Identification of the bacterial biodiversity in koumiss by denaturing gradient gel electrophoresis and species-specific polymerase chain reaction

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ABSTRACT

Bacterial biodiversity in traditional koumiss fermented milk was studied by denaturing gradient gel electrophoresis (DGGE). Target DNA bands were identified according to the reference species ladder, constructed in this study. Comigrating bands present in the DGGE profiles were resolved by species-specific PCR. The results revealed a novel bacterial profile and extensive bacterial biodiversity in koumiss. The dominant lactic acid bacteria included *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, and *Lactobacillus kefiranofaciens*. Frequently encountered bacterial species were *Enterococcus faecalis*, *Lactococcus lactis*, *Lactobacillus paracasei*, *Lactobacillus kitasatonis*, and *Lactobacillus kefir*. *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *Lactobacillus buchneri*, and *Lactobacillus jensenii* were occasionally found in this product. In addition, *L. buchneri*, *L. jensenii*, and *L. kitasatonis*, which were never previously isolated by culture-dependent methods, were identified for the first time in the Xinjiang koumiss. Furthermore, conventional cultivation was performed by plating samples on M17, de Man-Rogosa-Sharpe, Halligan-Pearce, and Kenner fecal media. The results revealed that lactobacilli were the dominant species in the koumiss ecosystem, which was consistent with the results obtained by the DGGE analysis. This is the first systematic study of the microbial composition in koumiss, and our findings will be helpful in selecting appropriate strains for the manufacture of this product at the industrial level.

Key words: bacterial biodiversity, koumiss, denaturing gradient gel electrophoresis, species-specific polymerase chain reaction

INTRODUCTION

Koumiss, which is made from unpasteurized fresh mare milk by nomadic families, is the most popular fermented dairy beverage in Xinjiang, Qinghai, and Inner Mongolia of China. This traditional fermented product is widely consumed in Mongolia, Kazakhstan, Kirgizstan, and some regions of Russia. For centuries, koumiss has been considered to be not only an essential food but also a nutriment and a medicinal remedy (Ishii and Konagaya, 2002). As has been previously reported, koumiss is beneficial for enhancing innate immunity and treating tuberculosis and cardiovascular disease (Montanar et al., 1996; Wang et al., 2008). Lactic acid bacteria (LAB) and yeasts were proven to be the main components in koumiss starter, similar to the microflora of traditional beverage kefir (Pintado et al., 1996). Moreover, the benefits of koumiss are mainly attributable to the indigenous LAB, which also were reported to play a major fermentative role on the aroma, texture, and acidity of this product (Wu et al., 2009). Hence, in recent years there has been much interest in identifying bacterial communities in koumiss samples.

To study the microbial populations in koumiss, culture-dependent methods have been used to isolate bacteria and identify them based on their morphology, physiology, and biochemical profile (An et al., 2004; Shuangquan et al., 2006; Uchida et al., 2007; Wu et al., 2009). These conventional methods are not only laborious and time-consuming, but are restricted to culturable bacteria, which may not accurately represent the microbial profile of koumiss samples (Akkermans et al., 1994; Hugenholtz et al., 1998). In recent years, rapid culture-independent methods have been developed to study microbial biodiversity, such as single-strand conformation polymorphism, temporal temperature gradient gel electrophoresis (TTGE), and denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Lee et al., 1996; Ogier et al., 2004). These methods are based on direct analysis of extracted DNA from the microbial environment and do not rely on cell culture methods. Both TTGE and DGGE are able to separate identically sized DNA fragments based on differing GC

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Figure 1. Map of Xinjiang Uighur Autonomous Region, China, showing the locations of Narat grassland and Kapu River pasture.

content. Separation is performed with polyacrylamide gels containing a linear gradient of chemical denaturant solution that consists of urea and formamide (DGGE) or a linear temperature gradient (TTGE). Recently, these approaches have been successfully applied to investigate the microflora of fermented milk products (Flórez and Mayo, 2006; El-Baradei et al., 2007; Kafili et al., 2009).

In this study, the DGGE fingerprinting technique and species-specific PCR were used to identify the bacterial microflora of several homemade koumiss varieties. At present, traditional koumiss is prepared by nomadic families at the small-scale level. In most cases, fermentation conditions are relatively uncontrolled because the manufacturing facilities are quite primitive. In particular, differences in bacterial composition of conventional starters originating from the respective family environment will result in koumiss quality instability (Shuangquan et al., 2006). The aim of this study was to identify the flora in several traditional homemade koumiss preparations. Our results will be helpful in selecting appropriate strains as commercial starters for the manufacture of this product at the industrial level using other types of more readily available milk.

MATERIALS AND METHODS

Koumiss Samples

Ten samples of koumiss were aseptically collected from nomadic families of Yili, located in the Xinjiang Uighur Autonomous Region of China (Figure 1). Based on region of origin, samples were grouped as follows: group A (samples 1–5), collected from the Narat grassland, and group B (samples 6–10), obtained from the Kapu River pasture. The pH values of the samples ranged between 3.6 and 4.3. All collected samples were transported to the laboratory in an icebox at 4°C and then stored at –80°C before use.

Bacterial Strains and Growth Conditions

For rapid determination of the DGGE bands, the identification ladder was constructed using known reference strains. These strains belonged to 13 LAB species that had previously been found in koumiss by culture-dependent methods (Uchida et al., 2007; Wang et al., 2008; Wu et al., 2009). The reference strains used in this study included *Lactobacillus plantarum* 05–19, *Lactobacillus pentosus* ATCC8041, *Lactobacillus fermentum* ATCC14931, *Leuconostoc mesenteroides* AS1.2141^T, *Lactobacillus acidophilus* 05–171, *Lactobacillus helveticus* AS1.1877^T, *Lactobacillus curvatus* CCM7271, *Enterococcus faecalis* AS1.2984, *Lactococcus lactis* MG1614, *Streptococcus thermophilus* 05–33, *Lactobacillus casei* ATCC393, *Lactobacillus paracasei* E4213, and *Lactobacillus rhamnosus* AS1.2466^T. These LAB were cultured in de Man-Rogosa-Sharpe medium at 30 or 37°C under anaerobic conditions. The genome DNA from reference strains was isolated using the TIANamp bacteria DNA kit (Tiangen Inc., Beijing, China) according to the manufacturer's instructions.

Genomic DNA Extraction in Koumiss

Total DNA extraction from the koumiss samples was performed as described previously with some modification (El-Baradei et al., 2008). Each koumiss sample (5 mL) was emulsified in 5 mL of 2% (wt/vol) sterile trisodium citrate. Pronase (10 mg; Roche, Shanghai, China) and 20 µL of β-mercaptoethanol were added to each sample, followed by 3 h incubation at 50°C. Bacteria were washed twice by centrifugation at 10,000 × *g* for 15 min at 4°C. First, pellets were washed with 5 mL of sterile water and then resuspended with 5 mL of TES buffer [25 mM Tris HCl, 0.1 M EDTA, 25% (wt/vol) sucrose; pH 8.0]. After centrifugation for 10 min, the cell pellets were resuspended in 500 µL of TES buffer and transferred into sterile Eppendorf tubes. For the lysis of bacteria, lysozyme (Amersco Inc., Solon, OH) was then added at a final concentration of 30 mg/mL and the suspension was incubated at 37°C for 1 h. Total DNA was extracted by the phenol chloroform method (de Los Reyes-Gavilán et al., 1992). Finally, the DNA pellet was dissolved in 20 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) with 0.1 mg/mL RNase (Sigma-Aldrich Co., St. Louis, MO) and examined by 1% (wt/vol) agarose electrophoresis. Extracted DNA was quantified by a Qubit fluorometer and a Quant-iT double-stranded DNA broad-range assay kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Fifty nanograms of DNA was used as the template for subsequent PCR reactions.

Table 1. Species-specific PCR primers used in this study

No.	Target	Primer	Sequence (5'-3')	Annealing temp. (°C)	Reference
1	<i>Lactobacillus plantarum</i>	planF	CCGTTTATGCGGAACACCTA	58	Torriani et al., 2001
		pREV	TCGGGATTACCAAACATCAC		
2	<i>Lactobacillus pentosus</i>	pentF	CAGTGGCGCGTTGATATC	62	Torriani et al., 2001
		pREV	TCGGGATTACCAAACATCAC		
3	<i>Lactobacillus helveticus</i>	PecF	CTGTTTTCAATGTTGCAAGTC	64	Fortina et al., 2001
		PecR	TTTGCCAGCATTAACAAGTCT		
4	<i>Lactobacillus acidophilus</i>	Aci16SI	AGCTGAACCAACAGATTCAC	62	Fortina et al., 2001
		16SII	ACTACCAGGGTATCTAATCC		
5	<i>Lactobacillus casei</i>	Casei	TGCACTGAGATTCGACTTAA	55	Ward and Timmins, 1999
		Y2	CCCCTGCTGCCTCCCGTAGGAGT		
6	<i>Lactobacillus paracasei</i>	Para	CACCGAGATTCAACATGG	62	Ward and Timmins, 1999
		Y2	CCCCTGCTGCCTCCCGTAGGAGT		
7	<i>Lactobacillus rhamnosus</i>	Rham	TGCATCTTGATTTAATTTTG	50	Ward and Timmins, 1999
		Y2	CCCCTGCTGCCTCCCGTAGGAGT		

Nested PCR Amplification

The DGGE samples were prepared by performing 2 successive PCR using a DNA Engine thermal cycler PTC-200 (Bio-Rad, Hercules, CA). First, a 700-bp fragment of the 16S rDNA including the V3 region (in *Escherichia coli*, which corresponds to position 339–539) was amplified with primers W01 (5'-AGA GTT TGA TC [AC] TGG CTC-3') and W012 (5'-TAC GCA TTT CAC C [GT] C TAC A-3'). Second, the V3 region of 200 bp was amplified with the primers GC-HDA1 (5'-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC** TCC TAC GGG AGG CAG CAGT-3'; the GC clamp sequence is in bold) and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA-3'). The PCR reaction mixtures and the 2 above amplification programs were the same as described previously (Ogier et al., 2002). The sizes and quantities of PCR products were determined by 2% agarose gel electrophoresis in comparison with a standard containing DNA fragments of defined length.

DGGE Analysis

Denaturing gradient gel electrophoresis analysis was performed with the D-code universal mutation detection system (Bio-Rad) using 16 cm × 16 cm × 1 mm gels. The denaturing gradient gel contained 6 to 12% (wt/vol) gradient of polyacrylamide and 40 to 60% denaturant gradient solution increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7 M urea. Twenty-microliter samples (approximately 500 ng) of 200-bp PCR products were deposited in wells; electrophoresis was conducted with 0.5 × Tris acetate EDTA buffer (TAE; 20 mM Tris base, 10 mM glacial acetic acid, and 0.5 mM EDTA) electrophoresis buffer (85 V at 60°C for 13 h). After electrophoresis, the gels were stained for

15 min with SYBR Green I (Amersco Inc.; diluted 1:10,000 in 0.5 × TAE), rinsed for 20 min in 1 × TAE buffer, and visualized by UV transillumination. Three replications were performed for each koumiss sample by DGGE analysis.

Species-Specific PCR

To differentiate comigrating bands present in the DGGE profiles, species-specific PCR was carried out on DNA directly extracted from the koumiss samples using species-specific primers (Table 1). A total of 7 pairs of species-specific primers were prepared at a final concentration of 60 μM in deionized autoclaved water. All PCR reactions were performed in DNA Engine thermal cyclers PTC-200 (Bio-Rad) following the conditions previously published (Table 1). Finally, the sizes of PCR products were resolved by 2% agarose gel electrophoresis.

Sequencing of DGGE Bands

Some DGGE bands that could not be identified by the identification ladder were excised from the denaturing gels, purified, and sequenced as described previously (Hu et al., 2009). Sequence annotation and database searches for similar sequences were performed using BLAST at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) to determine the closest known relative species (Altschul et al., 1990).

Enumeration of the Bacteria Groups

Koumiss samples (1 mL) were mixed in 9 mL of sterile 0.85% (wt/vol) NaCl and agitated for 2 min. Serial dilutions were prepared in sterile 1% (wt/vol) peptone (Merk, Leicestershire, UK), plated on selective media

using a spiral plater, and incubated at the appropriate temperatures. Lactococci and streptococci, lactobacilli, *Leuconostoc*, and enterococci were enumerated on M17, de Man-Rogosa-Sharpe (pH adjusted to 5.4), Halligan-Pearce (Pearce and Halligan, 1978), and Kenner fecal (Devriese et al., 1990) agars, respectively.

RESULTS

Construction of the DGGE Identification Ladder

To best interpret the DGGE bands, the identification ladder was prepared by mixing the PCR products obtained from the 13 reference strains. Comigration in *L. plantarum* group (*L. plantarum* 05–19, *L. pentosus* ATCC8041), *L. acidophilus* group (*L. acidophilus* 05–171, *L. helveticus* AS1.1877^T), and *L. casei* group (*L. casei* ATCC393, *L. paracasei* E4213, and *L. rhamnosus* AS1.2466^T) was observed as described previously (Walter et al., 2000). The DNA fragments from the remaining 6 reference strains that we tested could be individually distinguished by DGGE (Figure 2).

Identification of Bacterial Biodiversity by DGGE

The DGGE pattern of 10 koumiss samples included 20 bands, 9 of which could be assigned by the identification ladder (Figure 3). The assigned bacterial species included the *L. plantarum* group (band a), *L. fermentum* (band b), *Ln. mesenteroides* (band d), *L. acidophilus* group (band f), *L. curvatus* (band g), *E. faecalis* (band k), *Lc. lactis* (band m), *S. thermophilus* (band o), and *L. casei* group (band r). The other bands (c, d', e, h, i, j, l, n, p, q, q') were excised and sequenced. These sequence alignments were carried out using the BLAST database (Table 2). It was observed that the bands f and h were found in all koumiss samples, which were respectively assigned to *L. acidophilus* group and *Lactobacillus kefiranofaciens* (Figure 3). It is noteworthy that the bands' intensity reflects the relative proportion of each species in the total bacterial population (Muyzer et al., 1993). Thus, the strong intensities and high frequencies of bands f and h indicated that *L. acidophilus* group and *L. kefiranofaciens* were the dominant bacterial species of the koumiss ecosystem. However, some species present in only 1 or 2 samples, such as *L. curvatus* (band g) and *Lactobacillus buchneri* (band q), indicated that these species were poorly represented in koumiss. These results demonstrated that the koumiss products of each family contained their special microflora that resulted from their unique environment.

Species-Specific PCR Assays of Koumiss

Species-specific PCR was carried out to further determine the comigrating bands on the DGGE profiles

Figure 2. Denaturing gradient gel electrophoresis profile of the reference strains used in the identification ladder. M: the identification ladder; lane 1: *Lactobacillus plantarum* 05–19; lane 2: *Lactobacillus pentosus* ATCC8041; lane 3: *Lactobacillus fermentum* ATCC14931; lane 4: *Leuconostoc mesenteroides* AS1.2141^T; lane 5: *Lactobacillus acidophilus* 05–171; lane 6: *Lactobacillus helveticus* AS1.1877^T; lane 7: *Lactobacillus curvatus* CCM7271; lane 8: *Enterococcus faecalis* AS1.2984; lane 9: *Lactococcus lactis* MG1614; lane 10: *Streptococcus thermophilus* 05–33; lane 11: *Lactobacillus casei* ATCC393; lane 12: *Lactobacillus paracasei* E4213; lane 13: *Lactobacillus rhamnosus* AS1.2466^T.

in all such samples. Seven pairs of species-specific primers were used to identify the comigrating bands, which were preliminarily assigned as *L. plantarum* group (band a), *L. acidophilus* group (band f), and *L. casei* group (band r). According to the results summarized in Table 3, band f was found to contain both *L. helveticus* and *L. acidophilus* except for 3 samples (1, 5, and 6), band r was identified as *L. paracasei*, and band a was found to be present only in sample 1. This fragment was confirmed to be *L. plantarum* and *L. pentosus* by the PCR. However, as shown in Table 3, these 2 species were present in some samples that did not have the corresponding band on the DGGE profiles. The reason for this discrepancy was that minority bacterial species can not be detected by DGGE alone if they account for less than 1% of the most dominant species (Ogier et al., 2002). Thus, the combination of DGGE and species-specific PCR is the best approach to identify bacterial species precisely and rapidly. To make it easier to compare the bacterial biodiversity between the different samples, the known species identified by the identification ladder and sequencing is summarized in Table 3.

Enumeration of Bacterial Groups

The viable bacterial counts of koumiss samples enumerated on different selective media are shown in Table

4. The population of lactobacilli ranged from 6.92 to 8.40 log₁₀ cfu/mL. The bacterial counts of *Streptococcus* and *Enterococcus* were either relatively low or absent in many samples (Table 4). Only 4 koumiss samples (2, 4, 6, and 7) out of 10 showed viable *Enterococcus*, with counts ranging from 3.30 to 4.69 log₁₀ cfu/mL. The counts of *Streptococcus* were obvious in only 2 koumiss samples (3 and 10) and ranged from 6.55 and 6.90 log₁₀ cfu/mL. On the other hand, no viable bacterial counts were detected on M17 (30°C, aerobic) and Halligan-Pearce media, which suggested that *Lactococcus* and *Leuconostoc* were under the detection levels in koumiss samples. Conventional culture-dependent methods indicated that lactobacilli were the dominant species in the koumiss ecosystem, which was consistent with the results obtained by the DGGE analysis.

DISCUSSION

Although traditional koumiss made by fresh mare milk has been widely consumed in China for centuries, little is known about the bacterial communities responsible for the texture and flavor of this product. This is partially because of the use of culture-dependent methods for bacteria identification in previous studies, which provides relatively poor representation of species present in this product. In particular, media used for isolation might fail to recover bacteria unable to grow outside the koumiss environment. In recent years, many molecular tools have been rapidly introduced to identify the bacterial biodiversity of the fermented foods. In DGGE method, double-stranded DNA fragments encounter increasing concentrations of the denaturing agents and partially melt in discrete regions called melting domains. The melting temperature of these domains is sequence-specific. Therefore, DNA fragments of the same size but different sequences will have melting domains with unique melting temperatures and will migrate accordingly in the DGGE gel (Ercolini, 2004). Occasionally, the sequences of the DNA fragments are

Figure 3. Denaturing gradient gel electrophoresis profiles of the bacterial community from koumiss samples (lanes 1–10). M: the identification ladder; A: *Lactobacillus plantarum* group; B: *Lactobacillus fermentum*; C: *Leuconostoc mesenteroides*; D: *Lactobacillus acidophilus* group; E: *Lactobacillus curvatus*; F: *Enterococcus faecalis*; G: *Lactococcus lactis*; H: *Streptococcus thermophilus*; I: *Lactobacillus casei* group.

different, but the melting temperature may be similar (Ogier et al., 2002). Thus, these fragments comigrate at the same position in denaturing gradient gels (Murray et al., 1996). To distinguish comigrating species on the DGGE, species-specific PCR was performed. Identical results in 3 replicates confirmed the reproducibility of amplifications and subsequent DGGE separations. The combination of DGGE and PCR allowed us to simultaneously identify 20 unique bacterial species in the koumiss samples.

According to the frequencies and intensities of the bands on the DGGE profiles, we divided the bacterial population of koumiss samples into 3 groups. The first comprised dominant bacterial species that were identified in each sample (e.g., *L. acidophilus*, *L. helveticus*,

Table 2. Identification by sequencing of V3 fragments excised from denaturing gradient gel electrophoresis bands of koumiss samples

Band no.	Closest relative	Identity (%)	Accession no.
c	Uncultured bacterium clone nbw741c09c1 16S ribosomal RNA gene	100	GQ011256.1
d'	Uncultured bacterium partial 16S rRNA gene, clone FC04D11	100	FM873249.1
e	<i>Lactobacillus jensenii</i>	95	DQ317562.1
h	<i>Lactobacillus kefiranofaciens</i>	100	AB372208.1
i	<i>Lactobacillus kitasatonis</i>	99	NR_024813.1
j	<i>Lactobacillus</i> sp. GTP5 16S ribosomal RNA gene	98	AF157035.1
l	Uncultured bacterium clone sic_6 16S ribosomal RNA gene	94	GQ175531.1
n	<i>Lactobacillus kefir</i>	92	AB42937.1
p	Uncultured bacterium clone 14LED04 16S ribosomal RNA gene	98	FJ163966.1
q	<i>Lactobacillus buchmeri</i>	92	FJ867641.1
q'	Uncultured bacterium clone 14LEJ05 16S ribosomal RNA gene	94	FJ163981.1

Table 3. Presence of bacterial species in the koumiss samples using denaturing gradient gel electrophoresis analysis and specific primer tests

Target species	Presence of bacteria in koumiss samples ¹										Frequency ²
	1	2	3	4	5	6	7	8	9	10	
<i>Lactobacillus plantatum</i> ³	+	+	+	–	+	+	–	+	+	–	7/10
<i>Lactobacillus pentosus</i> ³	+	–	–	–	–	+	–	+	–	–	3/10
<i>Lactobacillus helveticus</i> ³	+	+	+	+	+	+	+	+	+	+	10/10
<i>Lactobacillus acidophilus</i> ³	–	+	+	+	–	–	+	+	+	+	7/10
<i>Lactobacillus casei</i> ³	–	–	–	–	–	–	–	–	–	–	0/10
<i>Lactobacillus paracasei</i> ³	–	–	+	+	+	+	+	–	+	–	6/10
<i>Lactobacillus rhamnosus</i> ³	–	–	–	–	–	–	–	–	–	–	0/10
<i>Lactobacillus fermentum</i>	+	+	+	–	+	+	+	+	+	+	9/10
<i>Leuconostoc mesenteroides</i>	–	+	–	+	–	–	+	+	–	–	4/10
<i>Lactobacillus curvatus</i>	–	–	–	+	–	–	–	–	–	–	1/10
<i>Enterococcus faecalis</i>	–	+	–	+	+	+	+	–	–	–	5/10
<i>Lactococcus lactis</i>	–	+	+	–	–	+	+	+	–	–	5/10
<i>Streptococcus thermophilus</i>	–	–	+	–	–	–	–	+	+	+	4/10
<i>Lactobacillus jensenii</i>	+	+	+	+	–	–	–	+	–	–	5/10
<i>Lactobacillus kefiranofaciens</i>	+	–	+	+	+	+	+	+	+	+	9/10
<i>Lactobacillus kitasatonis</i>	+	+	–	+	+	+	–	+	–	–	6/10
<i>Lactobacillus kefir</i>	+	+	–	–	–	–	–	+	+	+	5/10
<i>Lactobacillus buchneri</i>	+	–	–	–	–	–	–	–	+	–	2/10

¹+: positive; –: negative.²Frequency: ratio of number of positive samples to total number of tested koumiss samples.³Species identified by specific PCR assays.

L. fermentum, and *L. kefiranofaciens*). The bacteria of this group may play a significant role in the fermentation and organoleptic properties of koumiss because of their commonality in the tested samples. The second group comprised frequently encountered bacteria species (e.g., *E. faecalis*, *Lc. lactis*, *L. paracasei*, *L. kitasatonis*, and *Lactobacillus kefir*). The third group comprised occasionally encountered bacterial species (e.g., *Ln. mesenteroides*, *L. curvatus*, *S. thermophilus*, *L. buchneri*, and *Lactobacillus jensenii*). The species belonging to the second and third groups may have a less crucial role in the fermentation process of koumiss. Although the bacterial microflora profiles were different in 10 samples, the dominant LAB species were very similar. The minor variations in the structure of the bacterial community in koumiss were likely related to the difference of manufacturing procedure and nomadic lifestyle in each family.

In the present work, several bacterial species were detected for the first time in the Xinjiang koumiss using DGGE analyses. These included *L. buchneri*, *L. jensenii*, and *L. kitasatonis*, which were never previously isolated from koumiss by culture-dependent methods. This might be attributed to choice of media, inability to culture injured or viable but nonculturable bacteria, or other factors. Furthermore, it is worth noting that some bacterial species were detected using DGGE method, but no viable cells were found on selective media (e.g., *Lc. Lactis*, *S. thermophilus*, and *Ln. mesenteroides*). For *Lc. lactis* or *S. thermophilus*, these results were mainly

attributable to DNA originating from dead bacterial cells that may have previously existed in some koumiss samples and were destroyed by low pH value. However, some *Leuconostoc* strains are particularly slow-growing bacteria and need at least 6 d to form visible colonies (Lonvaud-Funel, 2000). These results further confirmed that the DGGE method was more efficient than the culture-dependent methods for identifying the bacterial biodiversity of the food ecosystems. In addition, *Lactobacillus delbrueckii* ssp. *bulgaricus* was not found in any of the samples analyzed by DGGE. This result was further confirmed by species-specific PCR (data not shown). This was unexpected because this species is considered a common species of fermented milk. The absence of *L. delbrueckii* ssp. *bulgaricus* in koumiss samples may be attributable to its low cell concentration in the starter (El-Baradei et al., 2008) or the low temperature during the fermentation of koumiss (Uchida et al., 2007). These results confirmed the ability of molecular methods to precisely characterize the complex microbial biodiversity in traditional fermented koumiss.

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Table 4. Viable counts of different lactic acid bacteria groups in koumiss fermented milk samples

Medium ¹	Group targeted	Incubation (°C), conditions ²	Bacterial count (log cfu/mL ± SD)									
			1	2	3	4	5	6	7	8	9	10
M17	<i>Lactococcus</i> spp.	30, A	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
M17	<i>Streptococcus</i> spp.	42, A	<2.0	<2.0	6.55 ± 0.08	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	6.90 ± 0.21
MRS	<i>Lactobacillus</i> spp.	30, AN	8.19 ± 0.06	8.21 ± 0.06	7.40 ± 0.16	7.60 ± 0.37	6.92 ± 0.21	7.34 ± 0.61	7.61 ± 0.15	8.40 ± 0.06	7.98 ± 0.12	8.09 ± 0.02
HP	<i>Leuconostoc</i> spp.	30, A	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
KF	<i>Enterococcus</i> spp.	37, AN	<2.0	3.30 ± 0.16	<2.0	4.69 ± 0.05	<2.0	3.57 ± 0.02	3.50 ± 0.02	<2.0	<2.0	<2.0

¹MRS: de Man-Rogosa-Sharpe; HP: Halligan-Pearce; KF: Kenner fecal.

²A: under aerobic conditions; AN: under anaerobic conditions.

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