Study of bacterial and fungal community structures in traditional koumiss from Inner Mongolia

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ABSTRACT

Koumiss is notable for its nutritional functions, and microorganisms in koumiss determine its versatility. In this study, the bacterial and fungal community structures in traditional koumiss from Inner Mongolia, China, were investigated. Our results demonstrated that 6 bacterial phyla represented by 126 genera and 49 species and 3 fungal phyla represented by 59 genera and 57 species were detected in 11 samples of artisanal koumiss. Among them, *Lactobacillus* was the predominant genus of bacterium, and *Kluyveromyces* and *Saccharomyces* dominated at the fungal genus level. In addition, there were no differences in the bacterial and fungal richness and diversity of koumiss from 3 neighboring administrative divisions in Inner Mongolia, and the bacterial and fungal community structures (the varieties and relative abundance of bacterial and fungal genera and species) were clearly distinct in individual samples. This study provides a comprehensive understanding of the bacterial and fungal population profiles and the predominant genus and species, which would be beneficial for screening, isolation, and culture of potential probiotics to simulate traditional fermentation of koumiss for industrial and standardized production in the future.

Key words: koumiss, bacterial, fungal, diversity

INTRODUCTION

Dairy products have been acknowledged as highly nutritious foods around the world. Koumiss, which originates from traditional fermentation of mare milk, is a very popular dairy foodstuff for the people of Mongolia, Kazakhstan, Kirgizstan, and some regions of Russia and Bulgaria (Danova et al., 2005). In China, koumiss has been used by the nomads who inhabit In-

ner Mongolia as a traditional Mongolian medicine to treat and heal intestinal dyspepsia, hypertension, and dyslipidemia (Rong et al., 2015; Gesudu et al., 2016).

Koumiss is produced by spontaneously fermenting mare milk in various containers made of wood, animal skin, and porcelain. The natural, homemade, palm-sized cloth bag that contains microbial flora from the previous year's koumiss serves as microbial starter culture for fermenting during horse lactation (June to September) during the current year. Fermentation continues spontaneously at ambient temperature (~20°C) for 1 to 3 d, and fermented mare milk is beaten and stirred with a wooden stick to remove carbon dioxide, ensure homogeneity, and speed up spontaneous fermentation for eliminating propagation of pathogens. According to personal preference (organoleptic evaluation of acidity and alcohol content) and commercial needs, koumiss is bottled and stored at 4°C to extend its shelf life. In view of the artisanal technique of production of koumiss, spontaneous fermentation, which is propelled by natural microbial flora, determines its nutritional and functional profiles. Nevertheless, the microbial investigation of koumiss has been the subject of a limited number of studies. *Lactobacillus casei* Zhang (Ya et al., 2008), *Lactobacillus fermentum* SM-7 (Pan et al., 2011), *Lactococcus lactis* KLDS4.0325 (Yang et al., 2014), *Lactobacillus helveticus* NS8 (Rong et al., 2015), and *Lactobacillus crustorum* MN047 (Yi et al., 2016), which were isolated from koumiss, demonstrated versatile probiotic attributes such as immunomodulation, cholesterol-lowering effects, inhibition of angiotensin-converting enzyme, anti-inflammatory function, and antibacterial activity. Moreover, the bacterial flora and yeasts in koumiss were isolated and identified using culture-independent methods, such as denaturing gradient gel electrophoresis, and species-specific PCR (Hao et al., 2010; Mu et al., 2012). Nonetheless, the bacterial and fungal community structures need to be simultaneously investigated in traditional koumiss. The comprehensive understanding of bacterial and fungal population profiles will be beneficial for simulating

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spontaneous fermentation of koumiss in laboratories and pilot plants for commercial production of artisanal koumiss.

The objective of this study was to investigate the bacterial and fungal community structures and to discover the predominant microbial genus and species during spontaneous fermentation of koumiss in Inner Mongolia. The conducted research would provide fundamental references for screening, isolation, and culture of microbial candidates with probiotic functions to simulate traditional fermentation of koumiss for industrial and standardized production in the future.

MATERIALS AND METHODS

Collection of Samples

Traditional production of koumiss originated from spontaneous fermentation of mare milk in Inner Mongolia. A total of 11 samples of artisanal koumiss were obtained from yurts of the Mongolian nomads across

the entire grassland of Inner Mongolia, and 3 samples of mare milk (X0, H0, and C0) were obtained from 3 administrative divisions of Inner Mongolia during horse lactation (June to September). Koumiss samples were also derived from these 3 areas: 4 samples (X1, X2, X3, and X4) from Xilin Gol, 4 samples (H1, H2, H3, and H4) from Hulun Buir, and 3 samples (C1, C2, and C3) from Chifeng (Figure 1). The precise locations of sampling were Xilinhot, West Sunit Banner, West Ujimuin Banner, and Abag Banner in Xilin Gol for X1 to X4, respectively; Hailar, Evenki Banner, New Barag Right Banner (West Banner), and New Barag Left Banner (East Banner) in Hulun Buir for H1 to H4, respectively; and Tianshan Town, Daban Town, and Hexigten Banner in Chifeng for C1 to C3, respectively. In addition, these sampling spots covered most areas of prairie in Inner Mongolia. These raw and fermented dairy products (1,000 mL) were collected into sterile, self-sealing bags as soon as possible, and bags were immediately placed on ice, stored at 4°C, and analyzed to investigate nutritional components. After this collec-

Figure 1. Sampling of koumiss from 3 administrative divisions of Inner Mongolia. The geographical position of Inner Mongolia in China is highlighted in black on the top left figure. The precise locations of sampling of X1, X2, X3, and X4 were Xilinhot, West Sunit Banner, West Ujimuin Banner, and Abag Banner, respectively, in Xilin Gol. The locations of sampling of H1, H2, H3, and H4 were Hailar, Evenki Banner, New Barag Right Banner (West Banner), and New Barag Left Banner (East Banner), respectively, in Hulun Buir. The locations of sampling of C1, C2, and C3 were Tianshan Town, Daban Town, and Hexigten Banner, respectively, in Chifeng.

tion of 11 samples from 3 different areas, the koumiss was subpackaged and stored at -80°C for analysis of microbial community structure.

Measurement of Lactose, Acidity, pH, Alcohol, Protein, and Fat of Mare Milk and Koumiss

The content of lactose (China National Food Safety Standard, 2010), acidity (China National Food Safety Standard, 2016a), and alcohol (China National Food Safety Standard, 2016b) was measured according to Chinese national food safety standards. The content of lactose was calculated using the titration method with Feline's solution (Sinopharm, Beijing, China) and methylene blue (Sinopharm). The acidity of dairy product was determined by titration method with phenolphthalein indicator. The pH value of mare milk and koumiss was quantified by a pH meter. The content of protein was determined using the Kjeldahl method. The content of fat was measured using the Soxhlet extraction method.

Microbial DNA Extraction and PCR Amplification

Microbial DNA was extracted from koumiss using an EZNA stool DNA kit (Omega Bio-tek, Norcross, GA) according to the manufacturer's protocols. The V3-V4 region of the 16S rRNA gene was amplified by PCR using primers 341F 5'-CCTACGGGNGGCWGCAG-3' and 806R 5'-GGACTACHVGGGTATCTAAT-3', where the barcode was an 8-base sequence to each sample. The internal transcribed spacer (ITS) region of the rRNA gene was amplified by PCR using primers ITS3-KYO2F 5'-GATGAAGAACGYAGYRAA-3' and ITS4R 5'-TCCTCCGCTTATTGATATGC-3', where the barcode was an 8-base sequence to each sample. The PCR reaction was performed in a mixture containing 5 μL of 10 $\times$ KOD buffer (Toyobo, Osaka, Japan), 5 μL of 2.5 mM dNTPs (Toyobo), 1.5 μL of each primer (5 μM), 1 μL of KOD polymerase (Toyobo), and 100 ng of template DNA extracted from koumiss and was executed under the following program: 95 $^{\circ}\text{C}$ for 2 min; 27 cycles of 98 $^{\circ}\text{C}$ for 10 s, 62 $^{\circ}\text{C}$ for 30 s, and 68 $^{\circ}\text{C}$ for 30 s; and a final extension at 68 $^{\circ}\text{C}$ for 10 min.

Illumina HiSeq2500 Sequencing

Amplicons were extracted from 2% agarose gel and purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega, Madison, WI). Purified amplicons were pooled in equimolar quantities and

paired-end sequenced (2×250) by Illumina (San Diego, CA) platform according to standard protocols.

Bioinformatics and Statistical Analysis

Raw data containing adapters or low-quality reads would affect the assembly and analysis. Thus, to get high-quality clean reads, raw reads were further filtered after removing the reads containing more than 10% unknown nucleotides and less than 80% bases with quality value >20 . Paired-end clean reads were merged as raw tags using FLASH version 1.2.11 (Magoč and Salzberg, 2011) with a minimum overlap of 10 bp and mismatch error rates of 2%. Noisy sequences of raw tags were filtered by QIIME version 1.9.1 (Caporaso et al., 2010) pipeline under specific filtering conditions (Bokulich et al., 2013) to obtain high-quality clean tags. Clean tags were searched against the reference database (http://drive5.com/uchime/uchim_download.html) to perform reference-based chimera checking using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html). All chimeric tags were removed, and some effective tags were finally obtained for further analysis. The effective tags were clustered into the operational taxonomic units (OTU) of $\geq 97\%$ similarity using the UPARSE pipeline (Edgar, 2013). The tag with the highest abundance was selected as a representative sequence within each cluster. Venn analysis was performed in R (<https://cran.r-project.org/doc/manuals/>) to identify unique and common OTU. The representative sequences were classified into organisms by the naive Bayesian model using RDP Classifier version 2.2 (Wang et al., 2007) based on the SILVA (Pruesse et al., 2007) database (<https://www.arb-silva.de/>) for 16S rRNA sequencing and based on the UNITE (Köljalg et al., 2005) database (<https://unite.ut.ee/>) for ITS sequencing. The OTU rarefaction curve, Chao1, Simpson, and all other alpha diversity indices were calculated in QIIME. Weighted and unweighted UniFrac distance matrixes were generated by QIIME. The principal coordinates analyses (PCoA) of weighted and unweighted UniFrac distances were calculated and plotted in R. Alpha and beta indices among groups were computed by the Kruskal–Wallis H test and Tukey's honest significant difference (HSD) test in R.

RESULTS AND DISCUSSION

Nutritional Characteristics of Traditional Koumiss from Inner Mongolia

The content of lactose in mare milk was $7.58 \pm 0.34\%$ and in koumiss was $3.51 \pm 2.54\%$ for Xilin Gol, $3.66 \pm$

2.32% for Hulun Buir, and $3.18 \pm 1.06\%$ for Chifeng (Table 1). The level of lactose significantly decreased during spontaneous fermentation of mare milk ($P < 0.01$). The characteristic of the high level of lactose in mare milk suggests that it is a valuable substrate for utilization by lactic acid bacteria and yeasts in the production of fermented dairy products (e.g., koumiss). Koumiss is a traditional drink usually made from mare milk by spontaneous fermentation of lactose to lactic acid and alcohol by lactic acid bacteria and yeasts. The acidity of mare milk was $4.39 \pm 0.23\%$, and the acidity of koumiss was $119 \pm 46\%$ for Xilin Gol, $226 \pm 114\%$ for Hulun Buir, and $184 \pm 89\%$ for Chifeng (Table 1). The content of alcohol in mare milk was 0% and in koumiss was $1.03 \pm 1.04\%$ for Xilin Gol, $0.46 \pm 0.31\%$ for Hulun Buir, and $1.07 \pm 1.05\%$ for Chifeng (Table 1). The acidity and the alcoholic content significantly increased during spontaneous fermentation of mare milk ($P < 0.01$). In addition, as shown in Table 1, the contents of protein and fat were uniform during production of koumiss from mare milk ($P > 0.05$). The dynamics of nutritional components during spontaneous fermentation of mare milk implied that these samples were appropriate to be authentic koumiss, with more acid and alcohol as well as less lactose compared with raw mare milk, for the following analyses of bacterial and fungal community structures.

Bacterial and Fungal Sequence Abundance and Diversity

A total of 314,728 bacterial and 136,687 fungal high-quality sequences were generated from 11 koumiss samples, with an average of 28,612 (SD = 4,591) and 12,426 (SD = 2,980) sequences for each sample, re-

spectively. The average lengths of bacterial and fungal sequences were 462 and 373 bp, respectively. The high-quality bacterial and fungal sequences were clustered into the OTU of $\geq 97\%$ similarity. As shown in Table 2, at a genetic distance of 3%, a total of 2,725 bacterial and 919 fungal OTU were identified from 11 koumiss samples, and the number of OTU per sample ranged from 178 to 340 for bacteria and 50 to 102 for fungi. Taxonomic classification was inferred from the closest matches of OTU to particular phylogenetic groups. Of the bacterial sequences, 93.4, 84, and 16.2% were assigned to phylum, genus, and species, respectively, for bacterial taxonomy. Of the fungal sequences, 96.9, 77.8, and 55.4% were assigned to phylum, genus, and species, respectively, for fungal taxonomy. More and more unclassified genus and species that were identified in the bacterial and fungal sequences resulted in decreasing in sequence assignment from phylum to species. From the analysis of alpha diversity for the bacterial and fungal sequences from 11 koumiss samples, the Shannon diversity curves, but not the rarefaction curves, reached the saturation phase (Figure 2). These results showed that the majority of bacterial and fungal phylotypes for samples had already been captured in the analysis, although some additional new bacterial and fungal phylotypes could possibly be identified by increasing the depth of sequencing. In addition, Good's coverage (values $> 99\%$) suggested that the majority of microbial phylotypes obtained from koumiss had already been captured.

In this study, the purpose of the Chao1 analysis was to evaluate the bacterial and fungal richness of koumiss, and the Shannon and Simpson indices were applied to indicate the bacterial and fungal diversities of koumiss (Table 2). The bacterial and fungal richness of koumiss

Table 1. Nutritional profiles of mare milk and traditional koumiss from Inner Mongolia

Type	Location	Sample	Lactose (g/100 g)	Acidity ¹ (°T)	pH	Alcohol ² (%, vol/vol)	Protein (g/100 g)	Fat (g/100 g)
Mare milk	Xilin Gol	X0	7.14	4.06	7.05	0	1.65	0.66
	Hulun Buir	H0	7.96	4.57	6.89	0	1.92	0.39
	Chifeng	C0	7.63	4.53	6.66	0	1.95	0.76
Koumiss	Xilin Gol	X1	0.32	188	3.39	2.4	2.03	1.50
		X2	6.54	95	3.89	0	1.83	1.09
		X3	3.67	98	3.69	0.5	2.10	0.66
		X4	3.50	95	3.79	1.2	2.02	1.41
	Hulun Buir	H1	6.47	97	4.02	0	2.56	0.21
		H2	4.23	165	3.30	0.8	1.88	0.61
		H3	2.99	301	3.21	0.5	1.92	0.74
		H4	0.94	340	3.30	0.5	2.13	1.09
	Chifeng	C1	4.13	282	3.26	0	2.08	0.49
		C2	3.37	164	3.39	1.1	1.93	0.48
		C3	2.04	107	3.71	2.1	1.86	1.11

¹Degrees Theurer.

²Measured at 20°C.

Table 2. Information inferred from 16S rRNA and internal transcribed spacer analyses and diversity indices of koumiss samples from Inner Mongolia

Target	Sample ¹	No. of reads	No. of OTU ²	Chao1 index	Shannon index	Simpson index	Good's coverage	No. of observed species
Bacterial analysis	X1	22,916	241	380	3.78	0.87	0.9989	241
	X2	33,992	178	221	3.97	0.90	0.9996	178
	X3	33,185	290	382	4.81	0.94	0.9992	290
	X4	26,578	265	330	3.88	0.87	0.9992	265
	H1	21,098	232	278	3.59	0.84	0.9992	232
	H2	32,188	185	254	2.45	0.59	0.9995	185
	H3	34,085	340	391	4.03	0.88	0.9993	340
	H4	23,751	216	263	3.69	0.85	0.9992	216
	C1	29,305	232	310	2.68	0.73	0.9993	232
	C2	29,512	251	276	2.80	0.76	0.9995	251
	C3	28,118	295	382	4.26	0.90	0.9990	295
Fungal analysis	X1	11,579	85	106	1.53	0.36	0.9998	85
	X2	14,747	102	130	2.25	0.59	0.9999	102
	X3	15,009	89	120	2.45	0.72	0.9998	89
	X4	11,370	85	125	1.17	0.33	0.9997	85
	H1	14,319	68	91	2.12	0.63	0.9999	68
	H2	4,818	50	59	0.03	0.00	1.0000	50
	H3	14,564	78	101	3.51	0.86	0.9999	78
	H4	13,053	83	110	3.03	0.82	0.9998	83
	C1	10,121	90	128	3.47	0.84	0.9996	90
	C2	12,988	97	100	3.26	0.79	0.9999	97
	C3	14,119	92	115	2.21	0.69	0.9998	92

¹See Table 1 for abbreviation definitions.²Operational taxonomic units.

was compared by the Chao1 index. The Chao1 index of bacteria (315 ± 61) was significantly higher than that of fungi (108 ± 20) in koumiss from Inner Mongolia ($P < 0.01$). The bacterial and fungal diversity of koumiss was compared by the Shannon and Simpson indices. The Shannon index of bacteria (3.63 ± 0.72) was significantly higher than that of fungi (2.27 ± 1.07 ; $P < 0.01$). The Simpson index of bacteria (0.83 ± 0.1) was significantly higher than that of fungi (0.6 ± 0.27 ; $P < 0.05$). Similarly, the observed species of bacteria (248 ± 48) were significantly more numerous than that of fungi (84 ± 14 ; $P < 0.01$). The above comparative alpha indicators illustrated that koumiss from Inner Mongolia had more bacterial phylotypes than fungus. In contrast, in tarag, a bacteria- and fungi-containing dairy product produced by traditional fermentation of cow milk in Inner Mongolia, the bacterial diversity was significantly lower than the diversity of fungi (Sun et al., 2014). We speculated that the difference in nutritional profile between mare and cow milks (e.g., high lactose content of mare milk) may arouse the predominance of bacteria or fungi in traditional fermented dairy products.

The samples were obtained from 11 banners in 3 administrative divisions of Inner Mongolia (Figure 1). The Kruskal–Wallis and Tukey HSD analyses were applied to evaluate the differences in the bacterial and fungal richness and diversity of koumiss from 3 areas (X: Xilin Gol; H: Hulun Buir; C: Chifeng). The results

($P > 0.05$) showed that there was no difference between these 3 divisions (Figure 3). Due to the inheritance of artisanal technique of traditional production of koumiss among Mongols in these 3 divisions, the procedure of spontaneous fermentation of koumiss and the nutritional and microbiological profiles of mare milk from the Mongolian breed of horse were prone to be relatively consistent, which imparted different divisions with stable microbial richness and diversity in koumiss.

Bacterial Community Profile of Koumiss

Based on the 16S rRNA sequences, the bacterial phyla were identified as belonging to 6 phyla—namely, *Firmicutes* (relative abundance: 64.45%), *Proteobacteria* (34.55%), *Actinobacteria* (0.91%), *Bacteroidetes* (0.07%), *Deinococcus–Thermus* (0.01%), and *Cyanobacteria* (0.01%). Of the 6 bacteria phyla, *Firmicutes* dominated in 7 samples (X1, X3, X4, H1, H4, C1, and C3), corresponding to 79.08, 74.34, 91.34, 94.71, 82.15, 61.48, and 79.38% of the reads from 3 divisions of Inner Mongolia, respectively. In contrast, *Proteobacteria* dominated in the remaining 4 samples (X2, H2, H3, and C2), corresponding to 60.5, 78.01, 51.1, and 54.87% of the reads, respectively. The above results were consistent with previous studies that demonstrated 4 major bacterial phyla (*Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*) in koumiss (Gesudu et al.,

2016), kefir (Dobson et al., 2011), and tarag (Sun et al., 2014), which are traditional fermented dairy products, and *Firmicutes* dominated in the bacterial community. In this study, *Proteobacteria* was the predominant bacterial phylum in 4 samples, which was different from the previous studies. These results implied that the transformation of predominant phylum could take place in different samples of artisanal koumiss from spontaneous fermentation.

As shown in Figure 4A, 126 bacterial genera were identified; the number of genera >1% was 8, including *Lactobacillus* (50.91%), *Acetobacter* (17.99%), *Exiguobacterium* (8.17%), *Lactococcus* (7.62%), *Kleb-*

siella (5.75%), *Acinetobacter* (2.69%), *Pseudomonas* (1.75%), and *Enterobacter* (1.67%). *Lactobacillus* was the predominant bacterial genus. It is well known that *Lactobacillus* plays a significant role in fermented dairy products (Karami et al., 2017a; Wang et al., 2018). Of the 126 bacteria genera, *Lactobacillus* dominated in 7 samples (X1, X3, X4, H1, H4, C1, and C3), corresponding to 77.45, 61.91, 70.02, 93.57, 77.66, 52.51, and 58.67% of the reads from these 3 areas, respectively. The richness of *Lactobacillus* determined the predominant phylum of *Firmicutes*. In contrast, *Acetobacter* and *Lactobacillus* dominated in H2 and H3, corresponding to 63.06 and 19.2% for H2 and 27.1 and 26.18% for

Figure 2. Shannon diversity curves (A and C) and rarefaction curves (B and D) for 16S rRNA (A and B) and internal transcribed spacer (C and D) sequencing analyses. OTU = operational taxonomic units.

Figure 3. Box plots showing the comparisons of Chao1 (A and D), Shannon (B and E), and Simpson (C and F) indices from 3 administrative divisions (X: Xilin Gol; H: Hulun Buir; C: Chifeng) of Inner Mongolia for 16S rRNA (A, B, and C) and internal transcribed spacer (D, E, and F) sequencing analyses. Box plot is a graphic representation of a distribution of value by a rectangle, the ends of which mark the first and third quartiles, and the maximum and minimum values are marked by whiskers. The middle line marks the median and outliers are marked by a dot.

H3, respectively. Due to the ability of *Acetobacter* with alcohol dehydrogenase to oxidize ethanol to acetic acid (Tian et al., 2017), *Acetobacter* was able to transform alcohol produced by yeasts to acetic acid. *Lactococcus* and *Klebsiella* dominated in X2, corresponding to 21.9 and 27.41%, respectively. *Lactococcus* is regarded as an important dairy microorganism (Casalta and Montel, 2008; Cavanagh et al., 2015). Although *Klebsiella* is a potential pathogen for humans and ruminants, *Klebsiella* species together with *Lactobacillus* could enhance the functional potential of fermented dairy products (Zadoks et al., 2011; Rosales-Bravo et al., 2017). *Exiguobacterium* and *Acinetobacter* dominated in C2, corresponding to 40.69 and 13.03%, respectively. The bacterial genus *Exiguobacterium* possesses stress-responsive genes that help it colonize and thrive in diverse ecological niches (Kasana and Pandey, 2018) and can be found in Latin-style cheeses (Lusk et al., 2012). These results suggested that the bacterial genus varied in 11 koumiss samples from 3 divisions of Inner Mongolia. Due to spontaneous fermentation of koumiss in the

individual's yurt, microbiological qualities of mare milk and traditional starter culture and sanitary conditions may influence bacterial community structure.

At the species level, 49 bacteria species were identified; the number of species >1% was 8, including *Lc. lactis* (61.58%), *Lactobacillus buchneri* (12.72%), *Enterococcus italicus* (5.87%), *Lactobacillus homohiochii* (5.12%), *Lactobacillus hilgardii* (4.88%), *Lb. helveticus* (2.56%), *Leuconostoc mesenteroides* (2.34%), and *Streptococcus parauberis* (2.32%). Of the 49 bacterial species, *Lc. lactis* dominated in 4 samples (X2, X3, X4, and C3), corresponding to 76.68, 88.84, 95.12, and 85.42% of the reads from 3 divisions of Inner Mongolia, respectively. *Lactococcus lactis* and *Strep. parauberis* dominated in H3, corresponding to 38.57 and 29.31%, respectively. *Lactobacillus helveticus* dominated in 3 samples (X1, H1, and H4), corresponding to 57.18, 57.81, and 75.16%, respectively; *Lb. buchneri* dominated in H2 (53.46%); and *Lb. hilgardii* dominated in C1 (54.08%). *Streptococcus thermophilus* and *Ent. italicus* dominated in C2, corresponding to 20.68 and 41.65%, respectively.

The microbes that belonged to lactic acid bacteria were predominant in koumiss in this study and exhibited a multifaceted significant function in fermented dairy products in the previous studies, such as starter cultures (Vinderola et al., 2002; Takeda et al., 2011; Yu et al., 2011), antimicrobial activity (Ayeni et al., 2009; Arqués

et al., 2015; Karami et al., 2017b), and probiotics and health promoting (Kamiya et al., 2016; Kimoto-Nira et al., 2017; Linares et al., 2017).

The varieties and relative abundance of bacteria genera and species were clearly different in different samples that were obtained from 11 banners in the 3

Figure 4. Relative abundance of bacterial (A) and fungal (B) sequences at the genus level in each koumiss sample from 3 administrative divisions (X: Xilin Gol, 4 samples; H: Hulun Buir, 4 samples; C: Chifeng, 3 samples) of Inner Mongolia.

administrative divisions. The above result of diversity corresponded to the previous study, which focused on the variation in the bacterial diversity in koumiss from different geographic locations (Gesudu et al., 2016). In terms of the overall technique of traditional fermentation of koumiss in Inner Mongolia, we speculated (1) that microorganisms in homemade starters, raw mare milk, environment, and appliances for fermentation were sources of microbial flora in koumiss and (2) that the nutritional quality of mare milk and artisanal technological parameters for production (e.g., temperature, time of duration for fermentation, and intensity of beating and stirring) likely influenced the varieties of bacterial and fungal communities in end-product koumiss from different areas. The bacterial community structures of koumiss from 3 divisions were compared as inferred from the unweighted and weighted UniFrac PCoA. As demonstrated in Figure 5A and B, the values of 11 samples from 3 divisions were largely separated in the both unweighted (accounting for 26.25 and 16.61% of the total variance by the 2 principal components, respectively) and weighted (accounting for 60.58 and 15.34% of the total variance by the 2 principal components, respectively) analyses. In addition, the Kruskal–Wallis and Tukey HSD analyses which were based on the unweighted and weighted UniFrac PCoA were applied to evaluate the differences in the bacterial community structures of koumiss from 3 areas (X: Xilin Gol; H: Hulun Buir; C: Chifeng). As shown in Figure 6A and B, the results ($P > 0.05$) showed that there was no difference in the bacterial community structure of koumiss from these 3 areas. When the sequence data of individual samples were pooled for the comparisons between each division, there was no difference in the bacterial diversity of koumiss from these 3 divisions. Due to neighboring and no distinct geographical barrier between these 3 divisions, the bacterial community structures of 11 koumiss samples were not significantly different.

Fungal Community Profile of Koumiss

Based on the ITS sequences, the fungal phyla were identified as belonging to 3 phyla—namely, *Ascomycota* (relative abundance: 91.64%), *Basidiomycota* (8.35%), and *Mucoromycota* (0.01%). *Ascomycota* ($91.64 \pm 8.22\%$) dominated all koumiss samples from Inner Mongolia. In previous studies, the predominant phylum was identified as *Ascomycota* in traditional dairy products (Sun et al., 2014; Gao et al., 2017) and beverages containing alcohol (Jung et al., 2012; Li et al., 2013).

As shown in Figure 4A, 59 fungal genera were identified; the number of genera $>1\%$ was 11, including *Kluyveromyces* ($20.11 \pm 32.75\%$), *Saccharomyces* (19.47

$\pm 23.89\%$), *Dipodascus* ($14.59 \pm 29.51\%$), *Kazachstania* ($13.85 \pm 16.21\%$), *Dekkera* ($10.94 \pm 20.03\%$), *Trichosporon* ($10.63 \pm 10.6\%$), *Penicillium* ($4.03 \pm 12.65\%$), *Pichia* ($1.81 \pm 4.5\%$), *Clavispora* ($1.27 \pm 2.95\%$), *Cladosporium* ($0.78 \pm 1.73\%$), and *Aspergillus* ($0.57 \pm 0.66\%$). Of the 59 fungal genera, *Kluyveromyces* dominated in 2 samples (X1 and X4), corresponding to 85.72 and 84.12% of the reads from these 3 areas, respectively. *Saccharomyces* and *Trichosporon* dominated in 4 samples (H3, H4, C1, and C2), corresponding to 61.1, 69.8, 69.57, and 85.37%, respectively. *Kazachstania* (43.86%) and *Dipodascus* (28.35%) dominated in X3, and *Kazachstania* (43.47%) and *Dekkera* (36.03%) dominated in C3. *Dekkera* (61.92%) dominated in H1, and *Dipodascus* (99.79%) dominated in H2. These predominant genera in 10 samples belong to yeasts, and *Kluyveromyces*, *Saccharomyces*, *Dipodascus*, *Kazachstania*, and *Dekkera* play important roles in the processes of proteolysis, lipolysis, flavor emergence, lactic acid utilization, alcohol production, and fermentation of dairy products (Narvhus and Gadaga, 2003; Landell et al., 2006; Sudun et al., 2013; Sun et al., 2014). Nonetheless, *Penicillium* (42.14%), a genus of filamentous fungus, was the major genus in X2. Moreover, all 11 samples contained *Penicillium*, *Cladosporium*, and *Aspergillus*, which were regarded as commonly filamentous fungi leading to dairy spoilage such as off odor and flavor and dairy waste as well as economic losses (Garnier et al., 2017). The presence of spoilage fungi in koumiss indicated that the fermentation of mare milk required the sanitation of tools and the tight control of environmental cleaning and hygiene. Similar to the diversity of bacterial genera in koumiss, the varieties and abundance of fungal genera were different among these 11 koumiss. We speculated that the microbiological profiles of raw milk and traditional starter culture and environmental and tool hygiene were likely to affect the fungal community structure of traditional koumiss.

At the species level, 57 fungal species were identified; the number of genera $>1\%$ was 8, including *Kluyveromyces marxianus* (25.22%), *Kazachstania unispora* (19.8%), *Dekkera anomala* (17.66%), *Saccharomyces cerevisiae* (17.55%), *Trichosporon asahii* (10.25%), *Penicillium carneum* (2.83%), *Pichia membranifaciens* (2.05%), and *Clavispora lusitaniae* (1.13%). Similar to the analysis of bacterial community, the varieties and relative abundance of fungal species were clearly distinct in individual samples. The variation in fungal diversity was also discovered in spontaneously fermented cow milk from different geographic locations (Sun et al., 2014). The unweighted and weighted UniFrac PCoA were introduced to compare the fungal community structures of koumiss from 3 divisions, and the values of 11 samples from these 3 divisions were largely

Figure 5. Unweighted (A and C) and weighted (B and D) UniFrac principal coordinate analyses (PCoA) of the bacterial (A and B) and fungal (C and D) diversity in 11 koumiss samples from 3 administrative divisions of Inner Mongolia. The blue (square), green (triangle), and red (circle) symbols represent the samples from Xilin Gol (X; $n = 4$), Hulun Buir (H; $n = 4$), and Chifeng (C; $n = 3$), respectively, in Inner Mongolia.

separated in the both unweighted (accounting for 25.78 and 13.29% of the total variance by the 2 principal components, respectively) and weighted (accounting for 47.02 and 28.19% of the total variance by the 2 principal components, respectively) analyses (Figure 5C and D). Moreover, we used the Kruskal–Wallis and Tukey HSD analyses, which were based on the unweighted and weighted UniFrac PCoA of the sequence data of the samples from different areas, to evaluate the differences in the fungal community structure of koumiss from 3

areas (X: Xilin Gol; H: Hulun Buir; C: Chifeng). The results ($P > 0.05$) demonstrated that there was no difference between the 3 neighboring divisions without a distinct geographical barrier.

CONCLUSIONS

Simultaneous investigation of bacterial and fungal community structures in traditional koumiss is a prerequisite for understanding the mechanisms of sponta-

Figure 6. Box plots showing the comparisons of unweighted (A and C) and weighted (B and D) UniFrac principal coordinate analyses of the bacterial (A and B) and fungal (C and D) diversity from 3 areas (X: Xilin Gol; H: Hulun Buir; C: Chifeng) of Inner Mongolia. Box plot is a graphic representation of a distribution of value by a rectangle, the ends of which mark the first and third quartiles, and the maximum and minimum values are marked by whiskers. The middle line marks the median and outliers are marked by a dot.

neous fermentation of koumiss, which is fundamental for the development of commercial starter cultures for industrial and standardized production of koumiss in the future. In this study, the bacterial and fungal floras were investigated in artisanal koumiss from Inner Mongolia using high-throughput sequencing. First, nutritional analyses illustrated that the acidity and the alcohol content significantly increased with the decreasing of lactose content during traditional fermentation of koumiss. Then, our results demonstrated that 6 bacterial phyla represented by 126 genera and 49 species were discovered, and 3 fungal phyla represented by 59 genera and 57 species were verified in koumiss samples. *Lactobacillus* was the predominant genus of bacterium, and *Kluyveromyces* and *Saccharomyces* dominated the fungal genus overall in koumiss. Last, the varieties and

relative abundance of bacterial and fungal genera and species were obviously distinct in individual samples, and there were no differences in the bacterial and fungal richness and diversity of koumiss from the 3 neighboring administrative areas in Inner Mongolia as inferred from the analysis of combined sequence data from all samples.

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