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# Pyrosequencing Analysis of the Microbial Diversity of Airag, Khoormog and Tarag, Traditional Fermented Dairy Products of Mongolia

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**Here, we used pyrosequencing to obtain a detailed analysis of the microbial diversities of traditional fermented dairy products of Mongolia. From 22 *Airag* (fermented mare's milk), 5 *Khoormog* (fermented camel's milk) and 26 *Tarag* (fermented milk of cows, goats and yaks) samples collected in the Mongolian provinces of Arhangai, Bulgan, Dundgobi, Tov, Uburhangai and Umnugobi, we obtained a total of 81 operational taxonomic units, which were assigned to 15 families, 21 genera and 41 species in 3 phyla. The genus *Lactobacillus* is a core bacterial component of Mongolian fermented milks, and *Lactobacillus helveticus, Lactobacillus kefiranofaciens* and *Lactobacillus delbrueckii* were the predominant species of lactic acid bacteria (LAB) in the *Airag*, *Khoormog* and *Tarag* samples, respectively. By using this pyrosequencing approach, we successfully detected most LAB species that have been isolated as well as seven LAB species that have not been found in our previous culture-based study. A subsequent analysis of the principal components of the samples revealed that *L. delbrueckii*, *L. helveticus*, *L. kefiranofaciens* and *Streptococcus thermophilus* were the main factors influencing the microbial diversity of these Mongolian traditional fermented dairy products and that this diversity correlated with the animal species from which the milk was sourced.**

**Key words: Airag, Khoormog, Tarag, Mongolian traditional dairy products, pyroseqencing, microbial diversity**

## INTRODUCTION

Traditional fermented dairy products play an important role in the Mongolian diet because of their nutrient richness and medicinal potential [1, 2]. The nomads of Mongolia produce various kinds of traditional fermented dairy products. *Airag* is a mildly alcoholic, sour-tasting fermented drink that is usually made from the raw milk of mares; it is called *Koumiss* in Kazakhstan, Kyrgyzstan and Russia and *Chigee* in Inner Mongolia, China [3, 4]. *Khoormog* is a traditional fermented mild alcoholic beverage made from raw camel milk [5]. *Tarag* is a yogurt-type traditional fermented milk made from heat-treated cow, yak or goat milk [6]. These products are prepared by time-honored methods that were developed by the nomadic people in their gers (portable houses) and are naturally fermented by adding the milk to a traditional container without the use of commercial starters. Therefore, these products have unique microbial compositions depending on the individual houses in which they are prepared.

There have been numerous analyses of the lactic acid bacteria (LAB) and yeasts in the traditional fermented dairy products of Mongolia that have used culture-based methods [7–9]. However, in these studies, the species were identified on the basis of their phenotypic features, and the information obtained has not always contributed to an accurate and detailed picture of the microbial diversity of these products. Recently, some studies reported the detailed microbial composition of Mongolian dairy products by using a combination of culture-based method and molecular biological identification [3, 10]. In our previous study, we used culture-based isolation and molecular-based identification—random amplified polymorphic DNA (RAPD) PCR for strain typing and 16S rRNA gene sequencing for species identification—and reported on the diversity of LAB and yeasts in 22 *Airag* and 31 *Tarag* samples collected from various regions of Mongolia. A total of 367 LAB strains isolated from these samples revealed significant differences in LAB diversity. Specifically, we found that the predominant LAB species of *Airag* were *Lactobacillus helveticus* and *Lactobacillus kefiranofaciens*, and those of *Tarag* were *Lactobacillus*

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*delbrueckii* subsp. *bulgaricus*, *Lactobacillus fermentum*, *L. helveticus*, *L. kefiranofaciens* and *Streptococcus thermophilus*. However, in that study, differences in the LAB composition of the *Tarag* samples in relation to the animal species from which the milk was sourced were not observed [3].

Takeda et al. [10] reported a result similar to our previous result but with the slight difference that *L. delbrueckii* subsp. *lactis* and *L. fermentum* were the predominant species in *Airag* rather than *L. kefiranofaciens*.

To evaluate the features of Mongolian dairy products and their utility as probiotics, it is very important to conduct detailed analyses of their microbial diversity. To avoid the inherent disadvantages of culture methods, such as underestimation of the viable cell counts, culture-independent methods, such as clone libraries or denaturing gradient gel electrophoresis (DGGE), are often used. Although there have been a few studies [11, 12] on the bacterial diversity of *Airag* and *Tarag* using the DGGE approach, too few culture-independent approaches have been performed to clarify the microbial diversity of these targets. The 16S rRNA gene sequence-based pyrosequencing method enables a detailed, comprehensive and high-throughput analysis of microbial ecology, and this method has been applied to various traditional fermented food research studies [13–15]. In particular, the V1−V2 hypervariable region of the 16S rRNA gene has high frequencies of sequence variability and an outstanding ability to reproduce the full-length 16S rRNA gene-based taxonomic classification [16, 17].

In this study, we used the pyrosequencing method based on the sequence of the V1−V2 hypervariable region of the 16S rRNA gene to obtain a detailed analysis of the bacterial diversity of the 22 *Airag*, 5 *Khoormog* and 26 *Tarag* samples that we used in our previous study [3].

## MATERIALS AND METHODS

### *Sample collection*

All samples were collected from the Mongolian provinces of Arhangai, Bulgan, Dundgobi, Tov, Uburhangai and Umnugobi in July 2004 [3]. About 3 mL of samples were collected and stored at 4°C in a vehicle-mounted refrigerator. Subsequently, all of the samples were transported by air to the Yakult Central Institute for Microbiological Research, Tokyo, Japan, at below freezing and stored at –20°C until they were used for DNA extraction. We used 22 alcoholic beverages (*Airag*), 5 *Khoormog* samples and 26 *Tarag* samples made from milks of domestic animals. Although camel fermented milk samples were categorized as *Tarag* in our previous study, we recategorized them as *Khoormog* in this study in accordance with the proper Mongolian name [5], because raw camel milk was used for these samples (Table 1).

### *DNA extraction*

DNA was extracted as described previously [3]. Collected samples were centrifuged at 20,000 × *g* for 3 min. The pellet was suspended in 250 µL of extraction buffer (100 mM Tris–HCl, 40 mM EDTA, pH 9.0) and 500 µL of benzyl chloride; 0.7 g of glass beads (0.1 mm in diameter) were added to the suspension, and the mixture was shaken vigorously for 30 sec with a FastPrep FP120 (Qbiogene, Carlsbad, CA, USA) at a speed of 6.5 m/sec. Subsequently, 50 µL of 10% SDS was added to the suspension, which was then vortexed vigorously at 50°C for 20 min in a MicroIncubator M-36 (Taitec, Tokyo, Japan). The mixture was cooled on ice for 15 min after the addition of 150 µL of 3 M sodium acetate. After centrifugation of the mixture at 20,000 × *g* for 15 min, the supernatant was collected, and DNA was obtained by isopropanol precipitation. Finally, the DNA was diluted to 10 µg/ml with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at –80°C until use.

### *Pyrosequencing based on the V1–V2 hypervariable region of the 16S rRNA gene*

The PCR conditions were designed as previously described [14] with slight modifications. To amplify the V1–V2 hypervariable region, we used the universal primers 27F-mod (5′-AGRGTTTGATYMTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′) [18]. For the first PCR step, 25 µL of the reaction reagent contained 10 ng of extracted DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP, 5 pmol of each primer, and 0.625 U Ex Taq® HS (Takara Bio, Shiga, Japan). The PCR conditions were as follows: 98°C for 2.5 min; 15 cycles at 98°C for 15 sec, 50°C for 30 sec and 72°C for 20 sec; and finally 72°C for 5 min. Subsequently, 27F-mod with 53 kinds of barcode-sequence tag, which comprised 10 bp nucleotides, provided by Roche Diagnostics (Basel, Switzerland), and 338R were used for the second PCR. Each primer also had an additional adapter sequence on its 5′ end, which was required for the subsequent pyrosequencing reactions. For the second PCR step, 50 µL of the reaction reagent contained 1 µL of the first step product, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP, 5 pmol of each primer and 1.25 U Ex Taq® HS. The PCR conditions were as follows: 98°C for 2.5 min; 12 cycles at 98°C for 15 sec, 54°C for 30 sec and 72°C

Table 1. Properties, pyrosequencing data and alpha diversity scores of the samples used

| No. | Fermented milk | | Sampling location | | | Unfiltered sequence | Filter passed sequence | Chimeric sequence No. (Percentage) | Observed OTUs | Chao 1 | Good's coverage | PD whole tree | H' |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Type | Species origin | Province | City | Region | | | | | | | | |
| A01 | Airag | Mare | Arhangai | Khashaat | Forest Steppe | 3346 | 2635 | 1 (0.04) | 15 | 20 | 99.8 | 0.85 | 0.56 |
| A02 | Airag | Mare | Arhangai | Tsagaannuur | Forest Steppe | 3224 | 2460 | | 14 | 34 | 99.7 | 0.77 | 0.56 |
| A03 | Airag | Mare | Arhangai | Tsagaannuur | Forest Steppe | 3499 | 2756 | | 11 | 12 | 99.7 | 0.66 | 0.28 |
| A04 | Airag | Mare | Arhangai | Tsagaannuur | Forest Steppe | 3382 | 2718 | | 10 | 10 | 100 | 0.61 | 0.39 |
| A05 | Airag | Mare | Arhangai | Tsagaannuur | Forest Steppe | 3639 | 2909 | 2 (0.07) | 17 | 18 | 99.6 | 0.90 | 0.41 |
| A06 | Airag | Mare | Arhangai | Tsagaannuur | Forest Steppe | 3381 | 2678 | | 18 | 19 | 99.9 | 0.89 | 0.98 |
| A07 | Airag | Mare | Arhangai | Tsenkher | Forest Steppe | 3415 | 2729 | | 11 | 16 | 99.9 | 0.68 | 0.20 |
| A08 | Airag | Mare | Arhangai | Tsenkher | Forest Steppe | 3405 | 2680 | | 11 | 16 | 99.9 | 0.62 | 0.40 |
| A09 | Airag | Mare | Arhangai | Tsenkher | Forest Steppe | 3206 | 2575 | | 21 | 23 | 99.9 | 1.14 | 1.32 |
| A10 | Airag | Mare | Arhangai | Tsenkher | Forest Steppe | 3570 | 2828 | 1 (0.04) | 18 | 18 | 99.8 | 1.01 | 1.14 |
| A11 | Airag | Mare | Arhangai | Tsenkher | Forest Steppe | 3578 | 2883 | 1 (0.03) | 16 | 17 | 99.9 | 0.78 | 0.71 |
| A12 | Airag | Mare | Arhangai | Tsenkher | Forest Steppe | 3731 | 2905 | | 19 | 20 | 99.9 | 1.00 | 1.60 |
| A13 | Airag | Mare | Bulgan | Khishig-Ondor | Steppe | 3630 | 2952 | 1 (0.03) | 19 | 30 | 99.8 | 1.04 | 0.45 |
| A14 | Airag | Mare | Tuv | Bayan-Onjuul | Steppe | 3109 | 2471 | 1 (0.04) | 16 | 17 | 99.8 | 0.86 | 0.68 |
| A15 | Airag | Mare | Tuv | Erdenesant | Steppe | 3703 | 2992 | | 16 | 19 | 99.9 | 0.86 | 0.27 |
| A16 | Airag | Mare | Uburhangai | Bat-Olzii | Forest Steppe | 3584 | 2883 | | 10 | 10 | 99.9 | 0.46 | 0.54 |
| A17 | Airag | Mare | Uburhangai | Bat-Olzii | Forest Steppe | 3040 | 2347 | 1 (0.04) | 26 | 34 | 99.7 | 1.35 | 2.25 |
| A18 | Airag | Mare | Umnugobi | Dalanzadgad | Gobi Desert | 3490 | 2612 | 4 (0.15) | 30 | 40 | 99.9 | 1.19 | 1.79 |
| A19 | Airag | Mare | Umnugobi | Dalanzadgad | Gobi Desert | 3695 | 2796 | 2 (0.07) | 29 | 31 | 99.6 | 1.33 | 1.76 |
| A20 | Airag | Mare | Umnugobi | Dalanzadgad | Gobi Desert | 3535 | 2840 | | 17 | 18 | 99.9 | 0.81 | 0.74 |
| A21 | Airag | Mare | Umnugobi | Dalanzadgad | Gobi Desert | 3337 | 2613 | | 24 | 45 | 99.9 | 0.91 | 1.11 |
| A22 | Airag | Mare | Umnugobi | Hanhongor | Gobi Desert | 3406 | 2713 | | 14 | 16 | 99.9 | 0.72 | 0.62 |
| *Subtotal for Airag* | | | | | | *75905* | *59975* | *14 (0.02)* | *60 (17.4 ± 5.8)* | | | | |
| K01 | Khoormog | Camel | Dundgobi | Mandalgovi | Gobi Desert | 3250 | 2573 | 2 (0.08) | 16 | 18 | 100 | 0.95 | 0.81 |
| K02 | Khoormog | Camel | Dundgobi | Mandalgovi | Gobi Desert | 3115 | 2459 | | 19 | 20 | 99.7 | 0.78 | 1.18 |
| K03 | Khoormog | Camel | Umnugobi | Hanhongor | Gobi Desert | 3294 | 2545 | | 22 | 24 | 99.8 | 1.09 | 2.04 |
| K04 | Khoormog | Camel | Umnugobi | Hanhongor | Gobi Desert | 3190 | 2418 | 15 (0.62) | 20 | 26 | 99.6 | 0.91 | 1.49 |
| K05 | Khoormog | Camel | Umnugobi | Hanhongor | Gobi Desert | 3176 | 2522 | | 17 | 23 | 99.7 | 0.76 | 1.43 |
| *Subtotal for Khoormog* | | | | | | *16025* | *12517* | *17 (0.13)* | *39 (18.8 ± 2.4)* | | | | |
| T01 | Tarag | Cow | Arhangai | Khashaat | Forest Steppe | 4004 | 3087 | | 7 | 7 | 99.9 | 0.68 | 0.50 |
| T02 | Tarag | Cow | Bulgan | Dasinchilen | Steppe | 3830 | 3050 | | 9 | 9 | 99.9 | 0.63 | 0.82 |
| T03 | Tarag | Cow | Tuv | Erdenesant | Steppe | 4086 | 3267 | | 9 | 9 | 99.9 | 0.64 | 0.78 |
| T04 | Tarag | Cow | Tuv | Erdenesant | Steppe | 3447 | 2709 | | 10 | 10 | 99.9 | 0.62 | 1.80 |
| T05 | Tarag | Cow | Tuv | Erdenesant | Steppe | 4096 | 3203 | 1 (0.03) | 14 | 14 | 99.9 | 0.78 | 1.07 |
| T06 | Tarag | Cow | Tuv | Erdenesant | Steppe | 3562 | 2833 | 1 (0.04) | 10 | 11 | 99.9 | 0.69 | 1.10 |
| T07 | Tarag | Cow | Tuv | Erdenesant | Steppe | 3584 | 2861 | | 10 | 11 | 99.8 | 0.70 | 1.35 |
| T08 | Tarag | Goat | Umnugobi | Dalanzadgad | Gobi Desert | 3582 | 2865 | | 14 | 21 | 99.8 | 0.76 | 0.56 |
| T09 | Tarag | Goat | Umnugobi | Dalanzadgad | Gobi Desert | 4316 | 3420 | | 9 | 10 | 100 | 0.56 | 0.48 |
| T10 | Tarag | Goat | Umnugobi | Dalanzadgad | Gobi Desert | 4256 | 3386 | | 6 | 6 | 100 | 0.40 | 0.68 |
| T11 | Tarag | Goat | Umnugobi | Dalanzadgad | Gobi Desert | 4204 | 3305 | 1 (0.03) | 9 | 9 | 99.9 | 0.59 | 0.83 |
| T12 | Tarag | Goat | Umnugobi | Dalanzadgad | Gobi Desert | 3494 | 2754 | 2 (0.07) | 25 | 27 | 100 | 1.14 | 1.35 |
| T13 | Tarag | Goat | Umnugobi | Dalanzadgad | Gobi Desert | 3450 | 2716 | | 13 | 15 | 99.7 | 0.77 | 0.77 |
| T14 | Tarag | Yak | Arhangai | Khashaat | Forest Steppe | 4054 | 3214 | 2 (0.06) | 11 | 13 | 99.9 | 0.68 | 1.17 |
| T15 | Tarag | Yak | Arhangai | Tsagaannuur | Forest Steppe | 4007 | 3247 | 1 (0.03) | 6 | 6 | 100 | 0.37 | 0.75 |
| T16 | Tarag | Yak | Arhangai | Tsagaannuur | Forest Steppe | 3820 | 3021 | | 8 | 9 | 99.9 | 0.49 | 0.89 |
| T17 | Tarag | Yak | Arhangai | Tsagaannuur | Forest Steppe | 4043 | 3230 | 2 (0.06) | 7 | 7 | 99.9 | 0.44 | 0.61 |
| T18 | Tarag | Yak | Arhangai | Tsagaannuur | Forest Steppe | 3770 | 3021 | | 7 | 7 | 100 | 0.49 | 1.05 |
| T19 | Tarag | Yak | Arhangai | Tsagaannuur | Forest Steppe | 4120 | 3288 | 1 (0.03) | 11 | 12 | 99.9 | 0.57 | 0.77 |
| T20 | Tarag | Yak | Arhangai | Tsenkher | Forest Steppe | 4326 | 3493 | 2 (0.06) | 7 | 7 | 100 | 0.48 | 0.91 |
| T21 | Tarag | Yak | Arhangai | Tsenkher | Forest Steppe | 4326 | 3471 | 1 (0.03) | 8 | 9 | 100 | 0.51 | 0.90 |
| T22 | Tarag | Yak | Arhangai | Tsenkher | Forest Steppe | 4251 | 3403 | 1 (0.03) | 7 | 7 | 100 | 0.48 | 0.60 |
| T23 | Tarag | Yak | Arhangai | Tsenkher | Forest Steppe | 4164 | 3257 | 1 (0.03) | 10 | 14 | 100 | 0.58 | 0.90 |
| T24 | Tarag | Yak | Arhangai | Tsenkher | Forest Steppe | 3105 | 2439 | | 10 | 10 | 100 | 0.76 | 1.00 |
| T25 | Tarag | Yak | Uburhangai | Bat-Olzii | Forest Steppe | 3772 | 2981 | | 10 | 10 | 100 | 0.61 | 0.79 |
| T26 | Tarag | Yak | Uburhangai | Bat-Olzii | Forest Steppe | 3289 | 2572 | | 12 | 15 | 99.9 | 0.67 | 2.00 |
| *Subtotal for Tarag* | | | | | | *100958* | *80093* | *16 (0.01)* | *45 (10.0 ± 3.8)* | | | | |
| **Total** | | | | | | **192888** | **152585** | **47 (0.03)** | **81 (13.9 ± 5.6)** | | | | |

for 20 sec; and finally 72°C for 5 min. The PCR products were purified by using an AMPure® XP Kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol. The purified products were quantified with a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). All samples were adjusted to $10^9$ molecules dsDNA/μL with TE buffer, and equal volumes were mixed. The mixed samples were then purified and quantified again with a MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and Quant-iT™ PicoGreen® dsDNA Assay Kit. The amplicon mixture was applied to a Genome Sequencer GS Junior Bench Top System (Roche Diagnostics).

*Pyrosequencing data processing*

Sequence data processing was performed by using the QIIME software package v1.6.0 [19]. Briefly, sequences were filtered according to the following limiting quality-check parameters: a minimum quality score of 25, a maximum forward primer mismatch of 0, a maximum reverse primer mismatch of 2, a minimum read length of 300 bp, a maximum of 6 homopolymers and a maximum number of ambiguous bases of 0. A batch of sequence reads was then sorted to each sample according to the barcode-sequence tag. The USEARCH algorithm [20] was used for sequence clustering to operational taxonomic units (OTUs) at the 97% similarity level and for chimera checks. Chimera analyses were performed by using de novo and reference-based chimera checks against the pre-built 16S rRNA reference database provided in the Microbiome Utilities (http://microbiomeutil.sourceforge.net). Then sequences that were determined to be chimeric were removed. The filtered and chimera-removed sequence reads were then used to calculate alpha diversity scores, Chao 1 indices [21], the phylogenetic diversity (PD) whole tree [22], Shannon indices (H′) [23] and Good's coverage [24], which were in turn used for the subsequent analyses of bacterial diversities.

*Analyses of bacterial diversities*

Reference sequences for each OTU were classified at the phylum, family, genus and species level by using DNASIS® Taxon (Hitachi Solutions, Tokyo, Japan) against the bacterial 16S rRNA gene sequences of the Ribosomal Database Project database (Release 10, Update 31). At the species level, OTUs were assigned to the species names that showed the highest similarity score with a threshold of 97%, and OTUs that did not show over 97% similarity against any known species were described as unknown species (e.g., *Lactobacillus* sp. A, *Lactobacillus* sp. B, *Lactococcus* sp.). Any OTUs whose relative abundances were below 0.1% in each sample were placed in the "low abundance" cluster. For OTUs that were not assigned to a known species, phylogenetic trees were drawn with their closest related known species. Multiple alignment and construction of phylogenetic trees were performed with ClustalX ver. 2.0.12. [25]. Approximately 300 bp of the 16S rRNA gene was used to construct the phylogenetic trees by using the neighbor-joining method [26]. The statistical reliability of trees was evaluated by bootstrap analysis of 1000 replicates [27]. Bacterial diversities were analyzed based on the relative abundance of OTUs at the species level by using principal component analysis (PCA) in the BioNumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

*Statistical analyses*

Tukey's test and the $\chi^2$ test were used to compare average scores and detection rates, respectively.

## RESULTS

*Pyrosequencing data*

The V1−V2 hypervariable region of the 16S rRNA gene was amplified from 22 *Airag*, 5 *Khoormog* and 26 *Tarag* samples, and a total of 192,888 sequence reads were obtained. The read number for each sample varied from 3040 to 4326, with the average being 3639. A total of 152,585 sequence reads passed the quality check and barcode-sequence tag sorting; 47 sequence reads (0.03% of filtered sequence reads) were detected as chimeras and were therefore removed. The sequence reads clustered into 81 OTUs, with an average of 13.9 ± 5.6 per sample. For most samples, the numbers of observed OTUs were close to the Chao 1 indices. In addition, Good's coverage at the 97% similarity level for the samples, which provides an estimate of sampling completeness, was above 99.6%, with the average being 99.9% ± 0.1% (Table 1). These results indicate that the majority of bacterial phylotypes could be identified.

*Alpha diversity scores*

The average alpha diversity scores, observed OTUs, Chao 1 indices and PD whole trees were significantly higher ($p<0.05$) in the *Airag* and *Khoormog* samples than in the *Tarag* samples, whereas no significant differences were observed between the *Airag* and *Khoormog* samples. Moreover, the average Shannon index score was not significantly different between the *Airag*, *Khoormog* and *Tarag* samples (Table 2). Among the *Tarag* samples, we observed no differences between the alpha diversity

Table 2. Comparison of average alpha diversity scores

| | Observed OTUs | Chao 1 | PD whole tree | H’ |
|---|---|---|---|---|
| *Airag* (n = 22) | 17.4 ± 5.8[a] | 21.9 ± 9.5[a] | 0.9 ± 0.2[a] | 0.9 ± 0.6 |
| *Khoormog* (n = 5) | 18.8 ± 2.4[a] | 22.1 ± 3.2[a] | 0.9 ± 0.1[a] | 1.4 ± 0.5 |
| *Tarag* (n = 26) | 10.0 ± 3.8[b] | 11.0 ± 4.8[b] | 0.6 ± 0.2[b] | 0.9 ± 0.4 |

a,b Different letters indicate statistically significant differences at p<0.05.

scores of any of the combinations of animal species of milk.

*Bacterial diversity*

After we had filtered out the OTUs with low relative abundance, we detected a total of 15 families, 21 genera and 41 species in 3 phyla (*Actinobacteria, Firmicutes* and *Proteobacteria*) in the *Airag*, *Khoormog* and *Tarag* samples. At the family level, the following 15 families were observed: *Acetobacteraceae*, *Aeromonadaceae*, *Bacillaceae*, *Bifidobacteriaceae, Caulobacteraceae, Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, Micrococcaceae, Moraxellaceae, Planococcaceae, Staphylococcaceae* and *Streptococcaceae*. Among these families, *Lactobacillaceae* (which comprises only genus *Lactobacillus*) predominated in all samples at a rate of 68.7%–99.6%. *Streptococcaceae*, which comprises 2 genera, *Lactococcus* and *Streptococcus,* was the second most common family (with 10%–29.6% in 7 samples and 1.1%–9.5% in 27 samples, respectively). The relative abundance (p<0.05) and detection rate (p<0.01) of *Streptococcus* in *Tarag* were significantly higher than those in *Airag* samples, whereas the relative abundance and detection rate of *Lactococcus* in *Airag* were significantly higher (p<0.05) than those in *Tarag*. *Acetobacteraceae*, which comprises the genus *Acetobacter,* was detected in 13 *Airag*, 5 *Khoormog* and 5 *Tarag* samples; the detection rates of *Acetobacter* in *Airag* (p=0.05) and *Khoormog* (p<0.01) were markedly higher than that in *Tarag*. *Caulobacteraceae* (which comprises genus *Brevundimonas*) was detected in almost all of the samples (22 *Airag*, 5 *Khoormog* and 22 *Tarag*) at a low abundance (0.1%–2.7%). *Leuconostocaceae* (which comprises genus *Leuconostoc*) was detected in only 8 *Airag* and 2 *Khoormog* samples. *Micrococcaceae,* which comprises the 3 genera *Arthrobacter*, *Citricoccus* and *Kocuria,* was detected in 17 *Airag*, 3 *Khoormog* and 10 *Tarag* samples; the detection rate of *Arthrobacter* in *Airag* (16 samples) was significantly higher (p<0.05) than that in *Tarag*. *Staphylococcaceae* (which comprises genus *Macrococcus*) was detected in only *Airag* (7 samples) and *Khoormog* (2 samples). *Aeromonadaceae*, *Bacillaceae*, *Bifidobacteriaceae, Clostridiaceae, Enterobacteriaceae, Enterococcaceae, Moraxellaceae* and *Planococcaceae* were detected in a few samples as minor microbial components (Table 3). Thus, at the family to genus level, significant diversity was not observed between the *Airag* and *Khoormog* samples.

At the species level, a total of 41 OTUs were detected in all of the samples, and 5 genera, *Acetobacter*, *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Streptococcus,* were classified into 4, 2, 13, 3 and 3 OTUs, respectively. *Acetobacter pasteurianus* was the predominant species in the genus *Acetobacter*, with a significantly higher detection rate (p<0.01) in *Airag* (11 samples) and *Khoormog* (5 samples) than in *Tarag* samples. The 13 OTUs in the genus *Lactobacillus* were assigned to 11 known species and 2 unknown species. In the 22 *Airag* samples, *L. helveticus* was predominant (at an average relative abundance of 85.9% across the 22 samples), and *L. kefiranofaciens* (2.4%, n = 20), *Lactobacillus kefiri* (1.2%, n = 21), *Lactobacillus parakefiri* (2.1%, n = 20) and *Lactobacillus diolivorans* (0.6%, n = 17) were dominant. In the 5 *Khoormog* samples, *L. kefiranofaciens* predominated (at an average relative abundance of 62.0%); *L. helveticus* (25.0%) and *L. kefiri* (4.5%) were dominant and detected in all 5 samples. Among the 26 *Tarag* samples, *L. delbrueckii* was the predominant species (74.6%, n = 24), whereas *L. helveticus* (19.2%, n = 24) and *L. fermentum* (2.7%, n = 11) were dominant. In the genus *Lactococcus* (*Lc.*), *Lactococcus lactis* was detected as the predominant species in *Arag* (1.3%, n = 13) and *Khoormog* (4.0%, n = 5), respectively, whereas *S. thermophilus* was predominant in *Tarag* samples (8.3%, n = 20).

Four OTUs showed less than 97% sequence similarity to any recognized species. These OTUs were assigned to *Citricoccus* sp., *Lactobacillus* sp. A, *Lactobacillus* sp. B and *Lactococcus* sp., respectively, because they showed similarity values of 96.4%, 92.2%, 93.1% and 94.8% with the type strains of their most closely related species—*Citricoccus zhacaiensis* FS24$^T$ (EU305672), *Lactobacillus kalixensis* Kx127A2$^T$ (AY253657), *Lactobacillus kitasatonis* JCM 1039$^T$ (AB107638) and *Lactococcus chungangensis* CAU 28$^T$ (EF694028),

Table 3. Bacterial diversity of *Airag*, *Khoormog* and *Tarag* at the phylum to genus level

| Taxon | A01 | A02 | A03 | A04 | A05 | A06 | A07 | A08 | A09 | A10 | A11 | A12 | A13 | A14 | A15 | A16 | A17 | A18 | A19 | A20 | A21 | A22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mare* (*Airag*) | | | | | | | | | | | | | | | | | | | | | |
| **Actinobacteria** | | | | | | | | | | | | | | | | | | | | | | |
| *Bifidobacteriaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Bifidobacterium* | | | | | | | | | | | | | | | | | 0.2 | | | | | |
| *Micrococcaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Arthrobacter* | 0.2 | | 0.2 | | 0.3 | 0.2 | 0.2 | 0.1 | | 0.2 | 0.1 | 0.2 | | 0.3 | 0.2 | 0.2 | 0.7 | 0.1 | | | 0.3 | 0.4 |
| *Citricoccus* | | | | | | | | | | | | | 0.1 | | | | | | | | | |
| *Kocuria* | | | | | | | | | | | | | | | | | | 0.3 | | | | |
| **Firmicutes** | | | | | | | | | | | | | | | | | | | | | | |
| *Bacillaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Anoxybacillus* | | | | | | | | | | | | | | | | | | | | | | |
| *Bacillus* | | | | | | | | | | | | | | | | | | | | | | |
| *Enterococcaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Enterococcus* | | | | | | | | | 0.1 | | | | | | | | | 0.3 | | | 0.2 | |
| *Lactobacillaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Lactobacillus* | 99.1 | 98.4 | 99.2 | 99.6 | 97.8 | 98.2 | 99.0 | 99.4 | 96.8 | 91.7 | 99.1 | 91.4 | 97.8 | 95.5 | 98.6 | 99.1 | 88.9 | 76.8 | 70.7 | 96.7 | 89.7 | 96.6 |
| *Leuconostocaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Leuconostoc* | | 1.1 | | | | | | | | | | 0.2 | 0.2 | | | | | 3.1 | 1.4 | 0.3 | 0.1 | 0.2 |
| *Staphylococcaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Macrococcus* | | | | | | 0.2 | | | 0.5 | 0.3 | | 0.1 | 0.3 | | 0.3 | | | | 0.3 | | | |
| *Streptococcaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Lactococcus* | | | | | 0.4 | 0.8 | | | 1.5 | 0.2 | 0.2 | 7.3 | 1.1 | 0.6 | | | 7.5 | 17.3 | 24.4 | 2.1 | 5.8 | 2.2 |
| *Streptococcus* | | | | 0.1 | 0.3 | | | | | 6.9 | 0.2 | | | 2.3 | | | | 0.1 | 0.3 | | | |
| **Proteobacteria** | | | | | | | | | | | | | | | | | | | | | | |
| *Acetobacteraceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Acetobacter* | 0.2 | | 0.1 | | 0.2 | 0.1 | | | 0.2 | 0.1 | | | | 0.4 | 0.2 | | | 1.3 | 2.1 | 0.3 | 3.0 | |
| *Komagataeibacter* | | | | | | | | | | | | | | | | | | | | | 0.2 | |
| *Aeromonadaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Aeromonas* | | | | | | | | | | | | | | | | | | | | | | |
| *Caulobacteraceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Brevundimonas* | 0.5 | 0.3 | 0.3 | 0.3 | 0.7 | 0.3 | 0.7 | 0.3 | 0.5 | 0.3 | 0.3 | 0.7 | 0.3 | 0.7 | 0.5 | 0.7 | 1.9 | 0.3 | 0.3 | 0.4 | 0.7 | 0.4 |
| *Clostridiaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Clostridium* | | | | | | | | | | | | | | | | | | | | | | |
| *Enterobacteriaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Shigella* | | | | | | | | | | | | | | | | | | | 0.2 | | | |
| *Yokenella* | | | | | | | | | | | | | | | | | | | | | | |
| *Moraxellaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Acinetobacter* | | | | | | | | | | | | | | | 0.1 | | | | | | | |
| *Enhydrobacter* | | | | | | | | | | 0.1 | | 0.1 | | | | | 0.3 | | | | | |
| *Planococcaceae* | | | | | | | | | | | | | | | | | | | | | | |
| *Kurthia* | | | | | | | | | | | | | | | | | | | | | | |
| Low No. | 0.2 | 0.2 | 0.1 | | 0.2 | 0.1 | 0.1 | 0.1 | 0.4 | 0.1 | 0.1 | | 0.2 | 0.2 | 0.1 | | 0.4 | 0.3 | 0.4 | 0.2 | | 0.1 |

| Taxon | K01 | K02 | K03 | K04 | K05 | T01 | T02 | T03 | T04 | T05 | T06 | T07 | T08 | T09 | T10 | T11 | T12 | T13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Camel* (*Khoormog*) | | | | | Cow* (*Tarag*) | | | | | | | Goat* (*Tarag*) | | | | | |
| **Actinobacteria** | | | | | | | | | | | | | | | | | | |
| *Bifidobacteriaceae* | | | | | | | | | | | | | | | | | | |
| *Bifidobacterium* | | | | | | | | | | | | | | | | | | |
| *Micrococcaceae* | | | | | | | | | | | | | | | | | | |
| *Arthrobacter* | 0.2 | | 0.1 | | 0.2 | | | | 0.6 | 0.2 | 0.7 | 1.4 | | | 0.2 | | 0.1 | 0.1 |
| *Citricoccus* | | | | | | | | | | | | | | | | | | |
| *Kocuria* | | | | | | | | | | | | | | | | | | |
| **Firmicutes** | | | | | | | | | | | | | | | | | | |
| *Bacillaceae* | | | | | | | | | | | | | | | | | | |
| *Anoxybacillus* | | | | | | | | | | | | | | | | | | 1.4 |
| *Bacillus* | | | | 0.4 | | | | | | | | | | | | | | |
| *Enterococcaceae* | | | | | | | | | | | | | | | | | | |
| *Enterococcus* | | | | | | | | | | | | | | | | | | |
| *Lactobacillaceae* | | | | | | | | | | | | | | | | | | |
| *Lactobacillus* | 94.5 | 92.0 | 85.8 | 91.6 | 94.4 | 99.0 | 91.5 | 94.2 | 68.7 | 91.3 | 96.9 | 95.7 | 99.3 | 97.2 | 99.6 | 80.4 | 96.6 | 96.5 |
| *Leuconostocaceae* | | | | | | | | | | | | | | | | | | |
| *Leuconostoc* | | 0.6 | | 0.1 | | | | | | | | | | | | | | |
| *Staphylococcaceae* | | | | | | | | | | | | | | | | | | |
| *Macrococcus* | | 0.1 | 0.2 | | | | | | | | | | | | | | | |
| *Streptococcaceae* | | | | | | | | | | | | | | | | | | |
| *Lactococcus* | 1.2 | 4.8 | 5.3 | 4.3 | 4.5 | | 0.2 | | | | | | | | | | 1.2 | 0.7 |
| *Streptococcus* | | | | 2.1 | | 0.4 | 8.0 | 4.8 | 29.6 | 7.8 | | | | 2.4 | | 19.2 | 0.5 | 0.3 |
| **Proteobacteria** | | | | | | | | | | | | | | | | | | |
| *Acetobacteraceae* | | | | | | | | | | | | | | | | | | |
| *Acetobacter* | 3.2 | 2.0 | 6.2 | 1.1 | 0.6 | | | 0.4 | | 0.1 | | | 0.3 | | | | 0.5 | |
| *Komagataeibacter* | | | | | | | | | | | | | | | | | | |
| *Aeromonadaceae* | | | | | | | | | | | | | | | | | | |
| *Aeromonas* | | | 1.1 | | | | | | | | | | | | | | | |
| *Caulobacteraceae* | | | | | | | | | | | | | | | | | | |
| *Brevundimonas* | 0.6 | 0.3 | 0.3 | 0.2 | 0.2 | 0.5 | 0.2 | 0.6 | 1.1 | 0.3 | 2.2 | 2.7 | 0.2 | 0.4 | 0.2 | 0.3 | 0.5 | 0.3 |
| *Clostridiaceae* | | | | | | | | | | | | | | | | | | |
| *Clostridium* | | | | | | | | | | | | | | | | | | 0.7 |
| *Enterobacteriaceae* | | | | | | | | | | | | | | | | | | |
| *Shigella* | | | 0.5 | | | | | | | | | | | | | | | |
| *Yokenella* | | | 0.3 | | | | | | | | | | | | | | | |
| *Moraxellaceae* | | | | | | | | | | | | | | | | | | |
| *Acinetobacter* | | | | | | | | | | | | | | | | | | |
| *Enhydrobacter* | | | | | | | | | | | | | | | | | | |
| *Planococcaceae* | | | | | | | | | | | | | | | | | | |
| *Kurthia* | | | | | | | | | | | | | | | | | 0.1 | |
| Low No. | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | | 0.2 | | | 0.1 | 0.2 | 0.2 | 0.2 | | | 0.2 | 0.4 | |

| Taxon | T14 | T15 | T16 | T17 | T18 | T19 | T20 | T21 | T22 | T23 | T24 | T25 | T26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Yak* (*Tarag*) | | | | | | | | | | | | |
| **Actinobacteria** | | | | | | | | | | | | | |
| *Bifidobacteriaceae* | | | | | | | | | | | | | |
| *Bifidobacterium* | | | | | | | | | | | | | |
| *Micrococcaceae* | | | | | | | | | | | | | |
| *Arthrobacter* | | | | | | 0.1 | | | | | | 0.2 | 0.4 |
| *Citricoccus* | | | | | | | | | | | | | |
| *Kocuria* | | | | | | | | | | | | | |
| **Firmicutes** | | | | | | | | | | | | | |
| *Bacillaceae* | | | | | | | | | | | | | |
| *Anoxybacillus* | | | | | | | | | | | | | |
| *Bacillus* | | | | | | | | | | | | | |
| *Enterococcaceae* | | | | | | | | | | | | | |
| *Enterococcus* | | | | | | | | | | | | | |
| *Lactobacillaceae* | | | | | | | | | | | | | |
| *Lactobacillus* | 90.1 | 98.3 | 95.2 | 98.1 | 99.1 | 86.2 | 95.2 | 94.0 | 92.8 | 80.6 | 99.1 | 92.4 | 75.7 |
| *Leuconostocaceae* | | | | | | | | | | | | | |
| *Leuconostoc* | | | | | | | | | | | | | |
| *Staphylococcaceae* | | | | | | | | | | | | | |
| *Macrococcus* | | | | | | | | | | | | | |
| *Streptococcaceae* | | | | | | | | | | | | | |
| *Lactococcus* | | | | | | | | | | | 0.2 | | |
| *Streptococcus* | 9.5 | 1.6 | 4.6 | 1.8 | | 13.3 | 4.6 | 5.8 | 7.1 | 18.9 | 0.2 | 3.7 | 22.7 |
| **Proteobacteria** | | | | | | | | | | | | | |
| *Acetobacteraceae* | | | | | | | | | | | | | |
| *Acetobacter* | | | | | | 0.2 | | | | | 0.3 | 3.1 | |
| *Komagataeibacter* | | | | | | | | | | | | | |
| *Aeromonadaceae* | | | | | | | | | | | | | |
| *Aeromonas* | | | | | | | | | | | | | |
| *Caulobacteraceae* | | | | | | | | | | | | | |
| *Brevundimonas* | 0.2 | | 0.1 | | 0.7 | | 0.2 | 0.1 | 0.1 | 0.3 | | 0.6 | 1.1 |
| *Clostridiaceae* | | | | | | | | | | | | | |
| *Clostridium* | | | | | | | | | | | | | |
| *Enterobacteriaceae* | | | | | | | | | | | | | |
| *Shigella* | | | | | | | | | | | | | |
| *Yokenella* | | | | | | | | | | | | | |
| *Moraxellaceae* | | | | | | | | | | | | | |
| *Acinetobacter* | | | | | | | | | | | | | |
| *Enhydrobacter* | | | | | | | | | | | | | |
| *Planococcaceae* | | | | | | | | | | | | | |
| *Kurthia* | | | | | | | | | | | | | |
| Low No. | 0.2 | | | | 0.2 | 0.1 | | | | 0.1 | 0.2 | | 0.1 |

0.1–1 % | 1–10 % | 10–50 % | 50–100 %

* Animal species from which the milk was sourced

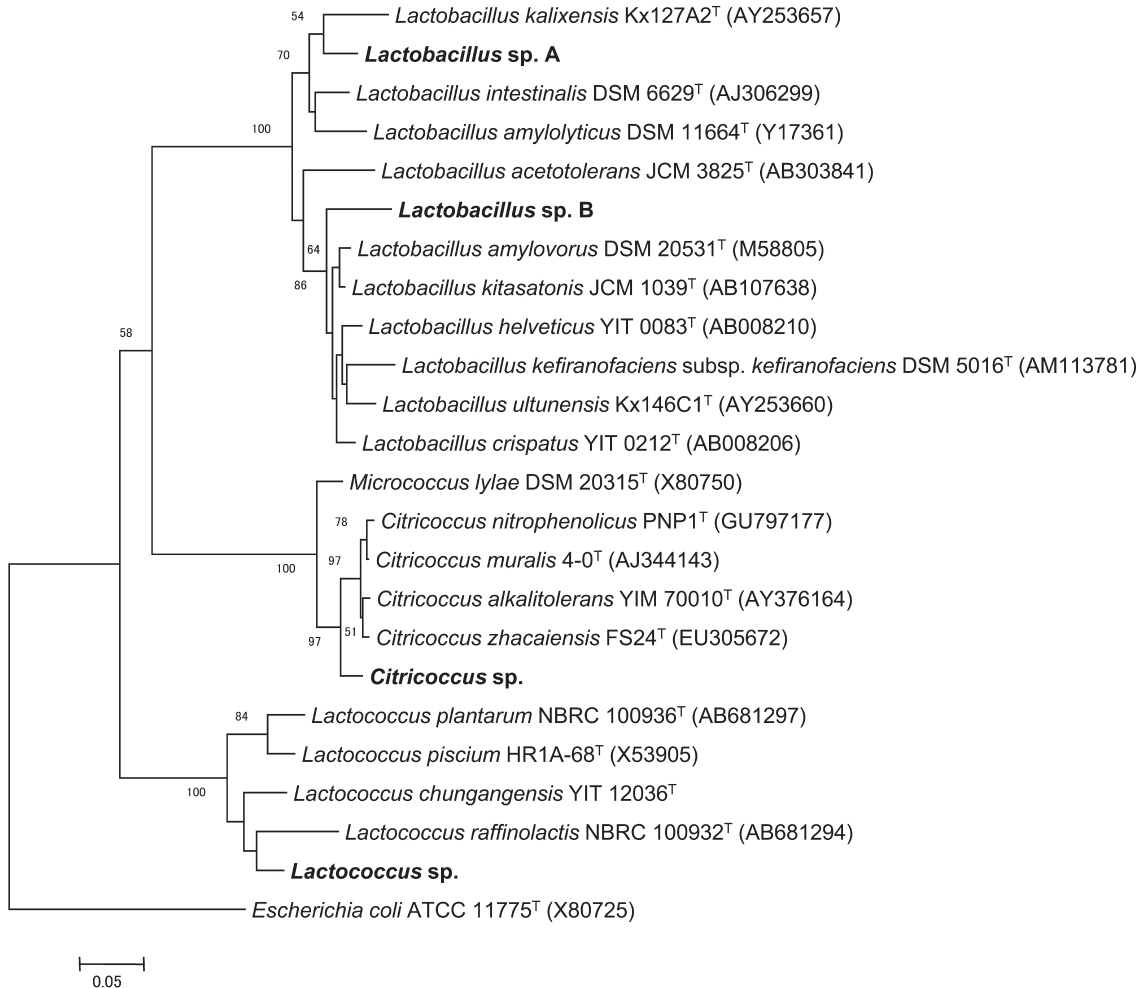


Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the taxonomic positions of the OTUs that showed less than 97% sequence similarities with their closest related species. The tree was constructed by using the neighbor-joining method on the basis of a comparison of approximately 300 bp; *Escherichia coli* ATCC 11775$^T$ served as an out-group. Bootstrap values (%) based on 1000 replicates are given at the nodes. Bar, 5% sequence divergence.

respectively (Fig. 1). Three of these four OTUs (the exception being *Lactobacillus* sp. B) were detected only in *Airag* samples.

The OTUs that were not assigned to LAB species, such as *Arthrobacter russicus, Bifidobacterium mongoliense*, *Brevundimonas nasdae*, *Clostridium perfringens*, *Enhydrobacter aerosaccus*, *Enterococcus durans*, *Enterococcus italicus*, *Macrococcus caseolyticus* and *Streptococcus parauberis*, which are regarded as environmental-origin microbes, were detected in only a few samples and at low levels, with the exception of *A. russicus* and *B. nasdae*, which were detected in 14 *Airag*, 1 *Khoormog* and 9 *Tarag* samples and in 22 *Airag*, 5 *Khoormog* and 22 *Tarag* samples, respectively (Table 4).

*Correlation between bacterial diversity and sample properties*

The bacterial species diversity of each sample was analyzed based on species relative abundance, by using PCA, and compared with the differences among the animal species (mare, camel, cow, goat or yak) from which the milk was sourced and with the geographic differences of *Airag*, *Khoormog* and *Tarag* in 3 regions: the Gobi Desert region (provinces of Dundgobi and Umnugobi), the forest-steppe region (provinces of Arhangai and Uburhangai) and the steppe region (provinces of Bulgan and Tov) (Fig. 2). The relative abundance of *L. delbrueckii*, *L. helveticus* and *L. kefiranofaciens* had significant loadings to determine the sample plot location; relatively high loading was also

Table 4. Bacterial diversity of *Airag*, *Khoormog* and *Tarag* at the species level

| Taxon | A01 | A02 | A03 | A04 | A05 | A06 | A07 | A08 | A09 | A10 | A11 | A12 | A13 | A14 | A15 | A16 | A17 | A18 | A19 | A20 | A21 | A22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mare* (*Airag*) | | | | | | | | | | | | | | | | | | | | | |
| *Acetobacter fabarum* | | | | | | | | | | | | | | | | | | | | | | |
| *Acetobacter ghanensis* | | | | | | | | | | | | | | | | | | | 0.2 | | 0.2 | |
| *Acetobacter malorum* | | | | | | | | | 0.2 | | | | | 0.2 | | | | 0.6 | 1.2 | 0.1 | | |
| *Acetobacter pasteurianus* | 0.2 | | 0.1 | | 0.2 | 0.1 | | | | 0.1 | | | | 0.2 | 0.2 | | | 0.6 | 0.6 | 0.2 | 2.8 | |
| *Aeromonas media* | | | | | | | | | | | | | | | | | | | | | | |
| *Anoxybacillus contaminans* | | | | | | | | | | | | | | | | | | | | | | |
| *Arthrobacter russicus* | 0.1 | | 0.2 | | 0.3 | 0.1 | 0.1 | 0.1 | | 0.2 | | 0.2 | | 0.3 | 0.1 | 0.2 | 0.7 | | | | 0.2 | 0.4 |
| *Bacillus cereus* | | | | | | | | | | | | | | | | | | | | | | |
| *Bifidobacterium mongoliense* | | | | | | | | | | | | | | | | | 0.2 | | | | | |
| *Brevundimonas nasdae* | 0.5 | 0.3 | 0.3 | 0.3 | 0.7 | 0.3 | 0.7 | 0.3 | 0.5 | 0.3 | 0.3 | 0.7 | 0.3 | 0.7 | 0.5 | 0.7 | 1.9 | 0.3 | 0.3 | 0.4 | 0.7 | 0.4 |
| *Citricoccus* sp. | | | | | | | | | | | | | 0.1 | | | | | | | | | |
| *Clostridium perfringens* | | | | | | | | | | | | | | | | | | | | | | |
| *Enhydrobacter aerosaccus* | | | | | | | | | | 0.1 | | 0.1 | | | | | 0.3 | | | | | |
| *Enterococcus durans* | | | | | | | | | 0.1 | | | | | | | | | 0.2 | | | 0.2 | |
| *Enterococcus italicus* | | | | | | | | | | | | | | | | | | 0.1 | | | | |
| *Kocuria rhizophila* | | | | | | | | | | | | | | | | | | 0.3 | | | | |
| *Komagataeibacter europaeus* | | | | | | | | | | | | | | | | | | | | | 0.2 | |
| *Kurthia gibsonii* | | | | | | | | | | | | | | | | | | | | | | |
| *Lactobacillus crustorum* | | | | | | | | | | | | | | | | | | | 0.1 | | | |
| *Lactobacillus delbrueckii* | | | | | | | | | | | 1.4 | | | | | | | | 0.1 | | | |
| *Lactobacillus diolivorans* | 0.6 | 1.1 | 0.6 | 1.3 | 0.5 | 1.3 | 0.2 | 0.4 | 1.1 | 0.1 | 0.5 | 0.5 | 0.8 | | 0.4 | 0.3 | 0.6 | | | | | 0.2 |
| *Lactobacillus fermentum* | | | | | | | | | | | | | | | | | | | | | | |
| *Lactobacillus helveticus* | 92.9 | 92.9 | 97.0 | 95.2 | 95.6 | 83.8 | 97.9 | 94.9 | 76.2 | 81.8 | 90.7 | 72.8 | 95.0 | 90.8 | 97.3 | 92.5 | 52.2 | 65.7 | 61.2 | 88.3 | 83.1 | 91.9 |
| *Lactobacillus hilgardii* | | | | | | | | | | | | 0.1 | | | | | 0.1 | | | | | |
| *Lactobacillus kefiranofaciens* | 2.1 | 1.1 | 0.3 | 0.6 | 0.2 | 0.1 | 0.3 | 2.6 | 0.3 | 0.4 | 0.5 | 4.5 | | 4.0 | | 3.4 | 2.1 | 5.1 | 7.1 | 7.6 | 4.2 | 2.5 |
| *Lactobacillus kefiri* | 0.6 | | 0.6 | 0.3 | 0.4 | 0.8 | 0.2 | 0.5 | 2.2 | 0.9 | 1.4 | 1.9 | 0.6 | 0.6 | 0.3 | 0.6 | 2.3 | 5.2 | 1.5 | 0.4 | 1.6 | 1.7 |
| *Lactobacillus paracasei* | | | | | | | | | 0.1 | 0.2 | | | | | | | | 0.5 | 0.3 | 0.2 | 0.4 | |
| *Lactobacillus parakefiri* | 1.5 | 2.6 | 0.7 | 2.2 | 0.9 | 2.4 | 0.4 | 1.0 | 2.9 | 1.3 | 2.1 | 3.2 | 1.5 | 0.2 | 0.6 | 2.1 | 15.0 | | 0.2 | | 0.2 | 0.3 |
| *Lactobacillus pentosus* | | | | | | | | | | | | | | | | | | 0.3 | 0.1 | | | |
| *Lactobacillus* sp. A | 1.3 | 0.7 | | | 0.2 | 9.8 | | | 13.8 | 6.8 | 2.6 | 8.3 | | | | 0.1 | 16.5 | | | | | |
| *Lactobacillus* sp. B | | | | | | | | | | | | | | | | | | | | | | |
| *Lactococcus lactis* | | | | | | 0.7 | | | 1.4 | 0.2 | 0.1 | 1.1 | 0.3 | 0.2 | | | 0.3 | 17.2 | 24.3 | 2.1 | 5.7 | 2.2 |
| *Lactococcus raffinolactis* | | | | | 0.3 | | | | | | 0.1 | 6.1 | 0.8 | 0.4 | | | 6.1 | | | | | |
| *Lactococcus* sp. | | | | | | | | | | | | | | | | | 1.2 | | | | | |
| *Leuconostoc mesenteroides* | | 1.1 | | | | | | | | | | 0.2 | 0.1 | | | | | 3.1 | 1.4 | 0.2 | 0.1 | 0.2 |
| *Macrococcus caseolyticus* | | | | | | 0.2 | | | 0.5 | 0.3 | | 0.1 | 0.3 | | 0.3 | | | | 0.3 | | | |
| *Shigella flexneri* | | | | | | | | | | | | | | | | | | | 0.2 | | | |
| *Streptococcus gallolyticus* | | | | | | | | | | | | | | | | | | | | | | |
| *Streptococcus parauberis* | | | | 0.1 | 0.3 | | | | | | | | | | | | | | | | | |
| *Streptococcus thermophilus* | | | | | | | | | | 6.9 | 0.2 | | | 2.3 | | | | 0.1 | 0.3 | | | |
| *Yokenella regensburgei* | | | | | | | | | | | | | | | | | | | | | | |
| Low abundance | 0.3 | 0.2 | 0.1 | 0.1 | 0.3 | 0.3 | 0.1 | 0.1 | 0.5 | 0.1 | 0.2 | 0.2 | 0.3 | 0.2 | 0.4 | 0.1 | 0.6 | 0.7 | 0.5 | 0.4 | 0.5 | 0.2 |

| Taxon | K01 | K02 | K03 | K04 | K05 | T01 | T02 | T03 | T04 | T05 | T06 | T07 | T08 | T09 | T10 | T11 | T12 | T13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Camel* (*Khoormog*) | | | | | Cow* (*Tarag*) | | | | | | | Goat* (*Tarag*) | | | | | |
| *Acetobacter fabarum* | | | | | | | | | | | | | | | | | | |
| *Acetobacter ghanensis* | 2.1 | 0.2 | 4.6 | | 0.2 | | | | | | | | | | | | | |
| *Acetobacter malorum* | | 0.5 | 0.2 | | | | | 0.4 | | | | | | | | | | |
| *Acetobacter pasteurianus* | 1.0 | 1.2 | 1.4 | 1.0 | 0.3 | | | | | | | | 0.2 | | | | 0.4 | |
| *Aeromonas media* | | | 1.0 | | | | | | | | | | | | | | | |
| *Anoxybacillus contaminans* | | | | | | | | | | | | | | | | | | 1.4 |
| *Arthrobacter russicus* | 0.2 | | | | 0.1 | | | | 0.6 | 0.2 | 0.7 | 1.4 | | | 0.2 | | 0.1 | |
| *Bacillus cereus* | | | | 0.4 | | | | | | | | | | | | | | |
| *Bifidobacterium mongoliense* | | | | | | | | | | | | | | | | | | |
| *Brevundimonas nasdae* | 0.6 | 0.3 | 0.3 | 0.2 | 0.2 | 0.5 | 0.2 | 0.6 | 1.1 | 0.3 | 2.2 | 2.7 | 0.2 | 0.4 | 0.2 | 0.3 | 0.5 | 0.3 |
| *Citricoccus* sp. | | | | | | | | | | | | | | | | | | |
| *Clostridium perfringens* | | | | | | | | | | | | | | | | | | 0.7 |
| *Enhydrobacter aerosaccus* | | | | | | | | | | | | | | | | | | |
| *Enterococcus durans* | | | | | | | | | | | | | | | | | | |
| *Enterococcus italicus* | | | | | | | | | | | | | | | | | | |
| *Kocuria rhizophila* | | | | | | | | | | | | | | | | | | |
| *Komagataeibacter europaeus* | | | | | | | | | | | | | | | | | | |
| *Kurthia gibsonii* | | | | | | | | | | | | | | | | | 0.1 | |
| *Lactobacillus crustorum* | | | | | | | | | | | | | | | | | | |
| *Lactobacillus delbrueckii* | | | | | | 92.0 | 86.1 | 88.2 | 44.7 | 81.1 | 70.1 | 56.4 | | 94.2 | 84.3 | 80.4 | 2.0 | |
| *Lactobacillus diolivorans* | | | | | | | | | | | | | | | | | | |
| *Lactobacillus fermentum* | | | | | | 6.1 | 5.4 | 0.9 | 0.2 | 2.0 | | 1.0 | 0.1 | 1.5 | | | | |
| *Lactobacillus helveticus* | 1.5 | 4.0 | 42.7 | 15.8 | 61.0 | 0.9 | | 5.0 | 23.7 | 8.3 | 26.8 | 38.2 | 91.7 | 1.5 | 15.2 | | 74.2 | 3.4 |
| *Lactobacillus hilgardii* | | | | | | | | | | | | | | | | | | |
| *Lactobacillus kefiranofaciens* | 88.9 | 81.6 | 38.2 | 70.9 | 30.4 | | | | | | | | 4.8 | | | | 16.6 | 88.8 |
| *Lactobacillus kefiri* | 4.1 | 6.0 | 4.8 | 4.7 | 2.9 | | | | | | | | 2.7 | | | | 2.9 | 4.3 |
| *Lactobacillus paracasei* | | | | 0.1 | | | | | | | | | | | | | | |
| *Lactobacillus parakefiri* | | | | | | | | | | | | | | | | | | |
| *Lactobacillus pentosus* | | 0.4 | | | | | | | | | | | | | | | | |
| *Lactobacillus* sp. A | | | | | | | | | | | | | | | | | | |
| *Lactobacillus* sp. B | | | | | | | | | | | | | | | | | 0.8 | |
| *Lactococcus lactis* | 1.2 | 4.8 | 5.3 | 4.3 | 4.5 | | 0.2 | | | | | | | | | | 1.2 | 0.7 |
| *Lactococcus raffinolactis* | | | | | | | | | | | | | | | | | | |
| *Lactococcus* sp. | | | | | | | | | | | | | | | | | | |
| *Leuconostoc mesenteroides* | | 0.6 | | | | | | | | | | | | | | | | |
| *Macrococcus caseolyticus* | | 0.1 | 0.2 | | | | | | | | | | | | | | | |
| *Shigella flexneri* | | | 0.5 | | | | | | | | | | | | | | | |
| *Streptococcus gallolyticus* | | | | 2.1 | | | | | | | | | | | | | | |
| *Streptococcus parauberis* | | | | | | | | | | | | | | | | | | 0.3 |
| *Streptococcus thermophilus* | | | | | | 0.4 | 8.0 | 4.8 | 29.6 | 7.8 | | | | 2.3 | | 19.2 | 0.5 | |
| *Yokenella regensburgei* | | | 0.3 | | | | | | | | | | | | | | | |
| Low abundance | 0.3 | 0.4 | 0.6 | 0.5 | 0.4 | | 0.2 | | | 0.2 | 0.2 | 0.2 | 0.3 | 0.1 | | 0.2 | 0.7 | 0.2 |

| Taxon | T14 | T15 | T16 | T17 | T18 | T19 | T20 | T21 | T22 | T23 | T24 | T25 | T26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Yak* (*Tarag*) | | | | | | | | | | | | |
| *Acetobacter fabarum* | | | | | | 0.1 | | | | | | 3.0 | |
| *Acetobacter ghanensis* | | | | | | | | | | | | | |
| *Acetobacter malorum* | | | | | | | | | | | 0.3 | | |
| *Acetobacter pasteurianus* | | | | | | | | | | | | | |
| *Aeromonas media* | | | | | | | | | | | | | |
| *Anoxybacillus contaminans* | | | | | | | | | | | | | |
| *Arthrobacter russicus* | | | | | | 0.1 | | | | | | 0.2 | 0.4 |
| *Bacillus cereus* | | | | | | | | | | | | | |
| *Bifidobacterium mongoliense* | | | | | | | | | | | | | |
| *Brevundimonas nasdae* | 0.2 | | 0.1 | | 0.7 | | 0.2 | 0.1 | 0.1 | 0.3 | | 0.6 | 1.1 |
| *Citricoccus* sp. | | | | | | | | | | | | | |
| *Clostridium perfringens* | | | | | | | | | | | | | |
| *Enhydrobacter aerosaccus* | | | | | | | | | | | | | |
| *Enterococcus durans* | | | | | | | | | | | | | |
| *Enterococcus italicus* | | | | | | | | | | | | | |
| *Kocuria rhizophila* | | | | | | | | | | | | | |
| *Komagataeibacter europaeus* | | | | | | | | | | | | | |
| *Kurthia gibsonii* | | | | | | | | | | | | | |
| *Lactobacillus crustorum* | | | | | | | | | | | | | |
| *Lactobacillus delbrueckii* | 76.2 | 84.9 | 82.7 | 90.0 | 65.3 | 85.5 | 82.4 | 82.7 | 92.1 | 80.4 | 77.7 | 88.8 | 23.2 |
| *Lactobacillus diolivorans* | | | | | | | | | | | | | |
| *Lactobacillus fermentum* | 1.2 | | | | | | | | | | 2.8 | | 8.2 |
| *Lactobacillus helveticus* | 12.6 | 13.4 | 12.5 | 8.1 | 33.9 | 0.8 | 12.7 | 11.4 | 0.7 | 0.2 | 18.6 | 3.6 | 44.3 |
| *Lactobacillus hilgardii* | | | | | | | | | | | | | |
| *Lactobacillus kefiranofaciens* | | | | | | | | | | | | | |
| *Lactobacillus kefiri* | | | | | | | | | | | | | |
| *Lactobacillus paracasei* | | | | | | | | | | | | | |
| *Lactobacillus parakefiri* | | | | | | | | | | | | | |
| *Lactobacillus pentosus* | | | | | | | | | | | | | |
| *Lactobacillus* sp. A | | | | | | | | | | | | | |
| *Lactobacillus* sp. B | | | | | | | | | | | | | |
| *Lactococcus lactis* | | | | | | | | | | | 0.2 | | |
| *Lactococcus raffinolactis* | | | | | | | | | | | | | |
| *Lactococcus* sp. | | | | | | | | | | | | | |
| *Leuconostoc mesenteroides* | | | | | | | | | | | | | |
| *Macrococcus caseolyticus* | | | | | | | | | | | | | |
| *Shigella flexneri* | | | | | | | | | | | | | |
| *Streptococcus gallolyticus* | | | | | | | | | | | | | |
| *Streptococcus parauberis* | | | | | | | | | | | | | |
| *Streptococcus thermophilus* | 9.5 | 1.6 | 4.6 | 1.7 | | 13.3 | 4.6 | 5.8 | 7.1 | 18.9 | 0.2 | 3.7 | 22.7 |
| *Yokenella regensburgei* | | | | | | | | | | | | | |
| Low abundance | 0.2 | | | 0.2 | 0.2 | 0.2 | | | | 0.1 | 0.2 | 0.1 | 0.1 |

0.1–1 % | 1–10 % | 10–50 % | 50–100 %

* Animal species from which the milk was sourced

observed for the relative abundance of *S. thermophilus*. Although the *Tarag* samples made from goat milk did not belong to any clusters, three clusters were found in the PCA plot on the basis of the animal species from which the milk was sourced (Fig. 2A). Cluster I comprised *Airag* samples made from mare' s milk; *L. helveticus* was the significant factor in the formation of this cluster. Cluster II comprised *Khoormog* samples made from camel milk mainly, and *L. kefiranofaciens* was the main factor influencing the formation of this cluster. Cluster III comprised *Tarag* samples made from the milk of cows and yaks; *L. delbrueckii* and *S. thermophilus* were the main factors that contributed to the formation of this cluster. No correlations were found between sample bacterial diversities and the geographic sampling locations (Fig. 2B).

## DISCUSSION

In this study, we used a pyrosequencing method based on the 16S rRNA gene sequence to obtain detailed analyses of the bacterial diversity of traditional fermented dairy products of Mongolia. Based on the sequence of the V1−V2 hypervariable region, we were able to detect a total of 81 OTUs with an average of 13.9 ± 5.9 OTUs per sample (Table 1). An alpha diversity investigation suggested that the *Airag* and *Khoormog* samples were more diverse than the *Tarag* samples both in terms of OTU richness and phylogenetic relationships (Table 2). The *Airag* and *Khoormog* samples were made from non-pasteurized milk [3, 5], but the milk used for *Tarag* was boiled to remove milk fats [6], which would contribute to the differences in microbial diversity between *Tarag* and the other two products.

At the species level, a total of 22 LAB OTUs were observed, which showed high relative abundance in every sample, with the average being 98.5% ± 1.6%, suggesting that LAB were the core bacterial components involved in the fermentation of our samples. All of the LAB species that were isolated in our previous study that used the culture method [3] were also detected and identified with the pyrosequencing method in this study, except for *Enterococcus faecium*, *Lactobacillus casei*, *Lactobacillus farciminis*, *Lactobacillus parafarraginis*, *Lactobacillus paraplantarum*, *Leuconostoc* (*Leuc.*) *pseudomesenteroides* and *Pediococcus pentosaceus*. On the basis of sequence similarities, *E. faecium*, *L. farciminis, L. paraplantarum* and *Leuc. pseudomesenteroides* were not distinguishable from *E. durans*, *Lactobacillus crustorum*, *Lactobacillus pentosus* and *Leuconostoc mesenteroides*, respectively, in the V1−V2 hypervariable region with a 97% cutoff. The species name of *L. casei* was used based on the old taxonomy [28] in our previous study [3]; it should be corrected to *Lactobacillus paracasei* in accordance with the present taxonomy [29]. Thus, most species detected by using the culture method were also found with the pyrosequencing method. In addition, the pyrosequencing method detected 7 LAB species that were not detected by use of the culture method. These species could not be cultured in our previous study because of biases such as nutrient requirement, growth conditions or detection limit. Such biases may also explain the dissimilarity in LAB populations between the results obtained by culture and those obtained by pyrosequencing. The non-LAB OTUs were regarded as environmental contaminants from sources such as soils, animals and nomads [30–34]. *Airag*, *Khoormog* and *Tarag* were prepared by adding milk to a traditional container, such as the wooden barrel or cow's skin bag, without the use of commercial starters. These containers were thought of as the main sources of the microbes involved in the fermentation of traditional fermented milks. The non-LAB OTUs, such as the species in the genera *Clostridium*, *Enterococcus*, *Macrococcus* and *Moraxella,* may have been transferred from the animals, because the milks for *Airag* and *Khoormog* were not heat-treated. Indeed, the average numbers of non-LAB OTUs in the *Airag* and *Khoormog* samples were higher than those in the *Tarag* samples. In this study, 4 OTUs did not show high 16S rRNA gene sequence similarities with known species, suggesting that these species were uncultured owing to specific culture requirements. In our previous study [35], we isolated two *B. mongoliense* species from two *Airag* samples at $5.5 \times 10^7$ cfu/ml and $4.0 \times 10^7$ cfu/ml, respectively, whereas in this study, *B. mongoliense* was found in only one *Airag* sample at a relative abundance of 0.2% (equal to about $10^6$ cfu/ml). This result means that the pyrosequencing method only detected one-tenth (or less) of the bifidobacterial cells in the samples. This discrepancy between the present result and our previous result may be due to differences in the analytical methods used; in particular, the forward universal primer, 27F-mod, used in this study has mismatched bases for the 16S rRNA gene of *Bifidobacterium* [36], resulting in an underestimation of the bifidobacterial population by PCR.

PCA analysis based on the abundance of OTUs revealed an apparent correlation between the animal species providing the milk and the bacterial diversities of the *Airag*, *Khoormog* and *Tarag* samples. The abundance of the predominant LAB species made a major contribution to the formation of the clusters. *Tarag* samples made from

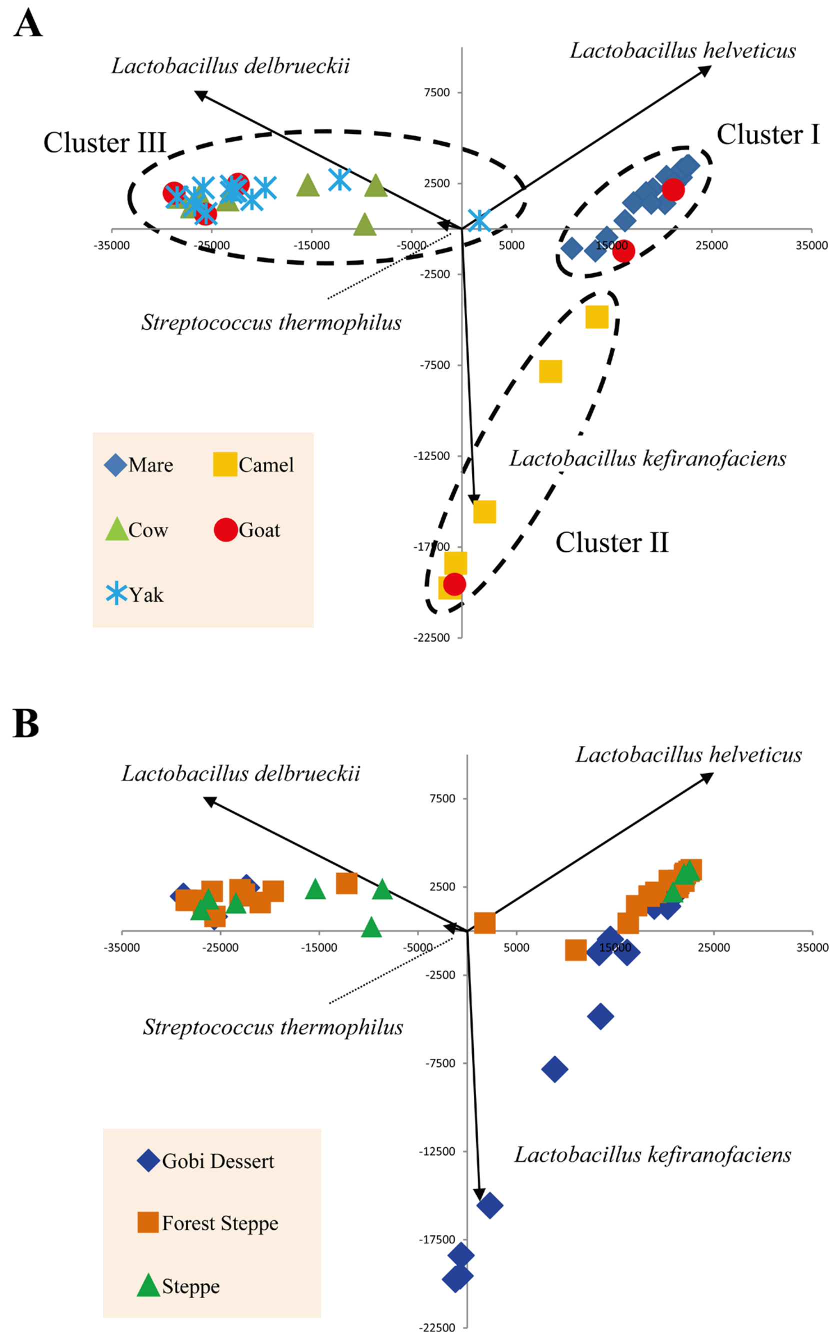


Fig. 2. Two-dimensional scatter plot of PC1 and PC2 scores from the principal component analysis of bacterial species diversity. The sample plots were assigned on the basis of (A) the animal species from which the milk was sourced for the preparation of the fermented dairy products and (B) the geographic sampling locations. The four largest loads of bacterial species are shown with arrows. The contributions of PC1 (horizontal axis) and PC2 (vertical axis) are 76.1% and 21.1%, respectively.

goat milk did not show a clear PCA pattern, indicating that there are unknown factors influencing the microbial diversity of the LAB composition in these samples.

In conclusion, we used a pyrosequencing method to evaluate the traditional fermented dairy products of Mongolia (*Airag*, *Khoormog* and *Tarag*) and

performed a detailed analysis of the microbial diversity of these products. We found a relationship between the predominant microbial component of these products and the animal species from which the milk was sourced for the preparation of the fermented dairy products. To our knowledge, this is the first study to apply a pyrosequencing method to these targets. Because pyrosequencing analysis was only able to reveal the microbial diversities at the time of sampling, further studies focused on manufacturing processes are needed to reveal the bacterial dynamics of these products.

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