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Identification and Characterization of the Dominant Lactic acid Bacteria Isolated from Traditional Fermented Milk in Mongolia

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ABSTRACT. Five samples of Airag and 20 of Tarag (both in Mongolia) were collected from scattered households. One hundred strains of lactic acid bacteria (LAB) were isolated and identified from these samples according to phenotypic characterization and 16S rRNA gene sequence analysis. Eighty-five isolates belonged to the genus *Lactobacillus*, 15 being classified as coccoid LAB. All isolates belonged to 5 genera and 11 to different species and subspecies. *Lactobacillus* (*Lb.*) *helveticus* was predominant population in Airag samples, *Lb. fermentum* and *Lb. helveticus* were the major LAB microflora in Tarag.

Abbreviations

CFU	colony forming units	CTAB	hexadecyltrimethylammonium bromide
DAP	*meso*-2,6-diaminopimelic acid	LAB	lactic acid bacteria
MRS	de Man–Rogosa–Sharpe (agar)	PDA	potato dextrose agar

E.	*Enterococcus*	*Lb.*	*Lactobacillus*	*Lc.*	*Lactococcus*
Le.	*Leuconostoc*	*P.*	*Pediococcus*	*S.*	*Streptococcus*

In Mongolia, lifes of nomadic Mongolians greatly depend on the domestic livestock such as horses, cows, yaks, goats, ewes and camels, and they have produced various kinds of traditional fermented dairy products from their milk. “Airag”, also called Koumiss, is a mildly alcoholic, sour-tasting fermented drink that is usually made from unpasteurised fresh mare milk. “Tarag” is a yogurt-like fermented milk from cows, ewes, goats or camels. They are produced from milk as a result of lactic acid and alcoholic fermentation by LAB and yeasts (Uchida *et al.* 2007; Watanabe *et al*. 2008). In Mongolia, the history of making and drinking Airag and Tarag is more than 1000 years, and rich microbial resources have been handed down (Danova *et al.* 2005; Uchida *et al*. 2007).

There are several reports on the LAB and yeasts in the traditional fermented milks of Mongolia (Uchida *et al*. 2007; Watanabe *et al*. 2008) and Inner Mongolia in China (An *et al*. 2004). However, papers on the isolates from Tarag made with fixed goat and camel milk in Ovorhangay and Govi Altay remain scarce. Therefore the identification of LAB from the traditional fermented dairy products in these regions will yield valuable knowledge.

Our aim was to enumerate, isolate, identify and characterize the dominant microorganisms from Airag and Tarag samples obtained from Mongolia, using combination the methods of conventional and 16S rRNA sequence analysis.

MATERIAL AND METHODS

Sample collection and enumeration of microorganisms. Five samples of Airag and 20 ones of Tarag were collected from scattered households. Samples of ≈500 mL fermented milk were taken aseptically into sterile glass bottles after thorough mixing in the bulk samples container. The pH values were measured with pH meter (pH100; *Extech*, USA).

Serial 10-fold dilutions were performed, and 0.1 mL aliquots of the appropriate dilutions (10^{-5}–10^{-8}) were inoculated in triplicate onto MRS (*Fluka*) agar anaerobically for 3 d at 30 °C. The counts of yeasts

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were determined using PDA (*Nissui Pharmaceutics*, Japan) agar, acidified to pH 3.5 with 10 % sterilized tartaric acid, and incubation for 5 d at 25 °C.

Isolation and identification of LAB. The colonies were randomly picked from MRS plates with 30–300 colonies, and transferred into 10 mL of MRS broth. The selected colonies were purified by repeated streaking on the MRS media. G^+ positive, catalase-negative isolates were purified and stored.

The isolates were tested for NH_3 production from arginine, gas production from glucose, salt tolerance, and growth temperature (Kozaki *et al*. 1992). The presence of DAP in the cell wall was determined according to Mathara *et al*. (2004). The type and amount of D and L antipods of lactic acid (produced from glucose) was assayed in modified MRS broth using a commercial kit (*Hoffman La Roche Diagnostic*; Germany). The determination of the saccharide fermentation patterns was performed according to Kozaki *et al*. (1992).

16S rRNA sequencing and molecular identification. Genomic DNA was extracted from 5 mL of culture at 37 °C in MRS broth by CTAB (Zhu *et al*. 1993). The 16S rRNA gene was amplified using primers 16S-FA 5′-GCA GAG TTC TCG GAG TCA CGA AGA GTT TGA TCC TGG CTC AG-3′ and 16S-RA 5′-AGC GGA TCA CTT CAC ACA GGA CTA CGG CTA CCT TGT TAC GA-3′ (Scarpellini *et al*. 2002) with some modifications. Nucleotides 1–21 of both primers are specific sequencing regions (underlined).

16S rRNA genes were amplified in a *MJ Research* RTC-200 thermocycler (*Biotech International*, Australia) with PCR buffer (Hacin *et al*. 2008). The PCR product was isolated from the agarose gel using a Huashun Gel Extraction Kit (*Huashun*, China). The purified PCR fragments were performed on ABI 3730 no. 1 DNA analyzers (*Applied Biosystems*) by the sequencing primers. The consensus sequences were compared with those available in *GenBank*. Sequence alignments and phylogenetic tree were done by using the MEGA software (ver. 4.0; Tamura *et al*. 2007).

RESULTS AND DISCUSSION

Enumeration of microorganisms. The average pH values of the fermented milk samples were 3.9 ± 0.2, 4.6 ± 0.8 and 4.5 ± 0.5 (Table I). The distribution of LAB and yeasts was shown according to their levels of viable counts. The LAB dominated in the microbial population of Airag, the viable counts ranging from 6.88 to 7.38 $\log_{10}$ CFU/g, whereas in the Tarag samples they were 6.72 ± 0.60 and 7.38 ± 0.34. In Airag the counts of yeasts ranged from 5.49 to 6.11 $\log_{10}$ CFU/g, the Tarag samples having yeast counts 4.98–6.17. Differences in the counts of LAB and yeasts were observed between Airag and Tarag samples. LAB counts of Airag were lower than that of Tarag from Govi Altay, and slightly higher than that from Ovorhangay, but yeasts counts of Airag were higher than that of both Tarag regions. A difference was found between our results and those of Watanabe *et al*. (2008) which can be explained by different environment and analytical methods.

Table I. General features of Airag and Tarag samples from three regions of Mongolia[a]

No.	Region	Sample	pH	LAB, $\log_{10}$CFU/g	Yeasts, $\log_{10}$CFU/g
1–5	Ulaanbaatar	Airag	3.9 ± 0.2 (3.7–4.1)	7.18 ± 0.26 (6.88–7.38)	5.89 ± 0.24 (5.49–6.11)
6–19	Govi Altay	Tarag	4.6 ± 0.8 (3.7–6.4)	6.72 ± 0.60 (5.09–7.23)	5.56 ± 0.49 (4.98–6.17)
20–25	Ovorhangay	Tarag	4.5 ± 0.5 (3.9–5.1)	7.38 ± 0.34 (6.89–7.80)	5.29 ± 0.12 (5.13–5.48)
Average			4.4 ± 0.7	6.97 ± 0.69	5.56 ± 0.43

[a]Twenty-five sites of 9 villages in 3 regions; in *parentheses* – the range of values.

As for Airag and Tarag, fermentation is of symbiotic nature and depends on the action of two distinct types of microorganisms (*i.e.* LAB and yeasts), the major microbial constituents being LAB. Moreover, indigenous microbiota plays the major role in dairy products making and ripening. Our counts of LAB and yeasts were slightly lower than those reported in some traditional naturally fermented milk from various parts of the world (Naersong *et al*. 1996; Abdelgadir *et al*. 2001; Mathara *et al*. 2004; Uchida *et al*. 2007; Watanabe *et al*. 2008), but they were higher than those of the other types of traditional naturally fermented milk, such as Qula (Duan *et al*. 2008), amasi (Gadaga *et al*. 2000) and Lben (Badis *et al*. 2004). This difference may be related to the differences in the milk of source animals, environment, fermentation time and temperature.

Identification of LAB. A total of 100 bacterial strains from 5 Airag and 20 Tarag samples were considered as presumptive LAB because of their G^+ and catalase-negative characteristics. By means of phenotypic tests, they were divided into 10 groups (Tables II and III). All isolates were able to ferment glucose but not starch and glycogen. Thirty-three isolates produced gas from glucose and seemed, therefore, to be obligatory heterofermentative strains.

Table II. Physiological and biochemical properties of LAB isolated from Airag and Tarag (groups *1–10*[a])[b]

Characteristics	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Number of isolates	39	2	1	2	8	33	4	5	5	1
Lactic acid isomer	DL	D	D(L)	L	L	DL	L	L	DL	L
Shape[c]	R	R	R	R	R	R	C	C	C	C
Gas from glucose	0/39	0/2	0/1	0/2	0/8	33/33	0/4	0/5	0/5	0/1
meso-2,6-DAP	0/39	0/2	0/1	0/2	8/8	0/33	–	–	–	–
Growth at (°C) 10	–	–	–	–	–	–	4/4	5/5	5/5	0/1
15	0/39	0/2	0/1	2/2	8/8	0/33	–	–	–	–
40	–	–	–	–	–	–	4/4	4/5	5/5	1/1
45	39/39	2/2	1/1	1/2	0/8	33/33	–	–	–	–
50	–	–	–	–	–	–	0/4	0/5	5/5	1/1
NaCl (%) 4.0	0/39	0/2	1/1	2/2	8/8	33/33	–	–	–	–
6.5	–	–	–	–	–	–	4/4	5/5	5/5	0/1
pH 3.5	39/39	0/2	0/1	2/2	8/8	4/33	0/4	0/5	3/5	0/1
4.5	39/39	2/2	1/1	2/2	8/8	33/33	4/4	5/5	5/5	0/1
9.0	0/39	2/2	0/1	0/2	0/8	33/33	4/4	5/5	0/5	0/1
NH_3	0/39	0/2	0/1	0/2	0/8	33/33	3/4	5/5	0/5	0/1

[a]Groups *1–10* were identified as *L. helveticus*, *L. delbrueckii*, *L. kefiranofaciens*, *L. casei*, *L. plantarum*, *L. fermentum*, *Enterococcus*, *Lactococcus*, *Pediococcus* and *Streptococcus*. [b]Number of positive strains to total number. [c]R – rod, C – cocci.

Table III. Saccharide fermentation properties of LAB isolated from Airag and Tarag (groups *1–10*[a])

Characteristics[b]	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Number of isolates	39	2	1	2	8	33	4	5	5	1
Arabinose	0/39	0/2	0/1	0/2	7/8	23/33	0/4	0/5	5/5	0/1
Xylose	0/39	0/2	0/1	0/2	8/8	0/33	0/4	0/5	5/5	0/1
Rhamnose	0/39	0/2	0/1	0/2	0/8	0/33	0/4	0/5	4/5	0/1
Ribose	0/39	0/2	0/1	2/2	8/8	33/33	3/4	5/5	0/5	0/1
Mannose	31/39	0/2	0/1	2/2	8/8	33/33	4/4	5/5	5/5	1/1
Fructose	33/39	2/2	1/1	2/2	8/8	33/33	4/4	5/5	5/5	1/1
Galactose	39/39	0/2	1/1	2/2	8/8	33/33	4/4	5/5	5/5	1/1
Sucrose	0/39	0/2	1/1	2/2	8/8	33/33	4/4	4/5	5/5	1/1
Maltose	39/39	0/2	1/1	2/2	8/8	33/33	4/4	5/5	0/5	0/1
Cellobiose	4/39	0/2	0/1	2/2	8/8	26/33	4/4	5/5	5/5	0/1
Lactose	37/39	2/2	1/1	2/2	8/8	33/33	4/4	5/5	4/5	1/1
Trehalose	19/39	0/2	0/1	2/2	8/8	27/33	4/4	5/5	3/5	0/1
Melibiose	1/39	0/2	1/1	0/2	8/8	33/33	0/4	0/5	0/5	0/1
Raffinose	0/39	0/2	1/1	0/2	8/8	33/33	0/4	0/5	3/5	0/1
Melezitose	1/39	0/2	0/1	2/2	7/8	0/33	0/4	0/5	0/5	0/1
Dextrin	0/39	0/2	0/1	2/2	8/8	0/33	0/4	5/5	2/5	0/1
Inulin	1/39	0/2	0/1	2/2	4/8	0/33	0/4	0/5	0/5	0/1
Mannitol	2/39	0/2	0/1	2/2	8/8	0/33	4/4	4/5	4/5	0/1
Glucitol	1/39	0/2	0/1	2/2	8/8	0/33	1/4	0/5	0/5	0/1
myo-Inositol	0/39	0/2	0/1	2/2	0/8	0/33	0/4	0/5	0/5	0/1
Aesculin	0/39	0/2	0/1	2/2	8/8	0/33	4/4	5/5	5/5	1/1
Salicin	5/39	0/2	0/1	2/2	8/8	0/33	4/4	5/5	3/5	0/1
Amygdalin	0/39	0/2	0/1	2/2	8/8	0/33	4/4	5/5	4/5	0/1

[a]*See* footnotes *a* and *b* of Table II. [b]All strains fermented glucose, no strain fermented starch and glycogen.

Thirty-nine isolates were identified as *Lb. helveticus* group. They grew well at 45 °C, produced DL-lactic acid, and most of them could utilize glucose, sucrose, mannose, maltose, galactose, lactose and

fructose. Eight DAP-positive facultatively heterofermentative isolates were identified as *Lb. plantarum*. They all could grow in the presence of 4.0 % NaCl, and ferment most sugars except for rhamnose, starch, *myo*-inositol and glycerol. Two isolates were found to be closely related to *Lb. delbrueckii*. They had no DAP in cell wall and produced D-lactic acid. Two isolates were identified as *Lb. casei*. Arabinose, xylose, rhamnose, melibiose, raffinose, starch and glycogen were not fermented by the two isolates. One isolate was identified as *Lb. kefiranofaciens*; it could ferment glucose, fructose, galactose, sucrose, maltose, lactose, raffinose and melibiose.

Heterofermentative bacilli (33) were identified as *Lb. fermentum* group. These strains could grow on MRS containing 4.0 % NaCl and, at pH 9.0, were DAP-negative and arginine-positive. They were able to ferment ribose, glucose, mannose, fructose, galactose, sucrose, maltose, lactose, melibiose, and raffinose.

Four coccoid strains were identified as *Enterococcus* group. They could grow at 10 or 40 °C and, at pH 9.0, produced NH_3 from arginine, most of them utilizing glucose, ribose, mannose, fructose, galactose, maltose, cellobiose, lactose, trehalose, aesculin, salicin, amygdalin *etc*. Five cocci were identified as lactococci. Most of them could grow at 10 and 40 °C, but not at 50 °C. Moreover, they produced L-lactic acid. Five coccoid strains were identified as *Pediococcus* group. They could grow at 50 °C and 6.5 % NaCl and produced DL-lactic acid. The last coccoid isolate was classified as *Streptococcus* according to its sugar fermentation and biochemical properties.

16S rRNA sequence identification and phylogenetic analysis. The obtained sequences (≈1.4 kbp) were deposited in *GenBank* and assigned the accession nos FJ640986–FJ641003, FJ844933–FJ845009, FJ91562–FJ915624 and EF536362.

Eighty-five rods isolated from Airag and Tarag were designated to 6 species, *Lb. casei*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. fermentum*, *Lb. helveticus*, *Lb. plantarum* and *Lb. kefiranofaciens* ssp. *kefiranofaciens* (Table IV). Moreover, 15 coccoid strains were characterized as *E. durans*, *E. saccharominimus*, *Lc. lactis* ssp. *lactis*, *P. acidilactici* and *S. thermophilus*.

Table IV. Numbers of LAB isolates from Airag and Tarag

Species	Airag	Tarag	Species	Airag	Tarag
E. durans	0	3	*Lb. kefiranofaciens* ssp. *kefiranofaciens*	0	1
E. saccharominimus	0	1	*Lb. plantarum*	6	2
Lb. casei	1	1	*Lc. lactis* ssp. *lactis*	0	5
Lb. delbrueckii ssp. *bulgaricus*	0	2	*P. acidilactici*	0	5
Lb. fermentum	0	33	*S. thermophilus*	0	1
Lb. helveticus	19	20	Total	26	74

Danilo *et al*. (2001) and Holzapfel *et al*. (2001) showed that the conventional method is limited for identification and characterization of strains to species and subspecies level, and is regarded uncertain, complicated, and time-consuming owing to the increasing number of species that vary in some of these characters. 16S rRNA sequence analysis was regarded as the most accurate and reliable method for species level identification. Based on physiological properties, nutritional requirements and growth conditions, we divided 100 isolates into 10 groups by conventional methods. However, the result of 16S rRNA sequence analysis showed that all these isolates belong to 11 species and subspecies. Group *7* containing 4 isolates was classified as an *Enterococcus* group by conventional methods but these isolates were identified as two species, *E. durans* and *E. saccharominimus*. As for *Lb. delbrueckii*, *Lb. kefiranofaciens* and *Lactococcus* groups, these isolates were identified to the genus and species level by conventional methods, but the 16S rRNA sequence analysis identified these isolates at the subspecies level. 16S rRNA sequence analysis is therefore more exact than conventional methods for some species identification of LAB.

Phylogenetic analysis revealed that the isolates and related type strains mainly consisted of two clusters belonging to 5 genera (Fig. 1). Six species of *Lactobacillus* spp. and *P. acidilactici* clustered into one large group; *E. durans*, *E. saccharominimus*, *Lc. lactis* ssp. *lactis*, and *S. thermophilus* formed another large group.

We determined LAB composition of Airag and Tarag by conventional and molecular methods. The 26 strains isolated from Airag were classified and characterized through a combination of 16S rRNA gene sequences and traditional methods. The 74 isolates from Tarag were identified by the same methods. The predominant LAB was *Lb. helveticus* in Airag. The same results were reported by Uchida *et al*. (2007) and Watanabe *et al*. (2008). However, we have not found coccoid isolates in Airag; this contradiction may be due to differences in the pH values of samples and analytical methods.

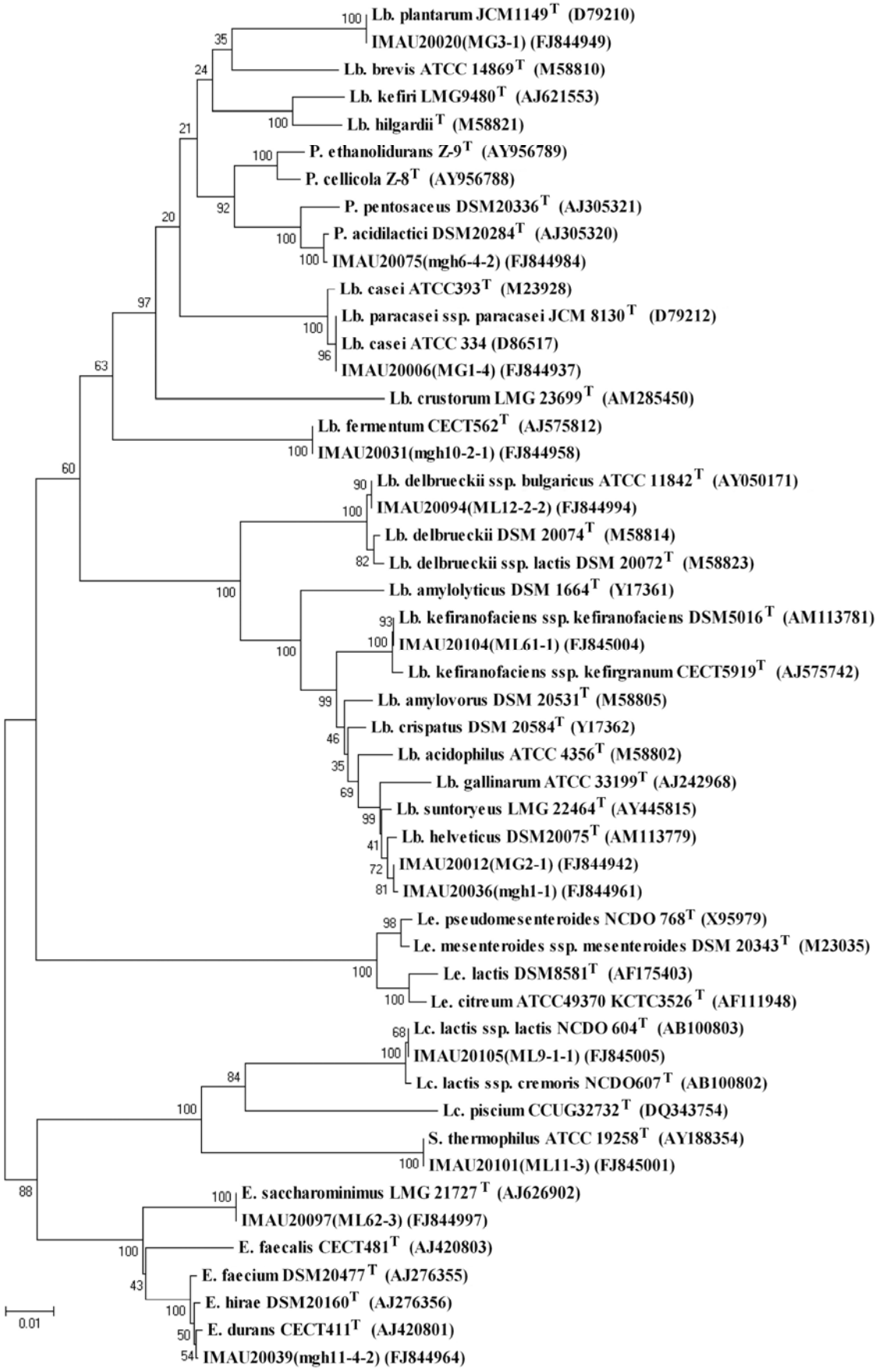

Fig. 1. Phylogenetic tree based on 16S rRNA sequence analysis, showing the phylogenetic placement of representative isolates from Airag and Tarag.

Otherwise, *Lb. fermentum* and *Lb. helveticus* were predominant LAB in Tarag, which represents 44.6 and 27.0 % of the isolates, respectively, but Uchida *et al*. (2007) and Watanabe *et al*. (2008) reported some different results. This can be due to the specific environmental factors of Govi Altay and Ovorhangay that can affect the composition of LAB in these samples.

Lactobacilli usually predominated in the traditional naturally-fermented milk (Naersong *et al.* 1996; An *et al*. 2004; Uchida *et al*. 2007). We have also considered lactobacilli as dominant LAB strains, which occupied 85.0 % of the total isolates. *Lb. fermentum*, *Lb. plantarum*, and especially *Lb. helveticus* were isolated with high frequency in most of traditional fermented milk such as koumiss (Watabe *et al.* 1998), Qula cheese (Duan *et al.* 2008), Parmigiano Reggiano cheese (Gala *et al*. 2008) and goat milk cheeses (Martin-Platero *et al.* 2009). However, the frequency of *Lb. helveticus* in Tarag was lower than in Airag, while the frequency of *Lb. fermentum* was much higher here than in Airag (Watabe *et al*. 1998; Watanabe *et al*. 2008).

Similar to our results, *E. durans*, *Lc. lactis* ssp. *lactis* and *S. thermophilus* were isolated from traditional fermented milk (An *et al*. 2004; Uchida *et al*. 2007; Watanabe *et al*. 2008; Khedid *et al*. 2009). However, *P. acidilactici* is rarely isolated from traditional fermented milk, such as Airag, Tarag, Qula and Pamigiano Regiano cheese (An *et al*. 2004; Duan *et al*. 2008; Watanabe *et al*. 2008). It is known that different raw milk and different making technology of dairy products resulted in various microorganism composition and different nutritional value. Thus, Tarag made from fixed goat and camel milk is a special dairy product in Mongolia with indigenous LAB microflora and high nutritional value.

Our results provide some raw data and strain resource for further study involved in probiotic strain selection and starter culture design concerning the industrialization production of traditional fermented milk.

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