactions and high densities of microorganisms are present in cheese ripening (Beresford *et al.*, 2001; Morsi and Soda, 1993). There are several factors which influence the development of the quality and organoleptic characteristics of cheese and yogurt, including the type of milk, its microbiological quality, the conditions of ripening and others (Herreros *et al.*, 2003). Some factors influencing growth of microorganisms in cheese, such as water content, salt concentration and pH. These factors are processed through a number of common steps such as gel formation, whey expulsion, acid production and salt addition, followed by a period of ripening (Beresford *et al.*, 2001). The primary changes involve the formation of peptides and amino acids from caseins, fatty acids from milk fat and the conversion of lactose to lactic acid and primary changes may also involve the degradation of citrate. The secondary changes involve the conversion of the end-products that result from the primary changes. For example amino acids yield the formation of amines, organic acids and sulfur compounds (Morsi and Soda, 1993). The micro flora of cheese may be divided into two groups include starter lactic acid bacteria that the most important of these is *Lactobacillus* and secondary microorganisms (Beresford *et al.*, 2001). However, the lactic acid bacteria (LAB) play an important role in cheesemaking. During cheesemaking, the

composition of LAB microflora undergoes several changes, according to modifications of environmental conditions, which frequently provide cellular stresses, such as heat shock, adverse pH, as well as reduction of oxidation–reduction potential, water activity and nutrient content (Elisabetta *et al.*, 2008). The LAB carries out the initial acidification of the milk which assists in gelation (Cogan *et al.*, 1997). Production of lactic acid by the starter flora during cheese manufacture results in a decrease in the pH of the milk (Beresford *et al.*, 2001). Some species of LAB are able to hydrolyze milk fat or at least some triglycerides (Morsi and Soda, 1993). Secondary flora include non-starter lactic acid bacteria (NSLAB), propionic acid bacteria (PAB) that grow in many cheese varieties during ripening, mould that are important in the ripening of a range of cheeses and yeast, however, the role of yeast in cheese ripening is unclear (Beresford *et al.*, 2001; Fleet, 1990). Some reports confirm the presence during cheese ripening of adventitious bacteria, referred to as NSLAB which play a major role in the organoleptic profile of the cheeses (Sancheza *et al.*, 2005). They are not part of the normal starter flora; they generally do not grow well in milk and do not contribute to acid production in the cheese vat (Cogan *et al.*, 1997). During ripening, starter organism's autolyze, releasing growth substrates and enzymes; at the same time, NSLAB numbers increase from low initial levels until they eventually become the predominant microflora in mature cheese (Sancheza *et al.*, 2005).

Lactobacillus is a genus of Gram-positive facultative anaerobic or microaerophilic bacteria. They are a major part of the lactic acid bacteria group, named as such because most of its members convert lactose and other sugars to lactic acid. Lactobacilli have many important roles in industry. Some *Lactobacillus* species are used for the production of yogurt, cheese, sauerkraut, pickles, beer, wine, cider, kimchi, chocolate, and other fermented foods, as well as animal feeds, such as silage (Makarova *et al.*, 2006; Man *et al.*, 1960). The lactic acid produced by Lactobacilli inhibits the growth of other organisms and lowers the pH of the product in these products

(Man *et al.*, 1960).

According to importance of Lactobacilli for flavour, colour, quality and texture of dairy products, the present study performed to molecular identification of the genotypic and phenotypic of *Lactobacillus* species in the traditional cheeses and yogurt produced in the Chaharmahal Va Bakhtiari province located in southwest Iran.

Materials and methods

Sampling

In present study, a total of 18 Dalameh cheeses and 15 traditional yogurt samples were collected from various sites and markets in the Chaharmahal Va Bakhtiari province located in southwest Iran and were analyzed.

Reference Strains

The reference strains viz. *Lactobacillus reuteri* NCDC AS17, AS83 and NRRL 14170, *Lb. delbrueckii* ssp. *delbrueckii* CCM 7191, *Lb. fermentum* NRRL 4524, *Lb. acidophilus* CCM 4833, *Lb. delbrueckii* ssp. *Lactis* CCM 2772, *Lb. delbrueckii* ssp. *bulgaricus* CCM 7190, *Lb. casei* CH1, *Lb. johnsonii* NRRL 2178, *Lb. plantarum* NRRL 1954, *Lb. helveticus* NRRL 4526, *Lb. brevis* NRRL 4527 and *Lb. rhamnosus* NRRL 442 were obtained from the Razi Institute, Tehran, Iran.

Genotypic Characterization of *Lactobacillus*

Samples (10 g) of each products and reference strains were mixed with 90 ml of 0.85% (w/v) sterile physiological saline and vortexed for 1 minute. Serial dilutions were made in the same solution and plated on to MRS agar. Plates were incubated for 72 h at 37°C, under aerobic conditions. Genomic DNA was extracted from 2 ml samples of overnight cultures grown in MRS broth at 30°C using DNP™ Kit (CinnaGen, Iran), according to the manufacturer's recommendation. The extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm. The extracted DNA of each sample was kept frozen at -20°C until used.

Gene Amplification

The oligonucleotide primers EGE1: 5'-AGAGTTTGATCCTGGCTCAG-3' and EGE2: 5'-CTACGGCTACCTTGTACGA-3' for *16S rDNA* gene described by Jensen *et al.* and conserved among various bacteria, including lactobacilli, were used as forward and reverse primers, respectively (Jensen *et al.*, 1993). *Lactobacillus acidophilus* 5 (Lb-5) was used as positive control. A negative-DNA control was performed by adding 1 µL of sterile ultrapure deionized water. For detection of *16S rDNA* gene the samples were amplified in a Gradient Palm Cycler (Corbett Research, Australia) and PCR reaction was performed in a total volume of 25 µL in 0.5 ml tubes containing 1 µg of genomic DNA, 1 µM of each primers, 2 mM MgCl₂, 200 µM dNTP, 2.5 µL of 10X PCR buffer and 1 unit of *Taq* DNA polymerase (Roche applied science, Germany). This solution was initially denatured at 95°C for 5 min, followed by 32 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min), elongation (72°C for 1 min) and a final extension at 72°C for 5 min. The amplified products were detected in 1% agarose gel electrophoresis. Aliquots of 10 µL of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, the gel was stained with Ethidium Bromide and images were obtained in UVIdoc gel documentation systems (UK).

RFLP Analysis of *16S rDNA* Gene

Restriction fragment length polymorphisms (RFLPs) were used for analysis of *16S rDNA* gene amplified from *Lactobacillus* strains. PCR products were digested by *TaqI* and *HaeIII* restriction enzymes (Roche applied science, USA) in a total volume of 20 µL (10 µL reaction solutions, 2 µL enzyme buffers, 0.2 µL of each enzyme, and 7.8 µL distilled water) and placed in the incubator at 65°C for *TaqI*, and 37°C for *HaeIII*. All of the reactions were performed overnight and additionally, *TaqI* restriction reactions were overlaid with mineral oil to avoid evaporation. Restriction fragments were analyzed electrophoretically in 2% agarose gel in TBE buffer.

Physiological and Phenotypic Characterization of Isolates

All isolates were tested for Gram stain and catalase activity. Also, they are examined for acid-producing ability and proteolytic activity. Gram-positive, catalase-negative isolates were checked for gas (CO₂) and NH₃ producing in modified MRS broth. API 50 CHL test strips were used for determination of sugar fermentation patterns of LAB isolates (BioMerieux, France). In addition, the presence of diaminopimelic acid (DAP) in the cell walls of *Lactobacillus* was determined on cellulose plates using a thinlayer-chromatography. Determination of the lactic acid produced system from glucose was performed enzymatically by using D-lactate and L-lactate dehydrogenase test kits (Roche Diagnostic, France).

Statistical Analysis

Analysis of data and polymorphic patterns of *16S rDNA* gene in *Lactobacillus* strains were performed using the SPSS version 17.0 computer software (SPSS, Chicago, IL).

Results

After DNA isolation the *16S rDNA* region was amplified by PCR reaction. For the amplification of *16S rDNA* region of isolates and reference strains EGE1 forward primer and EGE2 reverse primer were used. Forward primer is complementary to the 5' end of *16S rDNA*, and the reverse primer is complementary to the 3' end of *16S rDNA* region. Then 10 µL of PCR products were visualized by agarose gel electrophoresis under UV light. The length of amplification products varied from 1500 to 2000 bp (Fig. 1).

Lactobacillus isolated from cheeses and traditional yogurt samples and reference strains were digested by *HaeIII* and *TaqI* restriction enzymes to obtain a restriction profile (Fig. 2 and 3). Different lactobacilli bacterial strains isolated from Dalameh cheeses (10 strains) and traditional yogurt (9 strains) shown in Table 1.

Table 1. *Lactobacillus* strains isolated from Dalameh cheeses and traditional yogurt in southwest of Iran.

<i>Lactobacillus</i> species	No. in Dalameh cheeses	No. in traditional yogurt
<i>Lactobacillus delbrueckii</i>	14	8
<i>Lactobacillus acidophilus</i>	12	5
<i>Lactobacillus brevis</i>	12	6
<i>Lactobacillus lactis</i>	10	6
<i>Lactobacillus helveticus</i>	7	0
<i>Lactobacillus casei</i>	4	9
<i>Lactobacillus plantarum</i>	5	2
<i>Lactobacillus rhamnosus</i>	3	2
<i>Lactobacillus fermentum</i>	1	3
<i>Lactobacillus reuteri</i>	0	2
<i>Lactobacillus johnsonii</i>	1	0
Total	69	43

Discussion

The LAB are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. These microbes are broadly used by us in the production of fermented food products, such as yogurt (*Streptococcus* spp. and *Lactobacillus* spp.), cheeses (*Lactococcus* spp. and *Lactobacillus* spp.),

sauerkraut (*Leuconostoc* spp.), and sausage (Jensen *et al.*, 1993; Tamang *et al.*, 2000). These organisms are heterotrophic and generally have complex nutritional requirements because they lack many biosynthetic capabilities. Most species have multiple requirements for amino acids and vitamins. Because of this, lactic acid bacteria are generally abundant

only in communities where these requirements can be provided (Tango and Ghaly, 1999). Lactic acid is a natural organic acid which has many applications in pharmaceutical, food and chemical industries (Tango and Ghaly, 1999). LAB microflora plays a fundamental role in the acidification of curd, as well as in other physical and chemical transformations that affect the development of curd and flavour (Elisabetta *et al.*, 2008).

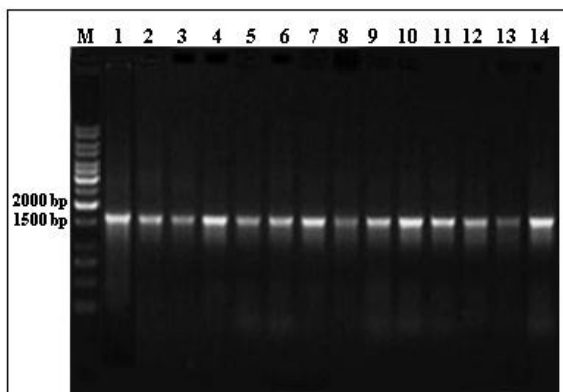


Fig. 1. PCR amplification products from *Lactobacillus* isolated and reference strains (Line M: 1 kb DNA marker (Fermentas, Germany), Lines 1-3: AS17, AS83, and NRRL 14170 *Lb. reuteri*, respectively, line 4: NRRL 4524 *Lb. fermentum*, line 5: CCM 4833 *Lb. acidophilus*, line 6: CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii*, line 7: CCM 2772 *Lb. delbrueckii* ssp. *lactis*, line 8: CCM 7190 *Lb. delbrueckii* ssp. *bulgaricus*, line 9: CH1 *Lb. casei*, line 10: NRRL 2178 *Lb. johnsonii*, line 11: NRRL 1954 *Lb. plantarum*, line 12: NRRL 4526 *Lb. helveticus*, line 13: NRRL 4527 *Lb. brevis*, and line 14: NRRL 442 *Lb. rhamnosus*).

The main genera of LAB include *Lactobacillus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Weissella* and *Vagococcus* (Guarneri *et al.*, 2001). Between this LAB microflora, *Lactobacillus* species play an important role in ripening of cheese, therefore, determination of *Lactobacillus* species isolated from traditional yogurt and cheese in Chaharmahal Va Bakhtiari province (southwest Iran) is useful in dairy industry. In present study the *Lactobacillus* species of 33 traditional cheese and yogurt samples were analyzed by cultivation on MRS media and then, genomic DNA

extracted and PCR reaction performed for amplification of *16S rDNA* gene. Then, PCR products were digested by *Hae*III and *Taq*I restriction enzymes.

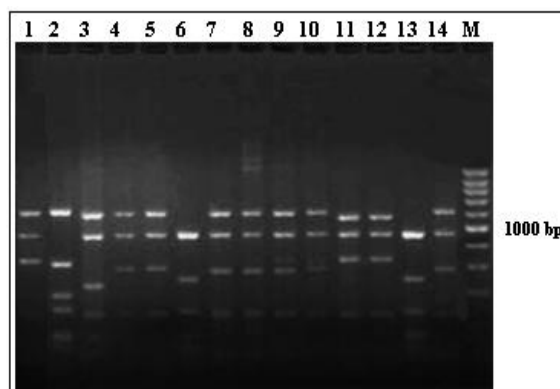


Fig. 2. Gel electrophoresis of PCR products after digested with *Hae*III restriction enzyme for detection of *Lactobacillus* strains in isolated and reference samples (Line M: 100 bp DNA ladder (Fermentas, Germany), line 1: NRRL 442 *Lb. rhamnosus*, lines 2-4: AS17, AS83, and NRRL 14170 *Lb. reuteri*, respectively, line 5: NRRL 4524 *Lb. fermentum*, line 6: CCM 4833 *Lb. acidophilus*, line 7: CCM 7191 *Lb. delbrueckii* ssp. *delbrueckii*, line 8: CCM 2772 *Lb. delbrueckii* ssp. *lactis*, line 9: CCM 7190 *Lb. delbrueckii* ssp. *Bulgaricus*, line 10: CH1 *Lb. casei*, line 11: NRRL 2178 *Lb. johnsonii*, line 12: NRRL 1954 *Lb. plantarum*, line 13: NRRL 4526 *Lb. helveticus*, and line 14: NRRL 4527 *Lb. brevis*).

In present study *Lactobacillus* species such as *Lb. delbrueckii*, *Lb. lactis*, *Lb. acidophilus*, *Lb. brevis*, *Lb. helveticus*, *Lb. casei*, *Lb. fermentum*, *Lb. rhamnosus*, *Lb. plantarum* and *Lb. johnsonii* were isolated from cheese and *Lb. delbrueckii*, *Lb. lactis*, *Lb. acidophilus*, *Lb. brevis*, *Lactobacillus casei*, *Lb. fermentum*, *Lb. rhamnosus*, *Lb. plantarum* and *Lb. reuteri* were detected in yogurt samples.

This work provides a first microbiological and molecular study of traditional cheese and yogurt in southwest Iran. The results of present study showed that witch strains of *Lactobacillus* species from traditional cheese and yogurt in southwest of Iran is important and more frequent in dairy industry and these findings can help us to have a better manufacturing of cheeses and yogurt.

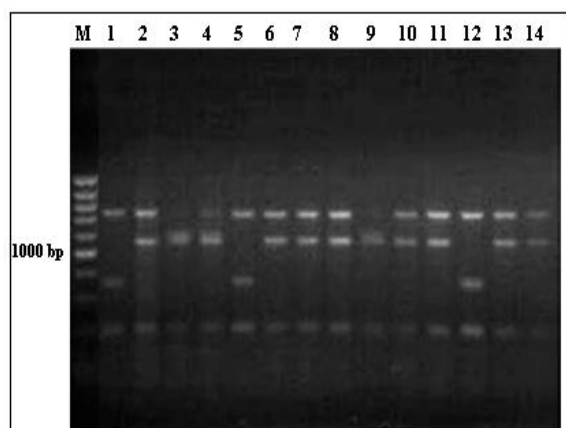


Fig. 3. Gel electrophoresis of PCR products after digested with *TaqI* restriction enzyme for detection of *Lactobacillus* strains in isolated and reference samples (Line M: Fermentas, Germany), lines 1-3: AS17, AS83, and NRRL 14170 *Lb. reuteri*, line 4: NRRL 4524 *Lb. fermentum*, line 5: CCM 4833 *Lb. acidophilus*, line 6: CCM 7191 *Lb. delbrueckii* ssp. *Delbrueckii*, line 7: CCM 2772 *Lb. delbrueckii* ssp. *lactis*, line 8: CCM 7190 *Lb. delbrueckii* ssp. *Bulgaricus*, line 9: CH1 *Lb. casei*, line 10: NRRL 2178 *Lb. johnsonii*, line 11: NRRL 1954 *Lb. plantarum*, line 12: NRRL 4526 *Lb. helveticus*, line 13: NRRL 4527 *Lb. brevis*, and line 14: NRRL 442 *Lb. rhamnosus*).

Some studies were performed about lactic acid bacteria and its correlation with fermented food products. Guarneri *et al.* in 2001 optimized PCR method to identify *Lactobacillus brevis*. Their study showed protocol was highly efficient and sensitive (Guarneri *et al.*, 2001). Tamang and Sarkar showed the predominance of *Lb. plantarum* and *Lb. brevis* in young bamboo shoots (Tamang *et al.*, 1996). Dickson *et al.* in year 2005 showed that PCR assay provides a more rapid, specific and sensitive alternative to conventional culture methods for the identification of *Lb. fermentum* in clinical specimen (Dickson *et al.*, 2005). The study of Ignacio Garabal and coworkers in 2008 on characterization of lactic acid bacteria isolated from raw cows' milk cheeses currently produced in Galicia (NW Spain) showed that microflora and lactic acid bacteria in cheese-making environments have undergone changes, with the most evident difference being the practical absence of *Enterococcal* strains among the current isolates (Ignacio Garabal *et al.*, 2008).

The research of Elisabetta *et al.* in 2008 on diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese showed that combination of PCR-denaturing gradient gel electrophoresis (DGGE) and sequencing can effectively describe the lactic acid bacteria population of Parmigiano Reggiano cheese in advanced stages of ripening, giving useful information for elucidating the role of LAB in determining the final cheese quality (Elisabetta *et al.*, 2008). In a study on fresh and fermented yak milk, Wu *et al.* reported the presence of *Lactobacillus fermentum*, *Lactobacillus helveticus* and *Lactobacillus curvatus* showing that lactobacilli could play an important role in the fermentation of yak milk (Wu *et al.*, 2009).

Some of the strains identified in this study displayed promising properties suggesting that they might be candidate strains to use as starter cultures in manufacturing of cheeses, therefore, the technological characterization of these isolated *Lactobacillus*, can employ as the appropriate selection as adjunct cultures for the standardization and improvement of the cheese and yogurt quality and safety

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