Phenotypic and genotypic diversity of dominant lactic acid bacteria isolated from traditional yoghurts produced by tribes of Iran

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Summary

Morphological, biochemical and molecular characteristics were studied to identify dominant lactic acid bacteria (LAB), isolated from traditional yoghurts produced by tribes of Iran. From 60 yoghurt samples, a total of 137 LAB isolates were determined, in which 66 and 71 were identified as lactic acid cocci and bacilli, respectively. Biochemical tests showed the occurrence of 9.76% mesophilic homofermentative, 10.98% mesophilic hetrofermentative, 26.83% thermophilic homofermentative and 47.56% mesophilic homofermentative cocci. As for lactic acid bacilli, mesophilic facultative hetrofermentative (26%); thermophilic obligate homofermentative (56%); mesophilic obligate hetrofermentative (18%) were found. Genetically the presence of the following species were verified: *E. faecium*; *E. faecalis*; *E. durans*; *L. lactis* subsp. *lactis*; *St. thermophilus*; *Lb. delbruecki* subsp. *bulgaricus*; *Lb. brevis*; *Lb. diolivorans*; *Lb. helveticus*; *Lb. jensenii*; *Lb. plantarum.* 9% of the *Lactobacillus* isolates showed incompatible results between phenotypic and genotypic characteristics. From the cocci isolates, 38.46% showed identical results between phylogenetic characteristics. The current study constitutes the first step in the designing process of LAB starter cultures, to protect the typical organoleptic characteristics of traditional yoghurt. The results could also be used to introduce new starter cultures for commercial use.

Key words: Lactic acid bacteria, Phenotype, Genotype, Yoghurt

Introduction

Fermentation is one of the oldest methods practiced by humans to transform milk into products with extended shelf lives (Pederson *et al*., 1979). Yoghurt, a fermented milk product containing a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (*Lactobacillus bulgaricus*) (Tamime and Marshall, 1997), has been very popular in Mediterranean countries (the Balkans, North Africa), central and southwest Asia (Mongolia, Turkey, Iraq, Iran and Syria) and central Europe. In many of countries, yoghurt is still manufactured using traditional procedures. Yoghurt consumption has steadily increased since World War II, not only in Europe, but also in the United States, and has gained popularity from health-concerned consumers worldwide.

Dairy starters are the most crucial components in the manufacture of high-quality fermented milks. Due to their probiotic activities, these organisms are claimed to impart nutritional and health benefits to consumers and are able to proliferate or even survive in the human gastrointestinal tract for a long period of time (Gardini *et al*., 1999).

Yoghurt starter cultures homofermentatively produce lactic acid from lactose and decrease a pH below 4.5 in 4-5 h at 42°C (fermentation time is longer if temperature is lower). The main characteristic and distinct flavor of yoghurt comes from the other metabolites produced by the symbiotic action of lactobacilli and streptococci. Another important characteristic of yoghurt, for consumer appeal, is the consistency or viscosity of the product, causing a desirable mouth feel. Milk proteins and/or stabilizers contribute to the viscosity of the product, but exopolysaccharides (EPS) produced by starter bacteria gain special importance, especially in countries where the use of stabilizers is not allowed (Chaves *et al*., 2002).

Several investigations have looked into the isolation, identification and application of lactic acid bacteria (LAB) which play significant roles in the manufacture of fermented dairy products. In one study (Dewan and Tamang, 2007), dominant LAB and the technological properties of Himalayan ethnic fermented milk products were identified based on phenotypic characterizations including API sugar test (fermentation ability). The researchers showed antagonistic properties against selected Gram-negative bacteria, and found that none of the strains produced bacteriocin and biogenic amines under the test conditions. In another investigation

(Giraffa *et al*., 2001), genotypic and phenotypic heterogeneity of *Streptococcus thermophilus* strains isolated from dairy products were identified and typed by a polyphasic approach. Phenotypic diversity was evaluated by a chemo metric model taking a number of biochemical characteristics (e.g. acidifying and peptidase activities) of technological interest into account. Genotypic diversity was evidenced by PCR fingerprinting.

Various kinds of yoghurt which could be a source of valuable starter bacteria with different organoleptic properties are traditionally produced in Iran. To our knowledge, works on phenotypic and genotypic diversities of dominant LAB in artisanal yoghurts of Iran have been scarce (Azadnia and Nazer, 2008). Therefore, the identification of naturally occurring LAB from traditional yoghurts from this region would yield valuable sources for yoghurt starter bacteria. The aim of this investigation was to isolate and identify dominant LAB occurring in Iranian artisanal yoghurts in order to set a preliminary description of LAB species responsible for the fermentation of these yoghurts.

Materials and Methods

Sample collection

During the spring and summer of 2010, a total of 60 yoghurt samples were collected from the tribes of four geographical regions of Fars, Khoozestan, Kerman, Yazd, Khorasan, Shahre Kord, Gilan and Mazandaran provinces of Iran. The samples were kept in sterile tubes, immediately transferred to the laboratory (at 4°C) and processed in less than 24 h.

Isolation of LAB

Samples were homogenized, serially diluted in normal saline and sub cultured in duplicate into MRS and M17 agars (Merck, Germany) and supplemented with 50 mg L^{-1} of natamycin (Beukes *et al*., 2001) to isolate lactobacilli and lactic acid cocci, respectively. MRS agar plates were incubated anaerobically using the Gas pack system (Merck Anaerocult type A) at 37°C for 72 h, and M17 agar plates were incubated aerobically at 37°C for 24 h (Dave and Shah, 1995; Shah, 2003). Dominant colonies were selected and transferred into the MRS or M17 broth mediums for enrichment. The enriched bacteria were purified using the streak plate technique. Purification was terminated when the appearance of colonies on the plate was homogeneous. The suspensions of purified isolates were kept at -80°C for further use in a growth medium with 15% glycerol.

Biochemical identification

Biochemical identification of the isolated bacteria was carried out using Bergey's manual of determinative bacteriology (David and John, 1994). All isolates were initially tested for Gram-reaction, catalase production and spore formation (Harrigan and McCance, 1976). Strains with Gram-positive and catalase negative reactions were selected for further identification (Sharpe *et al*., 1979). Primary identification of lactobacilli isolates were performed as shown in Table 1.

CO_2 production from glucose was detected by cultivating isolates in the MRS broth (citrate excluded) containing inverted Durham tubes (Schillinger and Lucke, 1987).

Growth of the lactobacilli at 15°C and 45°C and cocci isolates at 10°C and 45°C were evaluated in the modified MRS and M17 media, respectively (Hammes and Vogel, 1995; Schillinger and Lucke, 1987).

Growth ability of the isolated bacteria in 4% and 6.5% NaCl concentrations was determined in NaCl test medium (Morata *et al*., 1999).

Lactobacilli isolates were screened for their ability to ferment L (+) Arabinose, D (+) Galactose, Lactose, Raffinose, Sucrose, D (-) Salicin, D (+) Trehalose, D (+) Xylose, D (+) Mannose, Glucose, D (-) Ribose, Fructose and Rhamnose (Dykes *et al*., 1994).

Molecular characterization

Genomic DNA isolation

Following PCR, assays were performed with biochemically identified LAB. Genomic DNA was extracted using the phenol extraction method as previously described (Sambrook *et al*., 1989; Araujo *et al*., 2004), and was kept at -20°C for further use.

Primers and specific PCR conditions

DNA amplification conditions were as follows: 5 µl genomic of isolated DNA, 15 µl PCR buffer, 3 µl dNTPs (10 mM) (Fermentas, Lithuania), 9 µl $MgCl_2$ (50 mM), 0.5 µl Taq polymerase 5 IU/ µl, 3 µl of 1200-bp primer 16S-FA 5´-AGAGTTTGATCCTGGCTCAG-3´and 16S-RA5´-AGGAGGTGZTCCAGCCGC-3´ (Rudi *et al*., 1997). DNAase free H_2O (CinnaGen Inc., Iran) was then added to a final volume of 50 µl. DNA amplification was carried out with a thermal cycler programmed as follows: an initial denaturation of 3 min at 94°C, 35 cycles (consisting of denaturation 45 s at 94°C, annealing 30 s at 58°C, 30 s at 52°C, and polymerization 5 s at 72°C), followed by a final extension at 72°C for 5 min. The PCR products were electrophoresed in 1.0% agarose gels, stained with ethidium bromide and photographed.

Table 1: Differential biochemical properties of lactobacilli

Obligate hetrofermentative lactobacilli	Facultative hetrofermentative lactobacilli	Homofermentative lactobacilli	Characteristics
+	+	-	Pentose fermentation
+	-	-	Gas production
Lactobacillus brevis		*Lactobacillus acidophilus*	
Lactobacillus fermentum	*Lactobacillus* plantarum	*Lactobacillus heleviticus* *Lactobacillus delbruckii*	Example

16S rDNA sequencing

The 1200-bp PCR product was purified from agarose gels with a HiYield™ gel extraction kit (Bioneer, South Korea). 500 µl digestion solutions (DF buffer) were added to the PCR product, mixed effectively and incubated at 50°C until the gel was completely digested. The aqueous phase was then transferred into a plasmid purification column and centrifugated at 1500 g for 9 min. The aqueous phase was then transferred into an upper column and centrifugated at 1500 g for 9 min and removed. 400 µl washing buffer was added and columns were centrifugated at 14000 g for 2 min after which the aqueous phase was finally removed. 600 µl plasmid washing solution containing 80% ethanol was added to the upper column and centrifugated at 14000 g for 2 min after which the aqueous phase was removed. The upper column was then transferred into a new tube where 75 µl DNAase free water was added and centrifugated at 14000 g for 2 min. The recovered PCR amplicons were subjected to sequencing analysis (CinnaGen Inc., Iran).

Sequence alignment

The sequences were BLAST in GenBank database for species assignment. The complete 16S rDNA sequence of the selected strains were fitted into alignments of almost complete primary structures available in the NCBI database nucleotide collection (nr/nt) using Megablast optimized for highly similar sequences.

Results

Isolation of LAB

Altogether, 60 traditional yoghurt samples were collected for the isolation of dominant bacteria. Seventy and 330 colonies were chosen from MRS and M17 agar plates, respectively.

Biochemical identification

From 400 Gram-positive isolates, 137 (34.3%) were catalase negative and non-spore forming bacteria, all belonging to the LAB family. Of those, 66 and 71 were identified as lactic acid cocci and bacilli, respectively. 87.4% of the cocci and 85% of the lactobacilli isolates were categorized as homofermentative due to the absence of their ability to produce CO_2 from glucose (Table 1).

Regarding their ability to grow at different salt concentrations, 6% NaCl was used to distinguish between *enterococci*, *lactococci*, and *streptococci*. Only 6.59% of the isolated cocci were found to be resistant to 4 and 6% NaCl concentrations. However, only 5.95% of *Lactobacillus* isolates showed resistance to 6% salt concentration (Table 2).

Homofermentative lactobacilli cannot grow at 15°C, but they can grow at 45°C (Mannu *et al*., 2000). In other words, 95% of lactobacilli isolates grow at 45°C, but not at 15°C. To classify cocci isolates, their growth abilities at 10 and 45°C were tested. *Streptococcus thermophilus* cannot grow at 10°C, but grows properly at 45°C. 40.66% of the cocci isolates showed the same results. The ability of lactobacilli isolates to ferment different carbohydrates is shown in Table 3.

Table 4 Shows identification results of the isolated LAB based on phenotypic characterization.

Genotypic identification

In order to genetically determine isolates, PCR assays with primers targeting the gene for 16S rDNA were used followed by the sequencing of amplicons. The genotypic identification of isolated LAB from traditional yoghurts produced by the Iranian tribes is shown in Table 5. 9% of the lactobacilli isolates showed incompatible results between phenotypic and genotypic characteristics. 38.46% of the cocci showed identical results between

Table 2: Phenotypic characteristics of cocci lactic acid bacteria isolated from traditional yoghurts produced by tribes of Iran

Streptococcus thermophilus	*Streptococcus acidominimus*	*Enterococcus gallinarum*	*Entrococcus durans*	*Entrococcus faecium*	*Entrococcus faecalis*	*Lactococcus lactis* ssp. *lactis*	Characteristics
-	-	+	+	+	+	+	Growth at 10°C
+	-	+	+	+	+	-	Growth at 45°C
-	-	+	+	+	+	+	Growth at 4% salt
-	-	+	+	+	+	-	Growth at 6.5% salt
-	-	-	-	-	-	-	Gas production
-	-	+	+	+	+	-	Growth at pH = 9.6

Table 3: Carbohydrates fermentation pattern among lactic lactobacilli isolated from traditional yoghurts produced by tribes of Iran

Lactobacillus species										
L. diolivorans	*L. jensenii*	*L. plantarum*	*L. fermentum*	*L. brevis*	*L. heleviticus*	*L. acidophilus*	*L. bulgaricus*	*L. lactic*	*L. delbueckii*	Carbohydrate
	-	+	+	+	-	-	-	-	-	Arabinose
	+	+	+	+	+	+	+	+	+	Fructose
	+	+	+	+	+	+	-	-	-	Galactose
	+	+	+	+	+	+	+	+	+	Glucose
	-	+	+	+	+	+	+	+	-	Lactose
	+	+	+	-	+	+	+	+	+	Mannose
	+	+	+	+	-	+	-	-	-	Raffinose
	-	-	-	-	-	-	-	-	-	Rhamnose
	-	+	+	+	-	-	-	-	-	Ribose
	-	+	-	-	-	+	-	+	-	Salicin
	+	+	+		-	+	-	+	+	Sucrose
	+	+	+	-	+	+	-	+	+	Trehalose
	+	+	+	+	-	-	-	-	-	Xylose

Table 4: Phenotypic identification of LAB strains isolated from Iranian traditional yoghurts

Isolated strains according to phenotypic methods	The percentage of lactic acid bacteria isolated from collected samples
Streptococcus thermophilus	26.8
Streptococcus acidominimus	11.0
Enterococcus spp.	47.6
Lactococcus spp.	9.8
Lactobacillus plantarum	26.0
Lactobacillus delbruecki subsp. *bulgaricus*	34.0
Lactobacillus lactis	2.0
Lactobacillus fermentum	12.0
Lactobacillus heleviticus	14.0
Lactobacillus acidophilus	6.0
Lactobacillus brevis	6.0

Table 5: Genotypic identification of lactic acid bacteria strains isolated from Iranian traditional yoghurts, based on 16S rRNA gene sequences

Isolated strains according to genotypic methods	The percentage of lactic acid bacteria isolated from collected samples
Enterococcus faecium	27.3
Enterococcus gallinarum	6.1
Enterococcus faecalis	12.1
Lactococcus lactis subsp. *lactis*	3.0
Enterococcus durans	48.5
Streptococcus thermophilus	3.0
Lactobacillus delbruecki subsp. *bulgaricus*	50.0
Lactobacillus brevis	10.0
Lactobacillus diolivorans	5.0
Lactobacillus helveticus	15.0
Lactobacillus jensenii	5%
Lactobacillus plantarum	5.0

phylogenetic characteristics.

Discussion

Important characteristics used for the identification of LAB genera are the mode of glucose fermentation under standard conditions, non limiting concentrations of glucose and growth factors (amino acids, vitamins and nucleic acid precursors), and limited oxygen availability. Under these conditions LAB can be divided into two groups: the homofermentative that convert glucose almost quantitatively to lactic acid and the hetero-fermentative that ferment glucose to lactic acid, ethanol/acetic acid and CO_2 (Sharp, 1979).

Other criteria for the identification are the ability to grow at different temperatures and solt concentration. For the identification of lactobacilli isolates, the criterion of growth at 15 and 45°C was used. *Lactobacillus delbrueckii* ssp. *bulgaricus* cannot grow at 15°C; however, it can grow at 45°C. In the same manner, for the classification of lactic acid cocci isolates, growth abilities at 10 and 45°C were investigated; *Streptococcus thermophilus* cannot grow at 10°C, but grows well at 45°C.

The ability to grow at different salt concentrations, particularly 6%, may be used to distinguish between *enterococci*, *lactococci*, and *Streptococci.*

The most useful test for the determination of strain differences is carbohydrate fermentation. Thirteen different carbohydrates were used for this identification purpose. According to biochemical tests, the isolates were finally characterized as: mesophilic homo-fermentative cocci (*Lactococcus lactis*) 9.76% of isolates, mesophilic hetrofermentative cocci (*Leuconostoc mesenteroides*) 10.98% of isolates, thermophilic homofermentative cocci (*Streptococcus thermophilus*) 26.83% of isolates, mesophilic homo-fermentative cocci (47.56%), mesophilic facultative hetrofermentative lactobacilli (*Lactobacillus plantarum*) 26% of isolates, thermophilic obligate homofermentative lactobacilli (*Lactobacillus helveticus*) 14% of isolates, *Lactobacillus delbrueckii* subsp. *bulgaricus* (34% of isolates), *Lactobacillus delbrueckii* subsp. *lactis* (2% of isolates) and *Lactobacillus acidophilus* (6% of isolates), which have a narrow fermentation profile. Strains belonging to this category were able to ferment lactose and fructose and are thus close to *Lactobacillus delbrueckii* subsp. *bulgaricus* and mesophilic obligate hetrofermentative lactobacilli. This group contained *Lactobacillus brevis* (6% of isolates) and *Lactobacillus fermentum* (12% of isolates).

The phenotypic properties of the strains conformed to former species reported in the literature, with some exceptions mainly related to salt, temperature tolerance and carbohydrate fermentation. This is not surprising given the variability of the properties of “wild” strains reported in the literature (Cogan *et al*., 1997; Parente *et al*., 1997; Corroler *et al*., 1998; Ayad *et al*., 1999). The cocci isolate of the LAB family with the extended temperature range may have been selected by the ecological conditions prevailing during the production of traditional yoghurt. The salt tolerance of some cocci

isolates from yoghurt may also reflect an adaptation to the environment (2-10% salt-in-moisture Gobbetti *et al*., 2002; Piraino *et al*., 2005).

The representative LAB isolated from Algerian raw goat's milk were *Lactococcus* spp., *Streptococcus thermophilus*, *Leuconostoc* ssp., *Lactobacillus curvatus*, *Lactobacillus helveticus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus delbruecki* subsp. *bulgaricus* and *Lactobacillus acidophilus* (Guessas and Kihal, 2004). To investigate different species isolated from Iran, Azadnia and Khan Nazer (2009) conducted a phenotypical study and determined the following species as (44.44%) *Lactococcus lactis* subsp. *cremoris* and (55.56%) *Leuconostoc mesenteroides* subsp. *cremoris* among lactic acid cocci. In the case of lactobacillus, *Lactobacillus helveticus* (15.3%); *Lactobacillus plantarum* (22.3%); *Lactobacillus brevis* (21%); *Lactobacillus casei* subsp. *casei* (15.5%) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (25.9%) were found to be phenotypically close to our study.

Danilo *et al*. (2001) and Holzapfel *et al*. (2001) showed that to identify and characterize strains at the species and subspecies level, the conventional method is limited, un reliable, complicated, and time-consuming. On the other hand, 16S rRNA sequence analysis is regarded as the most accurate and reliable method for species level identification.

Olarte *et al*. (2000) noted that the presence of *Lb. plantarum* in goat milk cheese (Cameros) decreased the number of the enterobactriacea and fecal coliforms in the final product. Lactobacilli isolated from household bushera was found to belong to *Lb. plantarum*, *Lb. brevis* and *Lb. delbrueckii* subsp. *bulgaricus* (Muyanja *et al*., 2003). In the characterization of microbiota of homemade semi hard white zlatar cheese, *Lactobacillus brevis* was found to be one of the main groups (Terzic-Vidojevic *et al*., 2007).

As starter cultures, LAB are ubiquitous in dairy manufacturing. Specific fermentation processes have been developed to encourage the growth of the desired species, some of which are fastidious organisms such as *Lb. delbrueckii* subspecies *bulgaricus* and *Lb. helveticus* (Bottazzi, 1988). Isolates belonging to the *Lb. plantarum* group were shown to be predominant members of the LAB flora of acid-fermented condiments (Tempoyak). Nevertheless, isolates belonging to the *Lb. brevis* group and *Leuconostoc mesenteroides* were also observed (Leisner *et al*., 2001). Beukes *et al*. (2001) found *Lb. plantarum*, *Lb. delbrueckii*, *Leuconostoc mesenteroides* and *Lactococcus lactis* to be dominant microorganisms of South African traditional fermented milks. The most abundant isolated species from raw goat's milk of four Algerian races were *Lb. helveticus*, *Lb. plantarum*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. brevis* and *L. lactis* subsp. *lactis* (Badis *et al*., 2004b). Leisner *et al*. (1999) identified the LAB of Chili Bo and found *Lb. plantarum* to be the most important predominant organism.

The results of this study demonstrate the usefulness of different phenotypic and genotypic taxonomic criteria for the identification of natural isolates of dairy lactobacilli and cocci lactic acid bacteria isolates, which often show wide microbial heterogeneity (Dykes and yon Holy, 1994). The ability to discriminate between closely related strains and the capacity to identify unusual or atypical isolates can be improved by combining different molecular techniques such as DNA-DNA hybridization, ARDRA and PCR-specific primers which do not necessarily agree with phenotypic classifications.

Although the present study is not the first to isolate and identify Iranian traditional yoghurts, the literature has been scarce so far. The subject gains particular importance due to the considerable annual economic loss resulting from the import of yoghurt starters. Given the increased demand for traditional fermented products, the results of the present study can potentially launch considerable native achievement pertaining to the production of fermented dairy products. The identified isolates can be used to establish the production of volatile compounds and to assess their potential as starter cultures for commercial uses.

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