Diversity of lactic acid bacteria associated with traditional fermented dairy products in Mongolia

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ABSTRACT

Spontaneous milk fermentation has a long history in Mongolia, and beneficial microorganisms have been handed down from one generation to the next for use in fermented dairy products. The objective of this study was to investigate the diversity of lactic acid bacteria (LAB) communities in fermented yak, mare, goat, and cow milk products by analyzing 189 samples collected from 13 different regions in Mongolia. The LAB counts in these samples varied from 3.41 to 9.03 log cfu/mL. Fermented yak and mare milks had almost identical mean numbers of LAB, which were significantly higher than those in fermented goat milk but slightly lower than those in fermented cow milk. In total, 668 isolates were obtained from these samples using de Man, Rogosa, and Sharpe agar and M17 agar. Each isolate was considered to be presumptive LAB based on gram-positive and catalase-negative properties, and was identified at the species level by 16S rRNA gene sequencing, multiplex PCR assay, and restriction fragment length polymorphism analysis. All isolates from Mongolian dairy products were accurately identified as *Enterococcus faecalis* (1 strain), *Enterococcus durans* (3 strains), *Lactobacillus brevis* (3 strains), *Lactobacillus buchneri* (2 strains), *Lactobacillus casei* (16 strains), *Lactobacillus delbrueckii* ssp. *bulgaricus* (142 strains), *Lactobacillus diolivorans* (17 strains), *Lactobacillus fermentum* (42 strains), *Lactobacillus helveticus* (183 strains), *Lactobacillus kefir* (6 strains), *Lactobacillus plantarum* ssp. *plantarum* (7 strains), *Lactococcus lactis* ssp. *lactis* (7 strains), *Leuconostoc lactis* (22 strains), *Leuconostoc mesenteroides* (21 strains), *Streptococcus thermophilus* (195 strains), and *Weissella cibaria* (1 strain). The predominant LAB were *Strep. thermophilus* and *Lb. helveticus*, which were isolated from all sampling sites. The results demonstrate that traditional fermented

dairy products from different regions of Mongolia have complex compositions of LAB species. Such diversity of LAB provides useful information for further studies of probiotic strain selection and starter culture design, with regard to the industrial production of traditional fermented milk.

Key words: traditional fermented dairy product, lactic acid bacteria, 16S rRNA gene

INTRODUCTION

In Mongolia, a large landlocked country in northeast Asia, approximately 40% of the population lives in the countryside as nomadic herders. The livelihood and well-being of Mongolians depend mainly on livestock and on milk in particular (Fratkin, 2005). Milk is sacred in Mongolia, where milk and milk products are staple foods that are produced in great abundance from the country's 30 million cattle, yaks, camels, horses, goats, and sheep (Sternberg, 2008). The traditional methods and processes for producing a range of specific products have been handed down through generations; thus, traditional milk products represent huge wealth and an important part of Mongolian heritage.

Fermented mare milk is a very popular traditional beverage in Mongolia, and it is rich in ferments (lactic acid bacteria and yeast), trace elements, antibiotics, vitamins A, B₁, B₂, B₁₂, C, D, and E, ethyl alcohol, lactic acid, and carbonic acid (Abdel-Salam et al., 2010). Mongolians use fermented mare milk for medical purposes, for recovering strength, and as a cure for diseases of digestive organs and illnesses such as tuberculosis (Solaroli et al., 1993; Lozovich, 1995; Geerlings et al., 2006). As the major livestock species, sheep and goats provide most of the milk and dairy products. Goat milk has been described as having greater digestibility and lower allergenic properties than cow milk, and it has been attributed with certain therapeutic values in human nutrition (Barrionuevo et al., 2002; Martín-Diana et al., 2003). Fermented yak milk and cow milk are widely consumed by local herders in Mongolia.

All of these milk products have typical features that depend on local and regional traditions. In addition,

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raw material-associated lactic acid bacteria (LAB) have an essential role in the nutritious and organoleptic properties of fermented milk production (Caplice and Fitzgerald, 1999). Spontaneous milk fermentation has a long history in Mongolia, and many milk products are still fermented on the household scale. The indigenous microorganisms, which depend on the raw milk, environment, and technology conditions, are considered to play a major role in determining the typical features of traditional fermented milk products. These microorganisms have the ability to lower the pH of a mixture by producing acid from sugars, leading to the development of the desirable organoleptic properties (Corsetti et al., 2007).

A few previous studies have examined LAB and yeasts isolated from samples of fermented milk in Mongolia. Uchida et al. (2007) analyzed the LAB composition of 3 “airag” (an alcoholic fermented horse milk), 2 “tarag” (fermented milk of cows, yaks, goats, or camels), 2 “is-gelen tarag” and “qoormog” (prolonged fermentation products of tarag), and some cheeses collected from 3 nomadic families in the Donto-Govi prefecture of Mongolia. Watanabe et al. (2008) used culture- and molecular biology-based methods to identify 367 LAB strains and 152 yeast strains isolated from 22 airag and 31 tarag samples in Mongolia. *Lactobacillus casei*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus kefiranofaciens*, *Lactobacillus plantarum* ssp. *plantarum*, *Lactococcus lactis* ssp. *cremoris*, and *Streptococcus thermophilus* were the most commonly isolated species.

To obtain a complete overview of the indigenous LAB involved in natural fermented milk from various

regions and different raw milks in Mongolia, we aimed to systematically study the diversity of LAB isolated from fermented yak, mare, goat, and cow products in Mongolia. Meanwhile, several molecular methods, such as 16S rRNA gene sequencing analysis, species- or subspecies-specific PCR, and RFLP analysis were applied for a more rapid and reliable identification of a large number of isolates.

MATERIALS AND METHODS

Collection of Samples

Fermented milk samples and their collection sites are shown in Table 1. One hundred eighty-nine samples of traditional fermented milk were collected from individual households in 12 provinces and 1 city of Mongolia. About 2 mL of each fermented milk sample was aseptically collected and transported to our laboratory in a 4°C vehicle-mounted refrigerator. Subsequently, all of the samples were analyzed within a few hours.

Enumeration, Isolation, and Phenotypic Characterization of Isolates

One milliliter of milk sample was mixed with 9 mL of 0.85% (wt/vol) sterile physiological saline to make an initial dilution (10^{-1}). Serial dilutions were made for each sample and 0.1 mL of the appropriate dilution (10^{-4} , 10^{-5} , and 10^{-6}) spread plated in triplicate on media. Plate count agar with bromocresol purple (BCP; Nissui Ltd., Tokyo, Japan) was incubated at 30°C for 48 h for enumeration of the total LAB under

Table 1. Samples, lactic acid bacteria (LAB) counts, and sampling locations in Mongolia

Sample type	Sample number	Samples (n)	LAB count (log cfu/mL)	Mean LAB count	Sampling location
Fermented cow milk	A6–A9	4	4.23–5.12	4.85	Dornogovi
	A10, A12–A33	23	3.41–8.00	7.00	Sükhbaatar
	A34–A43	10	4.36–7.81	7.18	Dornod
	A44–A51	8	5.58–7.51	6.94	Khentii
	B1–B10, B12–B16	15	4.22–8.02	7.39	Selenge
	B17–B22	6	4.45–7.88	7.21	Hovd
	B25–B37	13	5.38–8.88	7.44	Bulgan
	B38–B53, B60–B61	18	4.32–8.30	7.31	Hovsgol
	B62–B65, B70–B86	21	4.49–7.64	7.08	Dzavhan
	B88–B106, B109–B110	21	5.81–8.48	7.51	Arhangay
	C1–C10	10	7.23–8.67	8.18	Ovorhangay
	C11–C14	4	5.38–8.88	7.44	Bulgan
	C15–C24	10	7.01–8.73	8.26	Tov
	D1–D10	10	8.25–9.03	8.54	Ulaanbaatar
Fermented yak milk	B54–B59	6	4.49–6.28	5.66	Hovsgol
	B66–B69	4	4.30–6.90	6.49	Dzavhan
	B107–B108	2	5.93–6.05	5.99	Arhangay
Fermented goat milk	A11	1	4.00	4.00	Sükhbaatar
Fermented mare milk	B11	1	5.40	5.40	Selenge
	B23–B24	2	7.13–7.91	7.67	Hovd

anaerobic conditions. Cycloheximide at a concentration of 0.01% (vol/vol) was added to the BCP plates to prevent fungal growth. After incubation, colonies were enumerated and recorded as colony forming units per milliliter of milk. de Man, Rogosa, and Sharpe agar (MRS; Difco Laboratories, Detroit, MI) and M17 (Oxoid Ltd., Basingstoke, UK) plates were incubated anaerobically at 30°C for 48 h. Colonies with distinct morphological differences (color, shape, and size) were selected and purified by streaking on the same medium. The catalase and Gram reactions of all the isolates were assessed, and gram-positive, catalase-negative isolates were preserved in milk containing 0.1% sodium glutamate and stored at -80°C. A representative isolate in each species were tested for gas production from glucose, salt tolerance (3.0% and 6.5%), growth at different temperature (5, 10, 45, and 50°C) and pH (3.0, 3.5, 4.0, and 7.0) in MRS broth, as described by Kozaki et al. (1992).

DNA Extraction and 16S rRNA Gene Sequencing

Total genomic DNA was extracted from overnight cultures by using a previously reported method (Yu et al., 2009). Purified DNA was diluted to a final concentration of 100 ng/μL for application. All gram-positive, catalase-negative bacterial isolates were preliminary identified by 16S rRNA sequencing. For each strain, the genomic DNA was used as a template for PCR amplification of a segment of 16S rRNA gene on the automatic thermal cycler (PTC-200, MJ Research, Waltham, MA). Primers and reaction mixtures are listed in Table 2. The sequencing of purified products was performed in Shanghai Sangni Biosciences Corporation of China (Shanghai). The sequences were analyzed and determined using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast>; Altschul et al., 1997) and were submitted to the NCBI (<http://www.blast.ncbi.nlm.nih.gov>). Consensus sequences were imported into MEGA version 4.0 software (<http://www.mega-software.net>; Tamura et al., 2007), with which a sequence alignment and phylogenetic trees were created based on the neighbor-joining (NJ) method. The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program, was calculated from 100 random resamplings. *Bacillus subtilis* was used as an outgroup.

Discrimination of Closely Related Species and Subspecies

According to the partial sequences of 16S rRNA gene, we used phylogenetic analysis to differentiate strains that belonged to the *Lb. casei* group (*Lb. casei*,

Lb. rhamnosus, and *Lb. zeae*), the *Lb. plantarum* group (*Lb. paraplantarum*, *Lb. pentosus*, and *Lb. plantarum* ssp. *plantarum*, *Lb. plantarum* ssp. *argentoratensis*), *Lb. delbrueckii*, *Lc. lactis*, and *Enterococcus* species. These isolates were further classified by using PCR-RFLP techniques or subspecies-specific primers to verify the results of 16S rRNA gene sequence analysis.

The PCR-RFLP analysis of the gene encoding the elongation factor Tu (*tuf* gene) and 16S rRNA gene were used to discriminate *Lb. casei* group and *Lc. lactis*, respectively. The PCR primers Tuf 1, Tuf 2, Y1, and Y2 were used to amplify the *tuf* gene and 16S rRNA gene (Table 2). Following amplification, the reaction mixtures were electrophoresed in 1% agarose gel in Tris-borate EDTA buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0) at 100 V for 1 h, and the gel was stained with ethidium bromide. Aliquots of 3 μL of each amplified product were digested with restriction endonucleases as recommended by the manufacturer's protocols. Two restriction enzymes, *RsaI* and *HaeIII* (Takara Bio-Company, Shiga, Japan), were used in separate reactions. Restriction digests were analyzed through 5.0% polyacrylamide gel at 150 V for 1.5 h in 0.1× Tris-borate EDTA buffer. A DL2000 marker (Takara Bio-Company) was used as a size marker. Fragments shorter than 100 bp were not considered in the analysis.

For further identification of *Lb. plantarum* group, a multiplex PCR assay was performed with the *recA* gene-based primers paraF, pentF, planF, and pREV (Table 2). For accurate classification of *Lb. delbrueckii*, representative strains within *Lb. delbrueckii* were identified by using subspecies-specific primers, namely the proline iminopeptidase (*pepIP*) gene-targeted primers LB1 and LLB1 (Table 2). For identification of *Enterococcus faecium*, *Enterococcus durans*, and *Enterococcus faecalis*, amplification of the 16S-23S rRNA gene intergenic transcribed spacer region (ITS) was performed as described previously (Fortina et al., 2003), using the primers G1 and L1 (Table 2).

RESULTS

Enumeration of Isolates and Phenotypic Properties

The viable counts of the LAB present in the samples are shown in Table 1. Total counts of LAB in 173 fermented cow milk samples on BCP agar varied in the range from 3.41 to 9.03 log cfu/mL. The LAB counts in 12 fermented yak milk samples, 3 fermented mare milk samples, and 1 fermented goat milk sample ranged from 4.30 to 6.90 log cfu/mL, 5.40 to 7.91 log cfu/mL, and 4.00 log cfu/mL, respectively.

Gram-positive and catalase-negative bacteria growing on MRS and M17 agar were considered as presumptive

Table 2. Oligonucleotide primers and PCR reactions used in this study

Primer	Oligonucleotide sequence (5'–3')	Composition of 50-μL reaction ¹	PCR cycles	Target (product size)	Reference
27f 1495R	AGAGTTTGATCCTGGCTCAG CTACGGCTACCTTGTTCACGA	5.0 μL of 10× buffer, 2 μL of dNTP mix, 2 μL of each primer, 200 ng of DNA, 2.5 U of Taq	94°C/1 min; 58°C/1 min; 72°C/2 min; 30 cycles ²	16S rRNA gene (1,500 bp)	Weisburg et al., 1991
Tuf1 Tuf2	GATGCTGCTCCAGAAGA ACCTTCTGGCAATTCAATC	5.0 μL of 10× buffer, 2 μL of dNTP mix, 5 μL of each primer, 200 ng of DNA, 1 U of Taq	95°C/30 s; 52°C/30 s; 72°C/2 min; 35 cycles ²	<i>tuf</i> gene (800 bp)	Ventura et al., 2003
paraF pentF planF pREV Y1 Y2	GTCACAGGCATTACGAAAAC CAGTGGCGCGGTTGATATC CCGTTTATGCGGAACACCTA TCGGGATTACCAAACATCAC TGGCTCAGGACGAACGCTGGCGGC CCTACTGCTGCCTCCCGTAGGAGT	5.0 μL of 10× buffer, 2 μL of dNTP mix, 2 μL of each primer, 1 μL of primer planF, 200 ng of DNA, 2.5 U of Taq	94°C/30 s; 56°C/10 s; 72°C/30 s; 30 cycles ³	<i>recA</i> gene	Torriani et al., 2001
L1 G1	CAAGGCATCCACCGT GAAGTCGTAACAAGG	5.0 μL of 10× buffer, 1.5 μL of dNTP mix, 2 μL of each primer, 200 ng of DNA, 2.5 U of Taq	94°C/45 s; 55°C/45 s; 70°C/1 min; 35 cycles ³	16S rRNA gene (348 bp)	Young et al., 1991
LB1 LLB1	AAAAATGAAGTTGTTTAAAGTAGGTA AAGTCTGTCCTCTGGCTGG	5.0 μL of 10× buffer, 1 μL of dNTP mix, 2 μL of each primer, 200 ng of DNA, 2.5 U of Taq	94°C/1 min; 55°C/2 min; 72°C 3 min; 25 cycles ²	16S–23S rDNA intergenic transcribed spacer region	Fortina et al., 2003
			94°C/45 s; 58°C/30 s; 72°C/30 s; 35 cycles ⁴	<i>pepIP</i> gene	Torriani et al., 1999

¹Contained 10 pM each primer; 10 mM dNTP mix (Takara Bio-Company, Shiga, Japan), Taq DNA polymerase (Takara Bio-Company).
²Initial denaturation at 94°C for 3 min and final extension of the amplified product at 72°C for 10 min.
³Initial denaturation at 94°C for 3 min and final extension of the amplified product at 72°C for 5 min.
⁴Initial denaturation at 94°C for 2 min and final extension of the amplified product at 72°C for 10 min.

LAB. In total, 668 presumptive LAB were isolated from 189 fermented milk samples. The majority of isolated strains were rod-shaped bacteria (398 isolates in total, with 307 isolates from MRS and 91 from M17 medium) and the remainders were cocci (270 isolates in total with 111 from MRS and 159 from M17 medium).

The characteristics of 16 representative LAB are shown in Table 3. All the isolates were homo- or heterofermentative rods or cocci, and these representative strains showed some different characteristics at the different growth conditions.

16S rRNA Gene Sequences Analysis

The partial 16S rRNA gene sequences (1,490 bp) of all the strains were determined. Then, the sequences were compared with related bacteria in GenBank and sequence similarities were determined using the BLAST program. According to the 16S rRNA gene sequences, all strains were divided into 16 groups. As shown in Figures 1 and 2, a representative isolate in each group and their related type strains were used to construct a phylogenetic tree by using the MEGA 4.0 software. Isolates IMAU20130, IMAU20619, IMAU20680, IMAU20686, IMAU20159, and IMAU20683 showed proximity to *Lb. kefir*, *Lb. buchneri*, *Lb. diolivorans*, *Lb. brevis*, *Lb. fermentum*, and *Lb. helveticus*, respectively, because they grouped on the phylogenetic tree together with the corresponding type strain. However, IMAU20113 appeared to be equally linked to both *Lb. plantarum* ssp. *plantarum* and *Lb. pentosus*, so the 16S rRNA gene sequence did not give discriminatory results for this group. Isolate IMAU20365 shared 100% identity with the 16S rRNA gene sequence of *Lb. casei*. Isolate IMAU20149 grouped with 4 subspecies *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. delbrueckii* ssp. *delbrueckii*, *Lb. delbrueckii* ssp. *indicus*, and *Lb. delbrueckii* ssp. *lactis*, and its 16S rRNA gene sequence showed a similarity of 100% to *Lb. delbrueckii* ssp. *bulgaricus* and over 99% to the other subspecies. As shown in Figure 2, IMAU20181, IMAU20309, IMAU20771, IMAU20141, IMAU20676, IMAU20243, and IMAU20244 were closely related to the type strains *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Weissella cibaria*, *Strep. thermophilus*, *Lc. lactis* ssp. *lactis*, *E. faecalis*, and *E. durans*, respectively.

The results confirmed that 668 strains belong to 6 genera (*Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Weissella*, and *Streptococcus*), 2 species groups, and 14 species: *Lb. casei* group, *Lb. plantarum* group, *E. faecalis*, *E. durans*, *Lb. brevis*, *Lb. buchneri*, *Lb. delbrueckii*, *Lb. diolivorans*, *Lb. fermentum*, *Lb. helveticus*, *Lb. kefir*, *Lc. lactis* ssp. *lactis*, *Leu. lactis*, *Leu. mesenteroides*, *Strep. thermophilus*, and *W. cibaria* (Table 4).

Nucleotide Sequence Accession Numbers

The GenBank/European Molecular Biology Laboratory (EMBL)/DNA Data Bank of Japan (DDBJ) accession numbers for the 16S rRNA gene sequences of 668 isolates were deposited under HM218005 to HM218007, HM218010 to HM218014, HM057851 to HM058157, HM058159 to HM058176, HM058178 to HM058309, HM058311 to HM058314, HM058316 to HM058322, HM058324 to HM058379, HM058381 to HM058516, respectively.

Identification of Closely Related Species

The partial *tuf* gene (800 bp) and 16S rRNA gene (348 bp) amplified from *Lb. casei* group, *Lc. lactis* ssp. *lactis*, and *Lc. lactis* ssp. *cremoris* were digested separately with the 2 restriction enzyme *Hae*III and *Rsa*I to ensure the identification of the isolates. The RFLP profiles are showed in Figure 3. The *Hae*III patterns of isolates showed great differences among *Lb. casei*, *Lb. zeae*, and *Lb. rhamnosus*. The Y1-Y2 amplified fragment from *Lc. lactis* ssp. *lactis* digested with *Rsa*I gave 2 products of 280 and 61 bp (Figure 3), but the amplified fragment from *Lc. lactis* ssp. *cremoris* was not digested with *Rsa*I. In addition, based on the 16S rRNA gene sequences analysis, the representative isolate IMAU20676 was closer to the type strain *Lc. lactis* ssp. *lactis* than to the other 2 subspecies on the phylogenetic tree. Therefore, based on the results of RFLP and 16S rRNA gene sequences analysis, 7 isolates were identified as *Lc. lactis* ssp. *lactis*.

The expected sizes of the amplicons were 318 bp for *Lb. plantarum* ssp. *plantarum*, 218 bp for *Lb. pentosus*, and 107 bp for *Lb. paraplantarum*. Seven isolates in *Lb. plantarum* group displayed a pattern characterized by the typical band of *Lb. plantarum* ssp. *plantarum* (318 bp). All the *Lb. delbrueckii* strains were identified as *Lb. delbrueckii* ssp. *bulgaricus* with specific amplification product (1,065 bp) by using *pepIP* gene-based primers (Figure 4). Primers G1 and L1 were used to amplify the 16S–23S rDNA ISR from genomic DNA of *E. faecium*, *E. durans*, *E. faecalis*, and 4 wild isolates (Figure 4).

DISCUSSION

The fermented milk samples analyzed in this study were produced using traditional and ancient methods of natural fermentation. These milk products vary depending on their quality, taste, ingredients, and production methods, which are unique among regions, provinces, and even families. Therefore, various beneficial microorganisms are present in different fermented milk products. We analyzed 189 samples to determine

Table 3. Phenotypic characteristics of 16 representative strains isolated from traditional fermented milk in Mongolia¹

Characteristic	Strain of each species ²															
	IMAU 20130	IMAU 20619	IMAU 20680	IMAU 20365	IMAU 20686	IMAU 20113	IMAU 20159	IMAU 20149	IMAU 20683	IMAU 20243	IMAU 20244	IMAU 20676	IMAU 20141	IMAU 20771	IMAU 20181	IMAU 20309
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	+	+	+	–	+	–	+	–	–	–	–	–	–	+	+	+
Catalase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth at pH																
3.0	–	–	–	–	–	w	–	–	+	–	–	–	–	–	–	–
3.5	w	+	w	+	+	+	+	–	+	w	+	–	–	–	–	w
4.0	+	+	+	+	+	+	+	+	+	+	+	w	–	+	+	+
7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth in NaCl																
3.0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.5%	–	+	–	w	–	+	w	–	–	+	+	–	–	+	–	+
Growth at temperature																
5°C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10°C	+	+	+	+	+	+	w	–	–	+	+	+	–	w	+	+
45°C	–	w	–	w	–	w	+	+	+	+	+	–	+	+	+	w
50°C	–	–	–	–	–	–	–	–	+	+	w	–	+	–	–	–

¹+ = positive, – = negative, w = weakly positive.
²IMAU20130 = *Lactobacillus kefir*; IMAU20619 = *Lactobacillus buchneri*; IMAU20680 = *Lactobacillus diolivorans*; IMAU20365 = *Lactobacillus casei*; IMAU20686 = *Lactobacillus brevis*; IMAU20113 = *Lactobacillus plantarum* ssp. *plantarum*; IMAU20159 = *Lactobacillus fermentum*; IMAU20149 = *Lactobacillus delbrueckii* ssp. *bulgaricus*; IMAU20683 = *Lactobacillus helveticus*; IMAU20243 = *Enterococcus faecalis*; IMAU20244 = *Enterococcus durans*; IMAU20676 = *Lactococcus lactis* ssp. *lactis*; IMAU20141 = *Streptococcus thermophilus*; IMAU20771 = *Weissella cibaria*; IMAU20181 = *Leuconostoc lactis*; IMAU20309 = *Leuconostoc mesenteroides*.

Figure 1. Neighbor-joining tree showing the phylogenetic relationships between 9 lactobacilli and the type strains based on 16S rRNA gene sequences. *Bacillus subtilis* is used as an outgroup. Numbers at the branch nodes are bootstrap values percentages (100 replicates) and the bar indicates 1% sequence divergence. *Lb.* = *Lactobacillus*.

Figure 2. Neighbor-joining tree showing the phylogenetic relationships between 7 cocci and the type strains of related genera based on 16S rRNA gene sequences. *Bacillus subtilis* is used as an outgroup. Numbers at the branch nodes are bootstrap values percentages (100 replicates) and the bar indicates 1% sequence divergence. *E.* = *Enterococcus*; *Lac.* = *Lactococcus*; *Leu.* = *Leuconostoc*; *S.* = *Streptococcus*; *W.* = *Weissella*.

their LAB composition, with the aim of improving our understanding of specific characteristics of fermented milk products in Mongolia.

Slight variations of the viable counts of LAB were observed among fermented milk products of cow, yak, sheep, and mare. The mean numbers of LAB in fer-

Table 4. Distribution of lactic acid bacteria (LAB) species in traditional fermented milk samples from different regions in Mongolia¹

LAB isolate	Sampling location													Total
	Dornogovi	Sükhbaatar	Dornod	Khentii	Selenge	Hovd	Bulgan	Hovsgol	Dzavhan	Arhangay	Ovorhangay	Tov	Ulaanbaatar	
<i>Enterococcus faecalis</i>				1 ^b										1 ^b
<i>Enterococcus durans</i>				1 ^b							1 ^a + 1 ^b			2 ^a + 1 ^b
<i>Lactobacillus brevis</i>											2 ^a + 1 ^b			2 ^a + 1 ^b
<i>Lactobacillus buchneri</i>					1 ^a					1 ^b				1 ^a + 1 ^b
<i>Lactobacillus casei</i>							1 ^a + 1 ^b	1 ^a	4 ^a + 8 ^b	1 ^b				6 ^a + 10 ^b
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>		8 ^a	16 ^a + 1 ^b	6 ^a + 1 ^b	14 ^a + 1 ^b	12 ^a + 1 ^b	12 ^a + 1 ^b	19 ^a + 1 ^b	9 ^a + 2 ^b	6 ^a + 3 ^b	4 ^a	5 ^a	17 ^a + 3 ^b	128 ^a + 14 ^b
<i>Lactobacillus diolivorans</i>		3 ^a					3 ^a + 2 ^b				5 ^a + 1 ^b	2 ^a + 1 ^b		13 ^a + 4 ^b
<i>Lactobacillus fermentum</i>		1 ^a + 1 ^b	5 ^b	2 ^a + 3 ^b		2 ^a + 1 ^b	5 ^a + 6 ^b	4 ^a + 2 ^b	2 ^b	3 ^b	1 ^a + 2 ^b	2 ^a		17 ^a + 25 ^b
<i>Lactobacillus helveticus</i>	1 ^a + 1 ^b	26 ^a + 3 ^b	7 ^a + 1 ^b	12 ^a + 4 ^b	5 ^a + 3 ^b	2 ^a + 1 ^b	11 ^a + 3 ^b	6 ^a	28 ^a + 12 ^b	20 ^a + 13 ^b	6 ^a + 2 ^b	8 ^a + 4 ^b	2 ^a + 2 ^b	134 ^a + 49 ^b
<i>Lactobacillus kefir</i>		1 ^a + 2 ^b	1 ^a		1 ^a		1 ^a							4 ^a + 2 ^b
<i>Lactobacillus plantarum</i> ssp. <i>plantarum</i>	2 ^a + 4 ^b						1 ^b							2 ^a + 5 ^b
<i>Lactococcus lactis</i> ssp. <i>lactis</i>					1 ^a				1 ^b		1 ^a + 1 ^b		2 ^a + 1 ^b	4 ^a + 3 ^b
<i>Leuconostoc lactis</i>		7 ^a + 5 ^b	1 ^a + 1 ^b	1 ^b	1 ^a + 1 ^b		1 ^a + 1 ^b		1 ^b	2 ^b	2 ^b			9 ^a + 13 ^b
<i>Leuconostoc mesenteroides</i>						2 ^b		1 ^a + 2 ^b	1 ^a + 1 ^b	2 ^b	3 ^a + 3 ^b		1 ^a + 3 ^b	7 ^a + 14 ^b
<i>Streptococcus thermophilus</i>	1 ^a	9 ^a + 9 ^b	1 ^a + 3 ^b	1 ^a + 7 ^b	1 ^a + 1 ^b	4 ^a + 12 ^b	5 ^a + 11 ^b	8 ^a + 22 ^b	24 ^a + 17 ^b	7 ^a + 13 ^b	1 ^a	4 ^a + 15 ^b	5 ^a + 14 ^b	70 ^a + 125 ^b
<i>Weissella cibaria</i>													1 ^b	1 ^b

¹a = isolated on de Man, Rogosa, and Sharpe agar; b = isolated on M17 agar.

Figure 3. Polymerase chain reaction-RFLP fingerprints of *Lactobacillus casei* group and *Lactococcus lactis* strains.

mented milk of yak and mare were almost identical and were significantly higher than those in fermented goat milk but slightly lower than those in fermented cow milk. These findings are consistent with the results of previous studies. Watanabe et al. (2008) reported that the LAB counts in traditional Mongolian fermented milks airag and tarag were 7.78 ± 0.50 and 8.35 ± 0.62 log cfu/mL, respectively. Notably, differences in the timing of milk ripening and transport or differences in sampling regions could lead to variations in LAB counts.

M17 and MRS media are used routinely for the isolation and counting of LAB from most fermented food products. Although described as a medium for the cultivation of lactobacilli, MRS has exhibited poor selectivity for lactobacilli (Ouadghiri et al., 2005). The MRS medium used in this study allowed the isolation of identified species found in fermented dairy products tested, except for *E. faecalis* and *W. cibaria*, which were isolated from M17 agar. However, more LAB isolates were obtained from MRS media (59.6%) than from M17 agar (40.4%), and the majority of lactobacilli (77.1%) were isolated from MRS media, whereas the majority of cocci (58.9%) were isolated from M17 media. Similar to our results, a prior study of LAB in yogurt demonstrated MRS as the medium of choice for differential counting of lactobacilli and M17 as the preferred medium for counting cocci (Coeuret et al., 2003).

Traditional microbiological assays are often time-consuming and can yield variable results for the identification of closely related LAB (Ercolini, 2004). The sequencing and analysis of 16S rRNA genes is considered a cornerstone of modern microbial taxonomy (Ventura and Zink, 2002). For this reason, all isolates in this

study were grouped and preliminarily identified based on their 16S rRNA gene sequences, and some closely related LAB were further examined using species- or subspecies-specific PCR primers and PCR-RFLP analysis. The results indicated that 16S rRNA gene sequences of strains in the *Lb. casei* group, *Lb. delbrueckii* group, and *Lb. plantarum* group were extremely similar; however, species-specific PCR and PCR-RFLP analysis could discriminate them at the species level.

The distribution of isolates among different sampling sites is presented in Table 4. Interestingly, *Strep. thermophilus* and *Lb. helveticus* were isolated from all sampling sites (representing 29.2 and 27.4% of all isolates, respectively), and *Lb. delbrueckii* ssp. *bulgaricus* was isolated from most sampling sites (representing 21.4% of all isolates). In previous studies, *Lb. helveticus* was found to be the predominant LAB component in airag, tarag (Watanabe et al., 2008), and Mongolian koumiss (a fermented mare milk drink) (Sun et al., 2010). In comparison, *Strep. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* were reported as the most frequently isolated bacteria in Columbian dairy products (Perea Veléz et al., 2007). *Lactobacillus delbrueckii* ssp. *bulgaricus* is the key organism used as a starter for the preparation of yogurt and may play a major role in coagulation of milk by rapidly increasing the acidity.

Other species isolated at relatively high frequencies included *Lb. casei*, *Lb. fermentum*, *Leu. mesenteroides*, *Leu. lactis*, and *Lb. diolivorans*. Several researchers have identified *Lb. fermentum* and *Lb. casei* from dairy products, such as Algerian arid zone raw goat milk and Nigerian fermented foods (Olasupo et al., 2001; Guessas and Kihal, 2004). *Leuconostoc mesenteroides* has also been identified in Maasai traditional fermented milk

Figure 4. Amplification patterns obtained with *recA* gene-based multiplex primers for the *Lactobacillus plantarum* group (a), the intergenic transcribed spacer-PCR profiles of *Enterococcus* species, and the *pepIP* gene-based multiplex primers for *Lactobacillus delbrueckii* ssp. *Bulgaricus* (b). Panel a: M = DL2000 DNA marker; 1 = *Lactobacillus pentosus* ATCC 8041; 2 = *Lactobacillus plantarum* ssp. *plantarum* ATCC 14917; 3 = *Lactobacillus paraplantarum* ATCC 700211; 4 = IMAU70004; 5 = IMAU70005; 6 = IMAU70010; 7 = IMAU70023; 8 = IMAU70035; 9 = IMAU70042; and 10 = IMAU70080. Panel b: M = 100-bp DNA ladder marker; 1 = *Enterococcus faecium* ATCC 19434; 2 = *Enterococcus faecalis* ATCC 19433; 3 = *Enterococcus durans* DSM20633; 4 = IMAU20244; 5 = IMAU20682; 6 = IMAU20243; 7 = IMAU20684; 8 = *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842; and 9 = IMAU20255.

(Mathara et al., 2004). *Leuconostoc* strains naturally contribute to the development of flavor in fermented products, although they exhibit a weak competitive ability during milk fermentation due to their complex nutritional requirements. Although *Lb. diolivorans* is typically isolated from maize silage (Krooneman et al., 2002), to our knowledge, it has never been reported in natural fermented milk. We isolated 17 strains of *Lb. diolivorans* in this study, probably due to their complex nutritional requirements, as well as weaker adaptation to the milk environment.

In addition, a few other isolates were classified as *E. faecalis*, *E. durans*, *Lc. lactis* ssp. *lactis*, *Lb. plantarum* ssp. *plantarum*, *Lb. kefir*, *Lb. brevis*, *Lb. buchneri*, and *W. cibaria*. *Enterococcus faecalis*, *E. faecium*, and to a lesser extent *E. durans* are found commonly in milk and cheese products (Franz et al., 1999). *Enterococ-*

cus faecalis has been isolated from kajmak samples (an artisanal Serbian dairy product) and Moroccan camel milk (Benkerroum et al., 2003; Jokovic et al., 2008), whereas *E. durans*, originally described as an isolate of milk and dairy products (Krooneman et al., 2002), has been associated traditionally with dairy products, including cheese and fermented milk. The presence of enterococci in traditional dairy products is probably due to fecal contamination of milk during milking; however, these species are used as an additive in starter cultures for the preservation of various fermented foods, including cheese and fermented vegetable products. In the current study, 7 strains were identified as *Lb. plantarum* ssp. *plantarum*. According to Wood and Holzappel (1995) and Stiles and Holzappel (1997), *Lb. plantarum* ssp. *plantarum* strains are commonly associated with plant-based food fermentations. *Weissella cibaria* was isolated from only one fermented cow milk sample in Ulaanbaatar city, suggesting that it is likely present in low quantities. According to Isono et al. (1994), *Weissella* strains are found occasionally in raw milk, but their technological role in fermented milk has not been described. In general, strains of *Lc. lactis* ssp. *lactis* are important commercial dairy starter cultures. In this study, the lowest frequency of *Lc. lactis* ssp. *lactis* was found in samples from Selenge, Dzavhan, Ovornhangay, and Ulaanbaatar provinces. *Lactobacillus kefir* was detected in Sükhbaatar, Dornod, Selenge, and Bulgan provinces. In previous research, *Lb. kefir* has been isolated mainly from kefir grains, but it has also been detected in some cheese varieties (Dolci et al., 2008). *Lactobacillus brevis* and *Lb. buchneri* have also been detected in fermented dairy products (Sumner et al., 1985; Rahman et al., 2009).

The complexity of the microbial composition in fermented milks appears to vary by region. We isolated 10 species of LAB in Ovornhangay province, 9 species in Bulgan province, 8 species in Dzavhan and Arhangay provinces, 7 species in Khentii, Sükhbaatar, and Selenge provinces, 6 species in Dornod and Hovsgol provinces and Ulaanbaatar city, 5 species in Hovd and Tov provinces, and only 3 species in Dornogovi. The presence of LAB with such variation of strains is not surprising because other factors may contribute to the variability of strains, such as different production methods, recipes, and raw materials. Furthermore, the environmental temperature and location differences may also cause some variation in strains.

CONCLUSIONS

This report describes the microbiological study and detailed identification of LAB involved in natural fermented milks. *Streptococcus thermophilus* and *Lb.*

helveticus were the dominant LAB species in fermented dairy products in Mongolia. Other species, including *E. faecalis*, *E. durans*, *Lb. brevis*, *Lb. buchneri*, *Lb. casei*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. diolivorans*, *Lb. fermentum*, *Lb. kefir*, *Lb. plantarum* ssp. *plantarum*, *Lc. lactis* ssp. *lactis*, *Leu. lactis*, *Leu. mesenteroides*, and *W. cibaria*, were identified at lower frequencies. The species distribution depends on the manufacturing processes, as well as on the specific ecological locality where the milk products were manufactured. This study provides raw data and a strain resource for further studies involving probiotic strain selection and starter culture design pertaining to the industrial production of traditional fermented milk.

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