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ORIGINAL RESEARCH

Biodiversity and origin of the microbial populations isolated from Masske, a traditional Iranian dairy product made from fermented Ewe's milk

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Masske is a traditional Iranian dairy product containing 50% butterfat made from fermented ewe's milk. Overall, 672 bacterial isolates were collected from ewe's milk, fermented milk (FM) and Masske samples that were produced in households located in southern regions of the Khorasan Province in Iran. To identify lactic acid bacteria in these samples, a total of 79 Gram-positive and catalase-negative isolates were analysed. The identification of isolates was achieved by phenotypic and sequential analysis of the 16S rRNA gene. Enterococcus faecium and Aerococcus viridans were the most frequently isolated species in the samples, but the most commonly present bacteria in Masske were Streptococcus thermophilus.

Keywords biodiversity, Masske, Phenotypic characterisation, 27FYM–PCR, Phylogenetic tree.

INTRODUCTION

Traditional dairy products have a wide range of phenotypic and genetic microbial diversity, so they may have many potential biotechnological applications (Palys *et al.* 1997; Wouters *et al.* 2002; van Hylckama *et al.* 2006; Topisirovic *et al.* 2006). Among all dairy products, various types of fermented milk products have played an important role throughout the world. Their nature depends on the type of milk used, and the conditions of fermentation and later processes. Historically, fermented dairy products have increased the shelf life of milk. These traditional foods have been consumed over centuries (Cogan and Accolas 1996; Tamime and Marshall 1997; Oberman and Libudzisz 1998; Stanley 1998; Zamfir *et al.* 2006). The use of starters improved the technological quality of dairy products, but limited their biodiversity and the organoleptic variation of their end products (Wouters *et al.* 2002; Leroy and De Vuyst 2004). Therefore, an increasing demand exists for new strains that will have desirable effects on the product characteristics (Fortina *et al.* 2003). Fermentation of milk by specific microorganisms changes the taste, texture, colour, flavour and the nutritional properties of milk and provides a wide range of end products (Oberman and Libudzisz 1998; Duboc and Mollet 2001). In addition, fermented milks contribute to human health (Oberman and Libudzisz 1998; Von Wright and Salminen 1998). They have been recommended for some gastrointestinal disorders. The use of these fermented dairy products is increasing in developing countries (Rastall *et al.* 2005).

Masske is a dairy product that is light yellow in colour with an attractive appearance, an integrated texture, a pleasant sour nutty aroma and is a semi-solid at room temperature (Sserunjogi *et al.* 1998; Gonfa *et al.* 2001; Ongol and Asano 2009). Its characteristic flavour serves as a major criterion for its acceptance (Sserunjogi *et al.* 1998). Masske is made by the fermentation of milk. It is produced in the northern and eastern regions of Iran, Mazandaran and Khorasan provinces, respectively, as well as in Turkey, Afghanistan and Balkans Peninsula. In Khorasan, Masske is manufactured in small rural households, and its sale is mainly limited to local markets. It is always produced in a

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traditional way handed down from past generations. It can be made from nonpasteurised ewe or goat milk or a mixture of the two (Jokovic *et al.* 2008).

During the production of dairy products, the growth of microbial profile, microorganisms, especially lactic acid bacteria (LAB), leads to the organoleptic characteristics of the final product (Caplice and Fitzgerald 1999). The microbial biodiversity of traditional dairy products mainly depends on the production technology used as well as the ecological localities where they have been made (Beukes *et al.* 2001; Mathara *et al.* 2004; Chammas *et al.* 2006; Zamfir *et al.* 2006; Dewan and Tamang 2007). The isolation and characterisation of indigenous lactic acid bacteria (LAB) from dairy products is very important for understanding the biochemical and microbiological properties that lead to the development of typical textures and flavours. (Wouters *et al.* 2002; Leroy and De Vuyst 2004). Therefore, this information can contribute to improving the process of Masske production, as well as understanding the conditions needed for obtaining a better quality product. The isolation of LAB having useful technological properties from traditionally fermented products is one of the ways in which the strains for industrial starters can be provided, which can help preserve the typical nature of the product.

The purpose of this research was to determine and analyse the microbial groups in the home-made Masske, the traditional Iranian butterfat product from the Southern Khorasan, Iran. The main aim of this study was to identify predominant species of LAB in analysed samples by classical culturing and molecular techniques and to provide a phylogenetic tree to evaluate the genetic relationships between those species.

MATERIALS AND METHODS

Masske preparation

The first step in the traditional process of Masske production is the gradual heating of the raw ewe's milk up to 55 °C, with occasional stirring. The milk is held at this temperature for another 3–5 min, and then, the warm milk is put through a process wherein a portion of traditionally fermented milk from a previous batch is added as an inoculum to initiate the fermentation process (Jokovic *et al.* 2008). The inoculated milk is poured into shallow pans where it stays for several hours or days. During that time, the pH decreases and the milk is transformed into a viscous yoghurt-like fermented milk (FM). The FM is placed in wooden tubs, with a goat skin or sheep skin bag called a tulum, with a little tap on the bottom of them. When the container is half filled with sour FM, tuluming is started with sharp convulsive movements and at the same time, some cold water is added into the tulum before the end of churning to improve the separation process and the firmness of the butterfat. Fermentation is completed by both natural microflora and the inoculum during the 30 to 60 minute tuluming operation. Towards the end of the tuluming step, the bottom tap is opened and a watery very sour liquid called Shallaghi, FM and a viscous elastic liquid called Shiraz are, respectively, removed from the tulum leaving the behind the Masske. The main steps involved in Masske production are presented in Fig. 1.

Sampling of Masske

Three samples of milk, two samples of FM and three samples of Masske, designated as MR1, MR2, MB, FB, FR, BBO, BB and BR, repectively, were collected from local producers in different regions of Gonabad (a southern city of Khorasan Province). The samples were intentionally collected in regions where it has been traditionally produced in households for centuries. All samples were made from ewe milk. The samples MR1 and MR2 (Milk of Riab), MB (milk of Beymorgh), FB (Fermented Milk of Beymorgh), FR (Fermented Milk of Riab), BBO (butterfat of Band Ozbak), BB (butterfat of Borjuk) and BR (butterfat of Riab) were collected from the farms situated in the south-western, south-eastern and eastern regions of Gonabad. Four samples MR1, MR2, FR and BR were taken from two farms in the south-western region of Gonabad. Three samples MB, FB and BBO were taken from two other farms in the eastern region of Gonabad, and one sample BB was taken from south-eastern region of Gonabad. Samples were taken from batches of Masske that had been manufactured independently in different periods of the year. The collected samples were kept in sterile bags at 4°C, and the analyses were performed within the following 24 h.

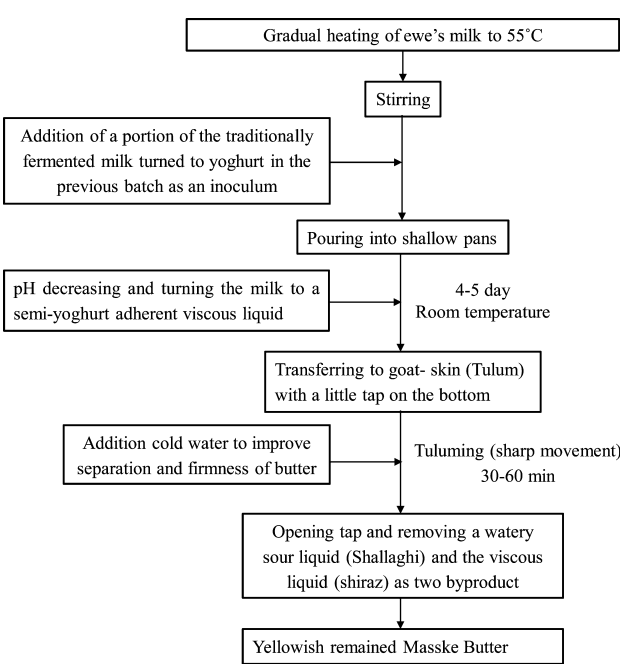

Figure 1 Flow scheme for the manufacturing process of Masske.

Chemical analyses

All the samples of milk, FM and Masske were analysed for pH, titratable acidity, total fat and NaCl content. The pH was measured by pH meter (Metrohm AG 774, Switzerland). Titratable acidity (TA) and pH were determined after the homogenisation of a 10 g sample in 90 mL of hot distilled water. For TA determination, the suspension was titrated with 0.1 M NaOH to a final pH 8.5 and was expressed as % acidity. The total fat content was determined by acido-butyrometric method of Van Gulik (ISO-3433, 1975). All analyses were performed in duplicate (Alegría *et al.* 2009).

Enumeration and isolation of microbial population

The milk and FM samples (10 mL) were homogenised in 90 mL of sterile 0.85% (w/v) NaCl (Merk, Germany) solution, after which 10-fold serial dilutions were prepared in the same solution. Masske samples (25 g) were homogenised in 225 mL of sterile 2% (w/v) tri-sodium citrate dihydrate solution (pH = 7.5) and preheated to 40 °C, and then, 10-fold serial dilution was made in peptone water (0.1%) (Merk, Germany). A volume of 0.1 mL of appropriate dilutions was spread plated in duplicate on the following media for isolation and enumeration of microbial population (Schleifer *et al.* 1985; Schleifer 1986 Schleifer and Kilpper-Briltz 1987; Van Hoorde and Verstraete 2008).

Lactic acid bacteria

Presumptive lactobacilli and lactococci were grown and enumerated on MRS agar (Liofilchem, Italy) and M17 agar (Fluka, USA) respectively after the incubation at 30 °C and 37 °C for 3 days. Anaerobic plate counts were also carried out at both temperatures (Anaerocult A, GasPak Merck, Germany) for 72 h on respective media (MRS for lactobacilli and M17 for lactococci) (Alegría *et al.* 2009).

For enrichment of the bacteria that were detected in a small number of the samples, 0.1 mL of the first dilution was transferred into 10 mL of MRS or M17 broth. Tubes were incubated at 30 °C and 37 °C for 48 h. After the incubation, a loopful of grown culture was streaked on MRS and M17 agar plates and incubated for additional 48 h at appropriate temperatures and under anaerobic conditions (Anaerocult A, GasPak Merck, Germany) (Alegría *et al.* 2009).

The pure Gram-positive and catalase-negative isolates were stored as frozen stocks at −80 °C in M17 or MRS broth supplemented with 15% glycerol. Working cultures were revitalised by two consecutive transfers in M17 or MRS broth at 30 °C for mesophilic LAB and at 37 °C for thermophilic LAB, respectively.

Total aerobic mesophilic

Aerobic mesophilic bacteria were grown on plate count milk agar (PCMA; Merck, Darmstadt, Germany) and enumerated after 48 h of incubation at 30 °C (Alegría *et al.* 2009).

Coliforms

Coliforms were grown on violet red bile lactose agar (VRBLA) (Merck, Germany), using the pour plate technique. Bacteria were enumerated after 24–48 h of incubation at 30 °C (Alegría *et al.* 2009).

In addition, for the detection and isolation of *E. coli* specifically, we inoculated 1 mL aliquots from three first dilutions into 3 mL lauryl sulphate (LS) broth (Merck, Darmstadt, Germany) containing inverted Durham tubes and incubated them at 35 °C. The subsequent tests were performed in EC broth and Tryptone water medium (Merck, Darmstadt, Germany). They were then streaked for isolation on Eosin Methylene Blue (EMB) agar (Merck, Darmstadt, Germany) plates and incubated for 24 h at 35 °C (Schleifer and Bell 2005).

Staphylococci

Dilutions were grown on Baird–Parker agar (B–PA; Merck) supplemented with egg yolk tellurite solution (Merck), and black colonies with or without, egg yolk clearing were recorded after 24 h of incubation at 37 °C (Schleifer and Bell 2005; Alegría *et al.* 2009).

Yeasts and moulds

Dilutions of milk, FM and Masske samples were plated on yeast extract glucose chloramphenicol agar (YGCA; Merck), and yeasts and moulds were enumerated after 3–5 days of incubation at 25 °C (Jokovic *et al.* 2008 & Alegría *et al.* 2009).

Phenotypic characterisation of isolates

Gram-positive and catalase-negative isolates were examined for cellular morphology by phase contrast microscopy on overnight cultures in M17 or MRS broth at 30 °C and 37 °C, respectively. In addition, all isolates were tested for growth at 10 °C for 10 days, 45 °C for 48 h and CO_2 production from glucose, growth in the presence of 6.5% NaCl and growth in two pH of 4.4 and 9.6. The cultures were classified as stated below into 50 different groups: Gram-positive and catalase-negative rods, which grew at 45 °C but not at 10 °C, were considered as thermophilic lactobacilli, while those that did not grow at 45 °C and those that grew at 10 °C were considered as mesophilic lactobacilli. Homofermentative, Gram-positive and catalase-negative cocci, which grew at 10 °C but not at 45 °C and 6.5% (w/v) NaCl, were considered as mesophilic lactococci. Homofermentative, Gram-positive and catalase-negative cocci, which grew at 45 °C but not at 10 °C and 6.5% NaCl, were considered as thermophilic cocci, while those that grew at 45 °C, 10 °C and 6.5% NaCl were considered as enterococci. Also, Gram-positive, catalase-positive and black colony groups on BPA and Gram-negative rods which are susceptible to be Staphylococci and *E. coli* are categorised, respectively. About 2–5 isolates were selected from

each group, and finally, 79 LAB indicator isolates were chosen for the molecular identification (Jokovic *et al.* 2008 & Alegría *et al.* 2009).

Molecular identification of microbial isolated

DNA extraction

Seventy-nine colonies from the MRS, M17, PCMA, BP and EMB agar plates were purified by subculturing on the same media, and the pure cultures were stored frozen at −80 °C until the analyses were performed. Cultures were recovered in the corresponding media, and the isolated colonies were suspended in 100 μL MilliQ water. About 5–6 glass beads were added to microtubes and left in the refrigerator at −20 for 5 min. Then, the tubes were mixed vigorously by vortex, and the microbial suspensions were transferred to the new tubes and heated for 20 min at 70 °C. After centrifugation for 10 min at 13 000 × g, 50 μL of supernatants (cell free extracts) were transferred into new tubes for the next steps, and 50 μL of new milliQ water was added to the rest (remaining pellet). The mentioned steps were repeated for the pellets, and 50 μL of supernatants was added to the previous supernatants and again centrifuged for 10 min at 13 000 × g. Then, 50 μL of upper liquid parts of supernatants was used as a source of DNA template to amplify a segment of the 16S rRNA gene by the polymerase chain reaction (PCR) technique (1 with some modification).

For those isolates that did not respond to the above protocol, the DNA extraction kits were used (Roche applied Science, USA). In this method, isolates were grown in liquid media, centrifuged, resuspended in PBS and mixed with an enzymatic solution (10 mg/mL lysozyme pH = 8, 10 mM Tris-HCl). Subsequent steps were performed according to the manufacturer's protocol (Palys *et al.* 1997; Wouters *et al.* 2002; van Hylckama *et al.* 2006; Topisirovic *et al.* 2006; Alegría *et al.* 2009).

Amplification of 16S rRNA genes

PCR amplifications were carried out in a 25 μL volume using a Thermal Lab cycler (Sensequest, Germany). The reaction mixture contained 2.5 μL 10 × PCR buffer, 2 μL dNTPs, 0.2 μL Taq DNA polymerase (1U) (Takara, Japan), 1.2 μL MgCl2 (50 mM), 1.25 μL of mixture of primers (5 pM), 18 μL ddH2O and 1.5 μL of DNA template (Lane 1991; Walter *et al.* 2000; Meroth *et al.* 2003; Gala *et al.* 2008).

The PCR primers used, 27FYM (5′-AGAGTTTGATY MTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACG ACTT-3′), were based on conserved regions of the 16S rRNA gene using the following cycling programme: initial denaturation at 95 °C for 5 min, 33 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, elongation at 72 °C for 2 min and final elongation at 72 °C for 10 min. PCR products were quantified by electrophoresis on 1.5% (w/v) agarose gel in 1 × TBE buffer and visualised by Green DNA viewer staining and visualised by BDR2/5/6 (Sony, Japan) (Lane 1991; Palys *et al.* 1997; Walter *et al.* 2000; Wouters *et al.* 2002; Meroth *et al.* 2003; van Hylckama *et al.* 2006; Topisirovic *et al.* 2006; Gala *et al.* 2008; Alegría *et al.* 2009). Amplicons were sequenced by Macrogan Advancing Thought Genomic Co., Korea.

Homological comparisons were performed using the Basic Local Alignment Search Tool (BLAST), and comparisons were performed online at the National Centre for Biotechnology Information (NCBI) homepage (http://www.ncbi.nlm.nih.gov) (Gala *et al.* 2008).

Bioinformatic analysis

To identify the bacteria under study at genus and species level, based on the gene sequence of 16S rRNA, an attempt was made to compare the sequences obtained with the ones already recorded in the gene bank. To this effect, the sequences were first prepared and edited and then compared in two stages.

Preparation and editing of the sequences

Because of the use of a common primer in the amplification of the intended gene region for various bacteria, the efficiency of the PCR for all the bacteria will not be the same, and under certain circumstances, they will not be amplified by the primer. Where they are amplified, the quality will not be suitable for sequencing, especially since the degenerate primers' amplification is itself low. On the other hand, the length of the amplified fragment of the universal primer utilised is about 1500 times, which is out of the operation range of the sequencing machines working on the basis of the Sanger sequencing method, the most commonly used method. As a result, there is always a possibility of failure in sequencing some specimens or the existence of parts of the sequences with a low quality. To increase the accuracy, avoiding the usage of such parts seems necessary.

To this effect, the sequences with < 200 adjacent bases of suitable quality will not be included in the analyses. About 9 of the 85 sequenced specimens were omitted due to their low quality. Moreover, all the nucleotides of each sequence were assessed and given points based on the Phred index of quality, and all the nucleotides with a margin of error higher than 1% were omitted from the end of the sequences. As a result of this procedure, most of the remaining sequences had a length of about 400–600 bases.

Comparison of the sequences with the help of blast

There are more than 300 000 sequence for each 16S rRNA gene recorded in the gene database. Therefore, this set is the most comprehensive reference for the comparison of the obtained sequences. To this end, each of the edited sequences in the gene bank was blast against the total bacterial genome and judged based on the similarity of the

obtained sequence with the ones already present in the gene bank in terms of the two characteristics of the degree of identity and coverage of the genes. At this stage, it was assumed that in the case of 100% identity and coverage between the intended sequences and the ones obtained, the two will probably belong to the same species.

Clearly, such a result is not without errors because there is a possibility that the gene region under investigation is shared between several species. In this case, there will be a need for further comparisons; therefore, in the next stage in this study, for a more accurate study of the sequences, the phylogeny study was used.

The phylogeny study of the 16S rRNA gene

The study of 300 000 bacterial sequences recorded in the gene bank for the 16S rRNA gene shows that these sequences generally belong to more than 1200 bacterial species (and subspecies). Therefore, for each bacterium, a sequence of optimal quality for the 16S rRNA gene was designated in the gene bank (a total of 1250 sequence). Then, the obtained sequences from this study were each aligned separately with this set and the best sequences identified in the bank in terms of their absolute identity as well as their partial identity were identified. In the next step, this set of identified sequences and the set of the sequences obtained from this study were aligned with each other and then, their lengths were adjusted. Alignment of the genes was carried out through the Clustal algorithm with its default parameters. Subsequently, with the help of the MEGA software (Tamura 2011), version 5, the phylogenetic tree was calculated based on the neighbour-joining (Saitou and Nei 1987) procedure with the maximum composite likelihood model (Tamura *et al.* 2004). The validity of the tree was also verified based on the random sampling (Bootstrap) method with a thousand repetitions (Felsenstein 1985).

RESULTS

Chemical characteristics

The chemical analyses of milk, FM and Masske samples showed that the pH value was higher in milk samples, and it decreased rapidly in FM and then increased a little in Masske. Mean pH values in Masske samples were similar to those reported in previous studies on butter (Sagdic and Arici 2002; Jokovic *et al.* 2008). The results of acidity detected in the Masske samples were the opposite. The lowest pH and the highest acidity values were determined in the FB. The values of dry matter ranged from 17.2% to 66.0% with a higher value in samples of Masske such as BR and BB. Total milk fat and fats in the dry matter varied among samples from 6% to 55% and from 33% to 83%, respectively. The highest content of fat in dry matter (83.3%) was found in BR Masske (Table 1).

Microbial diversity and dynamics of samples

Enumeration of microbial population

The viable counts of the main microbial groups present in the milk, FM and Masske samples are shown in Table 2. Total counts of aerobic mesophilic bacteria in the three samples of Masske on plate count agar (PCA) plates were higher than those in others and varied in the range of 7.0–7.6 log cfu per g.The sample of BBO Masske had the highest total bacterial count, while the lowest number was found in the sample of FR Fermented Milk (3.7 log cfu per g).

The highest counts of bacteria were found in the MRS media (30 °C & 37 °C), and M17 had the lowest bacterial count. The samples of milk (MB) and the three samples of Masske (BBO, BB and BR) had significantly higher bacterial counts than the other samples of milk and FM (6.2–7.8, cfu per g). As a whole, samples of FM had the lowest viable microbial count because of their low pH. (Table 2).

Numbers of hygienic-indicator populations were high throughout the whole process, especially in Masske samples, reaching their highest levels (7.6 log cfu per g) at sample BBO in its total count, sample BB for coliforms (5.6 log cfu per g) and *E. coli* (3.3 log cfu per g) and sample BR for staphylococci (6.5 log cfu per g) and yeast and moulds (7 log cfu per g). Although numbers of staphylococci were also high, strains of *Staphylococcus aureus* were never detected in the BPA plates., except in one case. The population of yeasts reached a maximum level in Masske samples, so it is necessary to make an appropriate decision to eliminate the existing pathogens without harming the nutritional value of the product.

Identification of isolates

A total of 672 bacterial isolates were collected from three samples of milk, two samples of Fermented Milk and three samples of Masske. They were grouped and preliminarily identified to the genus level based on physiological tests (data not shown). These isolates from different phenotypic groups were classified into 50 groups, and some representatives of each group were subjected to 16S rRNA sequencing to test the validity of their previous identification. The results showed that among the coccal LAB isolates, the majority belonged to the genera *Enterococcus* and *Aerococcus* (Table 3).

Coccal isolates were divided into three different phenotypic groups. The isolates of coccal group I grew very well at 10 °C and in the presence of 6.5% NaCl. In the 16S rRNA sequencing, they belonged to the genera *Enterococcus* and *Aerococcus*. Coccal group II was composed of the isolates that could not grow in the broth with a pH of 4.4 and that were not able to grow at 10 °C. The sequencing of 16S rRNA of one representative isolate from this group showed that it belonged to the species *Streptococcus thermophilus*.

Table 1 Chemical characteristics of the milk, fermented milk (FM) and Masske samples

	Samples							
Chemical Characteristic	*MR1*	*MR2*	*MB*	*FB*	*FR*	*BBO*	*BB*	*BR*
pH	6.7 ± 0.01	6.6 ± 0.04	6.6 ± 0.02	3.5 ± 0.08	4.0 ± 0.03	4.6 ± 0.31	4.2 ± 0.33	4.4 ± 0.23
Dry matter (%)	17.9 ± 0.21	18.1 ± 0.90	19.1 ± 0.18	16.7 ± 0.07	19.2 ± 0.05	62.0 ± 1.1	63.2 ± 1.6	66.0 ± 1.4
Fat (%)	6.49 ± 0.23	7.52 ± 0.38	8.06 ± 0.20	5.65 ± 0.03	7.80 ± 0.05	50 ± 2.1	52 ± 1.5	55 ± 1.7
Fat in dry matter (%)	36.2	41.4	42.2	33.9	40.6	80.6	82.5	83.3

±, standard deviation of two independent measurements; %, 100 cc milk or 100 gr Butterfat and FM.

All measurements are reported based on (g/100 g) Masske or FM and (g/100 mL) for milk.

MR, Milk of Riab; MB, Milk of Beymorgh; FB, FM of Beymorgh; FR, FM of Riab; BBO, Butterfat of Band Ozbak B; BB, Butterfat of Borjuk and BR, Butterfat of Riab.

Table 2 Average microbial counts (in Log_{10} cfu per g or mL) and standard deviation of diverse microbial groups of milk, fermented milk and Masske

	Samples							
Microbial group (media)	*MR1*	*MR2*	*MB*	*FB*	*FR*	*BBO*	*BB*	*BR*
MRS (30 °C)	5.1 ± 0.31	5.3 ± 0.23	7.2 ± 0.31	5.1 ± 0.33	3.5 ± 0.24	7.8 ± 0.23	7.1 ± 0.51	6.2 ± 0.11
MRS- anerobic (30 °C)	4.3 ± 0.2	4.4 ± 0.7	5.6 ± 0.3	3.2 ± 0.19	4.0 ± 0.1	5.9 ± 0.28	6.2 ± 0.24	4.4 ± 0.16
M17 (30 °C)	3.5 ± 0.24	3.3 ± 0.26	5.8 ± 0.99	3.5 ± 0.2	4.1 ± 0.39	5.1 ± 0.56	4.3 ± 0.32	5.5 ± 0.14
MRS (37 °C)	6.2 ± 0.88	6.1 ± 0.2	8.11 ± 0.9	6.0 ± 0.15	4.2 ± 0.12	8.0 ± 0.8	8.1 ± 0.97	6.7 ± 0.23
MRS- anerobic (37 °C)	4.2 ± 0.24	6.1 ± 0.16	7.3 ± 0.12	2.8 ± 0.3	4.4 ± 0.1	6.0 ± 0.71	7.6 ± 0.88	5.0 ± 0.15
M17 (37 °C)	5.5 ± 0.3	4.2 ± 0.2	6.0 ± 0.23	4.5 ± 0.14	4.2 ± 0.15	4.3 ± 0.39	5.3 ± 0.11	5.3 ± 0.11
Total aerobic counts (PCA)	5.4 ± 0.42	5.9 ± 0.57	5.5 ± 0.11	4.0 ± 0.38	3.7 ± 0.2	7.6 ± 0.2	7.4 ± 0.32	7.0 ± 0.46
Coliforms (VRBL)	3.7 ± 0.47	3.3 ± 0.23	2.1 ± 0.35	3.4 ± 0.12	2.0 ± 0.1	5.5 ± 0.18	5.6 ± 0.11	4.9 ± 0.87
E. Coli (EMBA)	4.5 ± 0.12	4.4 ± 0.36	3.2 ± 0.10	2.8 ± 0.23	2.3 ± 0.22	3.0 ± 0.24	3.3 ± 0.29	2.5 ± 0.10
Staphylocci(BPA)	3.2 ± 0.48	3.9 ± 0.56	3.0 ± 0.43	nd	nd	6.0 ± 0.76	5.7 ± 0.19	6.5 ± 0.47
Yeast and moulds (YGCA)	1.2 ± 0.10	1.0 ± 0.12	nd	2.8 ± 0.26	2.3 ± 0.33	6.7 ± 0.17	6.3 ± 0.47	7.0 ± 0.45

nd, not detected.

±, standard deviation of two independent measurements.

MR, Milk of Riab; MB, Milk of Beymorgh; FB, FM of Beymorgh; FR, FM of Riab; BBO, Butterfat of Band Ozbak; BB, Butterfat of Borjuk and BR, Butterfat of Riab.

Coccal group III was composed of heterofermentative coccoids with mucosal colonies that could not grow in the broth with a pH of 9.6 and at 45 °C. The sequencing of 16S rRNA of one representative isolate from this group showed that it belonged to *Leoconostoc mesenteroides*.

Rod-shaped isolates were classified into three groups. Two groups were homofermentative *Lactobacillus* that did not have the ability to grow under pH of 9.6 and in the presence of 6.5% NaCl. All of them could grow at 45 °C. The third group of rod-shaped isolates was heterofermentative lactobacilli that could grow at pH of 4.4. The 16S rRNA sequencing of the two isolates from this group revealed that they belonged to the species *Lactobacillus fermentum*.

To maximise the recovery of LAB bacteria from samples, two different culture media and two different culture conditions (aerobiosis and anaerobisis) were assayed. Although statistically not significant, MRS showed higher recovery numbers than M17 specifically. *Aerococcus viridans*, *Aerococcus urinaeequi* and *S. thermophilus* were isolated from all the media used. *Leuconostoc* spp. were mostly isolated from MRS agar, together with all the enterococci and lactobacilli (Table 3). Although MRS is described as a medium for the cultivation of lactobacilli, it showed a low degree of selectivity for this genus (Tamime and Marshall 1997; Stanley 1998; Zamfir *et al.* 2006).

BPA was used to isolate Staphyloccoci, which comprised four species, three of which were harmless to humans (*S. epidermis*, *S. hominis* and *S. haemolyticus*).

Main LAB population in samples

The identification of the isolates enabled us to characterise the main species of LAB present in different samples in this study. The isolation frequency of the main LAB species isolated is shown in Fig. 2.

Table 3 Major micro-organisms identified from milk, fermented milk and Masske samples isolated in two different culture media

Species	*Stage of production*			*Medium of isolation (number of isolated)*	*Total*
	Masske	*FM*	*Milk*		
Lactobacillus delbrueckii subsp. *delbrueckii*	4	–	–	MRS(4)	4
Lactobacillus fermentum	2	–	–	MRS(2)	2
Lactobacillus plantarum	1	–	–	M17(1)	1
Enterococcus faecium	5	2	8	MRS(15)	15
Enterococcus durans	–	1	3	MRS(4)	4
Enterococcus lactis	–	–	1	MRS(3)	3
Aerococcus viridans	1	–	8	MRS(5),M17(4)	9
Aerococcus urinaeequi	–	–	4	MRS(3),M17(1)	4
Streptococcus thermophilus	6	5	–	MRS(3),M17(8)	11
Lactococcus lactis	–	–	1	MRS(1)	1
Leuconostoc mesenteroides subsp. *mesenteroides*	–	–	3	MRS(3)	3
Staphylococcus epidermis	–	–	1	MRS(1)	1
Staphylococcus hominis	16	–	–	MRS(10),M17(6)	16
Staphylococcus haemolyticus	1	–	–	M17 (1)	1
Staphylococcus aureus	1	–	–	MRS(1)	1
Escherichia coli	–	–	2	MRS(2)	2
Acinetobacter junii	–	–	1	MRS(1)	1
Total	37	7	34	MRS(58) - M17(21)	79

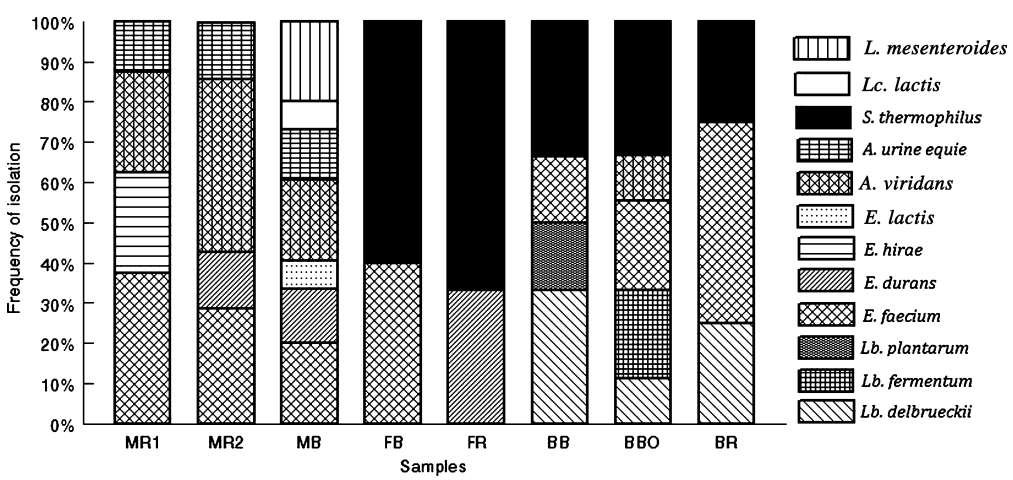

Figure 2 Distribution of LAB species in samples.

S. thermophilus was a predominant species in samples of FB, FR, BBO and BB. This species was also found in the BR sample, but in a considerably lower percentage (25%). *E. faecium* was isolated from all samples except from FR. Another enterococcal species, *E. durans*, was found in most of the samples, but with a lower isolation frequency (Fig. 1). Lactococci were present in only one milk sample (MB), at 6%. All lactobacilli (*Lb. delbrueckii*, *Lb. fermentum* and *Lb. plantarum*) were isolated from Masske samples, respectively. All milk samples had the different species of *A. viridans* and *A. urinaeequi* with a percentage of 53% and 26.6% of the whole micro-organisms in these two samples, respectively.

DISCUSSION

As observed in Fig. 3, the phylogram has 6 clades and each represents the group of organisms that are classified together as descendants of a common ancestor. These six clades comprise Enterococci, Aerococcci, Lactobacilli, *Leuconostoc*, Lactococci and Streptococci.

The first clade discussed is Enterococci. We put three reference species in this clade to compare. They are completely

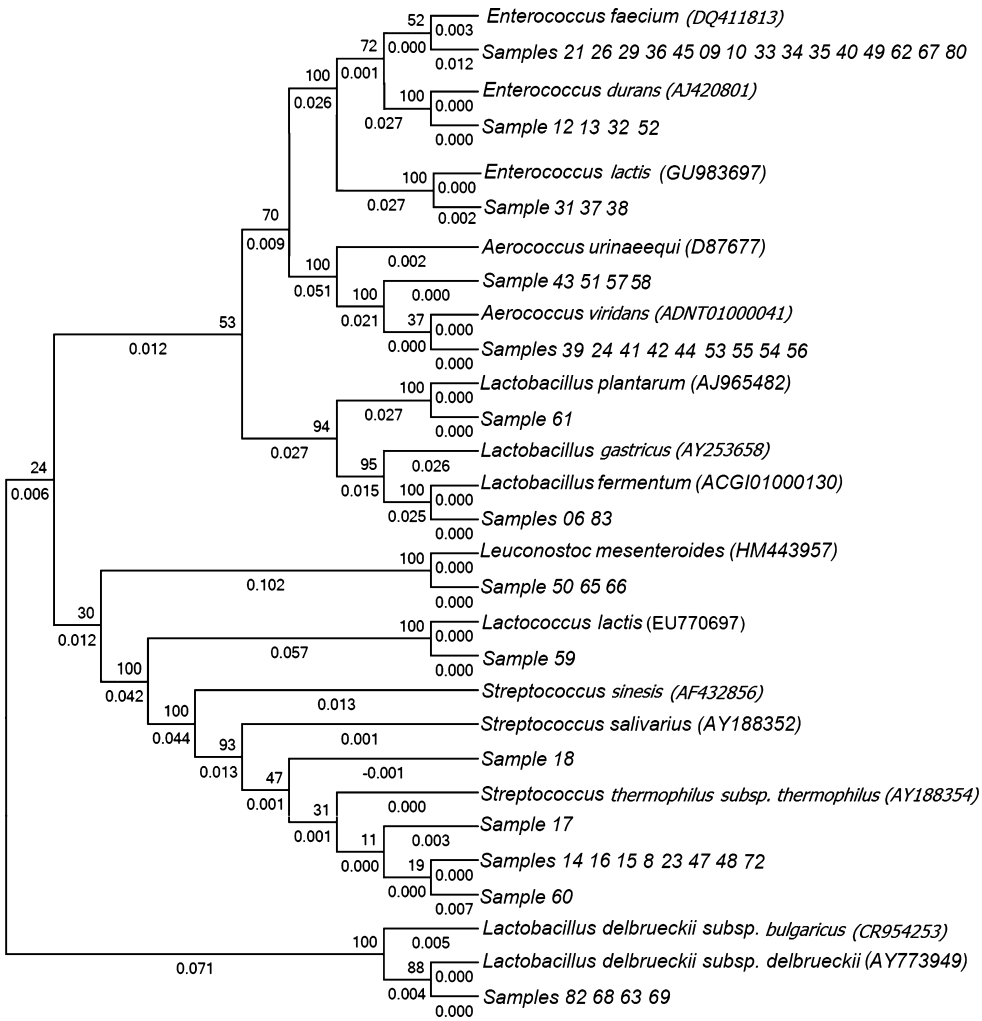

Figure 3 Genetic phylogram based on bioinformatical analysis of the 16s rRNA region of aligned sequences of lactic acid bacteria with some indicator micro-organisms.

the same as isolates for *Enterococcus durans* and *Enterococcus lactis* and the evolutionary distances (ED) of 0.003 for *Enterococcus faecium.*

The second clade represents the genus *Aerococcus* which is divided into two groups. The first comprises samples 43, 51, 57 and 58 that are located at a distance of about 0.002 from *A. urinaeequi*, and the second subclade comprises samples 24, 39, 41, 42, 44, 53, 54, 55 and 56 that are at null distances from *A. viridans.*

The third clade is divided into two completely separate subclades. One of these subclades includes only *Lactobacillus delbruekii* and some related sub species. Four samples of 63, 68, 69 and 82 have been put in this subclade with no ED (ED = 0). So in terms of taxonomical classification, they certainly belong to the aforementioned place. The second sub clade includes some species that do not have any genetical differences in the studied gene region. Sample 61 and *Lactobacillus plantarum*, *Lactobacillus paraplantarum* (not shown in this figure) and *Lactobacillus pentosus* (not shown in this figure) are taxonomic sisters. In other words, we cannot say precisely to which species these three reference species belong. This was also the same for *Latobacillus fermentum* and *Lactobacillus gastricus* (as reference species) and samples 83 and 6.

And finally, the last clade belongs to Streptococci. As shown in Fig. 3, although we have used *S. thermophilus* and *Streptococcus sinesis* as reference species in this figure, there is no evolutionary distance between the samples (isolates) and *S. thermophilus* either. So we reported *S. thermophilus* for these isolates.

In this research, the diversity of predominant species of LAB in analysed samples was identified through classical culturing and molecular techniques, and this study provides a phylogenetic tree for the evaluation of the genetic relationships between those species. To evaluate dramatic changes in the microbial community and the microbial genetic biodi-

versity during the process of Masske production, we should take into consideration two main factors which influence the microbial community during this process.

Firstly, the microbial growth conditions in terms of chemical parameters along with the main compounds present in every sample during the Masske production caused some of the microbial species to be predominant.

Secondly, the microbial exit in every stage of the process of Masske production can influence its microbial community.

All the milk samples under study contained the range of bacterial species. But, *A. viridans, A. urinaeequi* and *E. faecium* were predominant in these milk samples, respectively. Natural chemical parameters along with environmental micro-organisms which can be transferred from the dairy farms and by the machinery to the raw milk cause these micro-organisms to be predominant; in fresh raw milk, *micrococci* and *streptococci* are dominant among the microbial population. This microbial population is a reflection of the normal udder and teat skin flora (Chambers 2002). This can justify the abundant presence of *E. faecium* as faecal streptococci in the analysed raw milk samples in the present study. On the other hand, an increase in the Gram-negative rods in raw milk samples is also observable, in which *A. viridans and A. urinaeequi* are dominant. In most cases, an increase in Gram-negative rods can be an indicator of unsanitary conditions within the milk collection and handling system (Thomas and Thomas 1975).

S. thermophilus was a predominant species in all the FM samples. It is also revealed that *Lactobacillus delbrueckii* subsp. *delbrueckii* and *L. fermentum* are the predominant Lactobacilli in FM production. Yoghurt is a product with thermophilic fermentation in which starter cultures are responsible for the fermentation of the milk under optimum growth temperatures of 37 °C to 45 °C. Among these genera, *Streptococcus, Lactococcus, Bifidobacterium and Enterococcus* are often used for the production of yoghurt or fermented milk. On the other hand, an increase in the lactic acid concentration (around pH 4.3–4.5) causes the growth and metabolism of *S. thermophilus* to be inhibited (Robinson *et al.* 2002). All FM samples in this study have a final pH of around 3.5–4. This phenomenon along with the presence of *Enterococcus* as an initial indigenous microflora in the raw milk can be the main reason for the dominance of *S. thermophilus* together with *E. faecium and E. durans* in the analysed FM samples.

Ripened butterfat such as Masske is subjected to a relatively severe heat treatment. This treatment can remove thermoduric bacteria such as enterococci, lactobacilli and micrococci. Also protein denaturation causes sulfhydryl compound to be released; thus, the E_h in the cream decreases. This situation provides a better condition for suitable microbial growth (Wilbey 2002) but, temperate heat treatment of about 50 °C during Masske production cannot remove these microflora specifically. This can be the main cause of the presence of *Streptococcus* enterococci and lactobacilli in Masske. On the other hand, contamination of wash water and air in the processing of Masske may be considered to be the most important reasons for the presence of *Aerococcus* species (Jay 1996).

To improve the separation process and the firmness of the butterfat during the Masske process, cold water is added to the goat skin. Also, in the last few minutes of the shaking, a watery very sour liquid called Shallaghi drink, fermented milk and a viscous elastic liquid called Shiraz are removed from the skin and only the Masske is left there. These processes cause the MSNF to be removed and thus produce a less attractive medium for LAB proliferation. Also, LAB growth would be inhibited by the relative lack of nutrients and space because of particle formation in the Masske. Furthermore, it is demonstrated that $< 0.1\%$ of the droplets can contain any organisms (Richards 1982; Wilbey 2002). These can be the main strategies used to reduce micro-organisms in the final product (Masske), although based on the results, microbial load has been increased in the Masske due to lack of sanitary conditions.

In order to determine the phenotypic and genetic data of micro-organisms occurring during the production of Masske, we adopted a polyphasic study (Corsetti and Settanni 2007). Although there are biases due to differences in growth rates between the different species on the culture medium, this culture-dependent method can generate an image of the diversity of all the colonies growing on the plate (Randazzo *et al.* 2009). Also, it seems that it will be necessary to use a polyphasic approach for a more accurate specification of the LAB in Masske because of the dilution of the samples and pour plating or spreading the highly diluted specimen on the selective media. This dilution method makes the isolation of the species with small numbers extremely difficult (Ercolini *et al.* 2003).

The molecular technique used in this study showed the great diversity of species found in Masske (*S. thermophilus, E. faecium*, *A. viridans*, *E. durance*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. plantarum*). Also, the genetic phylogram based on bioinformatical analysis of the 16s rRNA region (Fig. 3) revealed the origin of *E. faecium* and *A. viridans* in Masske as the raw milk used, whereas *S. thermophilus* and *E. durans* originated from FM during the processing of Masske.

In the majority of Masske samples, a large number of enterococci were found. *E. faecium* was isolated in a higher percentage than other enterococcal strains. *E. faecium* is commonly isolated from traditional dairy products (Giraffa 2003). Although the presence of enterococci in dairy products is controversial due to the presence of virulence determinants and antibiotic resistance, many studies have shown that enterococci play an important role in the ripening of dairy products due to their proteolytic and lipolytic activities and the production of aromatic compounds without any compromise on safety (Moreno *et al.* 2006).

CONCLUSION

The survey of the microbial populations in the analysed collected samples of Masske showed that it consisted of four main genera: *Streptococcus*, *Enterococcus*, *Lactobacillus* and *Aerococcus*. The results from this study demonstrated a high distribution of different microbial genera among the Masske samples and the effect of the locality where Masske was manufactured (the abundant presence of Enterococci). The results obtained from this study also suggest the need to study different households in the same location and in various geographical regions in order to get more representative samples of Masske and to carry out further experiments to determine the presence of virulence determinants and antibiotic resistance among enterococci strains.

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