Investigation of bacterial and fungal diversity in tarag using high-throughput sequencing

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ABSTRACT

This is the first study on the bacterial and fungal community diversity in 17 tarag samples (naturally fermented dairy products) through a metagenomic approach involving high-throughput pyrosequencing. Our results revealed the presence of a total of 47 bacterial and 43 fungal genera in all tarag samples, in which *Lactobacillus* and *Galactomyces* were the predominant genera of bacteria and fungi, respectively. The number of some microbial genera, such as *Lactococcus*, *Acetobacter*, *Saccharomyces*, *Trichosporon*, and *Kluyveromyces*, among others, was found to vary between different samples. Altogether, our results showed that the microbial flora in different samples may be stratified by geographic region.

Key words: tarag, pyrosequencing, bacterial diversity, fungal diversity

INTRODUCTION

In northeast Asia, lives of nomadic people greatly depend on the domestic livestock such as horses, cows, yaks, goats, ewes, and camels, and they have produced various kinds of naturally fermented dairy products from the milk of these animals (Shuangquan et al., 2006; Sun et al., 2010b; Yu et al., 2011; Bao et al., 2012b; Liu et al., 2012a). Tarag, fermented milk of cows, is a special naturally fermented dairy product in Mongolia and the northwest of China. The history of making and drinking tarag in these regions dates back several thousand years (Airidengcaike et al., 2010). Tarag was generally made by natural fermentation without the use of any commercial starter cultures, and the natural cultures were subsequently used to inoculate fresh milk in the next day. To produce acidity, alcohol, and flavor to the desired level, tarag would be fermented for at least 5 to

8 d at ambient temperature around 10 to 25°C. In this way, a stable microbial phase was naturally formed, preserved, and handed down through generations together with the traditional tarag.

The naturally fermented dairy products, such as koumiss and tarag, have been considered not only as a kind of food, but also a complete nutriment with medicinal properties (Ishii and Konagaya, 2002). In China and Mongolia, nomads created the “koumiss therapy,” which combined traditional medicine with koumiss consumption to help in the treatment of hepatitis, chronic ulcer, tuberculosis, and so on (Hasisurong et al., 2003). Before we are able to understand the mechanism of such kinds of folk therapies, an in-depth characterization of the microbial communities in tarag is necessary to provide valuable information. It has been reported that lactic acid bacteria and yeasts play key roles during tarag production. By conventional culture and molecular-biology methods, Watanabe et al. (2008) reported that *Lactobacillus*, *Streptococcus*, and alcoholic-fermenting yeasts such as *Kluyveromyces* and *Saccharomyces* were the predominant microbial species in tarag.

Recently, more and more molecular technology based on culture-independent methods has been applied to the evaluation of microbial diversity in different fermented dairy products, namely, cheese (El-Baradei et al., 2007; Van Hoorde et al., 2008), tarag (Liu et al., 2012b), and kefir (Chen et al., 2008). Although these methods, such as denaturing gradient gel electrophoresis (Chen et al., 2008; Van Hoorde et al., 2008), temporal temperature gel electrophoresis (El-Baradei et al., 2007), and Sanger sequencing of 16S rRNA gene clone library (Liu et al., 2012b) have been used to investigate the microbial diversity, they may provide an inaccurate description of the total microbial structure.

Pyrosequencing, a next-generation DNA sequencing platform, based on the detection of pyrophosphate released during nucleotide incorporation, was developed by Margulies et al. (2005). Recently, this technique has successfully been used to detect the microbial communities in various fermented foods, such as cheese (Masoud

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et al., 2011; Alegría et al., 2012; Quigley et al., 2012), fermented seafood (Roh et al., 2010; Jung et al., 2013), fermented soybeans (Nam et al., 2012), pearl millet slurries (Humblot and Guyot, 2009), kefir (Dobson et al., 2011; Leite et al., 2012), fermented mung beans (Chao et al., 2013), and sourdoughs (Lattanzi et al., 2013). However, to our knowledge, this approach has, so far, not been applied in the investigation of microbial diversity in tarag. Meanwhile, the diversity of yeast in tarag has never been studied using culture-independent methods.

Therefore, we intended to investigate the microbial communities of the bacterial and fungal diversity in tarag, which would provide new insights into the microbiomes of tarag at the metagenomic level. In this study, the bacterial and fungal diversities in 17 tarag samples were evaluated with 454 pyrosequencing, and the microbial phase was systematically analyzed.

MATERIALS AND METHODS

Sample Collection

Seventeen tarag samples were collected from different regions of Mongolia and China. Detailed information of sampling location is listed in Table 1. Samples were aseptically collected and kept in ice boxes during transportation.

Extraction of Microbial DNA and PCR Amplification

Microbial DNA extraction was carried out according to the protocol of Liu et al. (2012b). The quality of the extracted DNA was assessed by 0.8% agarose gel electrophoresis, and the ratio of absorbance at 260 and 280 nm [optical density (OD) 260/280] was measured by spectrophotometry. All of the DNA samples were stored at -20°C until further processing.

A 195-nucleotide sequence of the V3 region of the 16S rRNA gene was amplified by PCR for bar-coded pyrosequencing. A set of 6-nucleotide barcodes was added to the universal forward primer 338F (5'-ACTCCTACGGAGGCAGCAG-3') and the reverse primer 533R (5'-TTACCGCGGCTGCTGGCAC-3'), which targeted the domain *Bacteria* (Huse et al., 2008). The internal transcribed spacer (ITS) 1 region of fungi was selected and amplified using the forward primer ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and the reverse primer ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'), to which another set of 6-nucleotide barcodes was added (Ghannoum et al., 2010). Polymerase chain reaction amplification of 16S rRNA V3 and ITS regions were performed as described in Humblot and Guyot (2009), and Ghannoum et al. (2010), respectively.

Amplicon Purification, Quantification, Pooling, and Pyrosequencing

Amplicons generated from PCR were purified using the AMPure XP purification system (Beckman Coulter Genomics, Takeley, UK), and the quality of the purified DNA was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). The quantity of amplicons was estimated using an Agilent DNA 1000 kit (Agilent Technologies Inc.). The PCR products were pooled in equimolar ratios to a final concentration of 100 nmol/L for pyrosequencing using a Roche GS FLX System (F. Hoffmann-La Roche Ltd., Basel, Switzerland) in the Advanced Studies in Genomics, Proteomics and Bioinformatics Laboratory, University of Hawaii (Honolulu).

Sequence Processing and Bioinformatics Analysis

Sequences generated from pyrosequencing were mainly analyzed with the QIIME (v1.5.0-dev; <http://qiime.org/>) suite of software tools for quality adjustment, barcode split, identification of the operational taxonomic units (OTU), taxonomic assignment, community comparison, and statistical analysis (Caporaso et al., 2010b; Kuczynski et al., 2011). Sequences were filtered to minimize the effects of poor sequence quality and sequencing errors by removing sequences with more than 1 ambiguous base calls and retaining only sequences that were ≥ 140 nucleotides. The extracted sequences were sorted into different samples according to the barcodes.

After the removal of the barcodes and primer, the extracted sequences were aligned using PyNAST software (Caporaso et al., 2010a) and the passed sequences were clustered based on a 97% sequence identity using the UCLUST algorithm (Edgar, 2010) to obtain a unique V3 and ITS sequence set, respectively. The taxonomy of each bacterial OTU representative sequence was assigned with the Ribosomal Database Project (RDP) II database using RDP classifier with an 80% confidence threshold (Wang et al., 2007). The representative fungal OTU sequences were phylogenetically classified by standalone Mega BLAST searches of the UNITE database (Abarenkov et al., 2010), a fungal ITS sequence database. Two de novo taxonomic trees were constructed using bacterial and fungal OTU representative sets using FastTree software (Price et al., 2009) for the downstream analysis, including the calculation of the α and β diversity.

The Shannon-Wiener index and Chao1 index were used to calculate the α diversity, and the UniFrac metric (Lozupone and Knight, 2005) was used to calculate the β diversity both in a weighted and unweighted fashion.

Table 1. Information on sequence analysis and diversity indices of tarag¹

Sample	Sampling location	No. of reads		No. of OTU ²		Chao1 index ³		Shannon index ⁴		ESC ⁵	
		Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
MG1	Bayandelger County, Sükhbaatar, Mongolia	3,291	4,951	72	212	129.6	251.0	1.0	3.3	97.3	93.9
MG2	Tawenduerwula County, Arkhangai, Mongolia	5,249	5,156	91	146	113.9	170.1	1.3	2.4	97.9	93.8
MG3	Hulunbuir County, Dornod, Mongolia	3,688	4,148	34	170	33.9	183.1	0.2	2.9	99.2	91.9
MG17	Wuergen County, Dornogovi, Mongolia	3,244	2,048	57	202	122.6	160.8	3.5	3.5	94.5	92.7
MG18	Tsetserleg City, Arkhangai, Mongolia	4,857	3,682	62	132	86.0	106.6	1.3	2.8	98.6	90.3
GS19	Xiahe County, Gansu, China	2,958	5,580	85	127	92.1	100.1	1.9	1.6	97.1	99.0
GS4	Luqu County, Gansu, China	2,843	3,606	63	151	48.5	113.9	1.2	2.3	98.7	98.1
GS5	Hezuo City, Gansu, China	2,777	1,128	112	111	147.1	49.5	2.1	2.4	98.7	95.9
GS6	Luqu County, Gansu, China	3,114	4,877	97	182	80.8	124.6	1.9	3.0	98.8	94.1
SC7	Nuogai County, Sichuan, China	2,026	2,961	46	173	80.4	135.6	0.7	3.3	98.9	90.3
SC8	Hongyuan County, Sichuan, China	5,002	6,952	156	144	241.6	94.6	3.9	1.3	96.8	99.1
SC9	Hongyuan County, Sichuan, China	3,373	11,175	89	160	164.1	84.5	1.8	1.6	97.7	99.4
NM10	Balinyou County, Inner Mongolia, China	3,367	7,520	57	278	61.0	174.2	2.7	3.1	96.7	97.9
NM11	Xinbaerhuyou County, Inner Mongolia, China	1,387	3,821	139	173	272.5	137.3	3.1	1.7	95.1	98.9
NM12	Zhenglan County, Inner Mongolia, China	4,146	9,266	73	135	87.5	110.0	2.4	1.5	97.1	98.3
NM13	Hailar City, Inner Mongolia, China	2,407	4,433	119	125	138.8	107.4	2.9	2.2	97.1	98.5
NM14	Alukerqin County, Inner Mongolia, China	1,257	6,370	125	243	205.2	192.2	2.4	3.2	97.4	97.9

¹OTU = operational taxonomic units; ESC = estimated sample coverage.²Number of OTU was calculated with the QIIME program (<http://qiime.org/>) at 3% distance level.³The Chao1 index was calculated with the QIIME program at 3% distance.⁴The Shannon index was calculated with the QIIME program at 3% distance.⁵ESC = estimated sample coverage; $G = (1 - n/N) \times 100$, where G is Good's coverage, which provides an estimate of sampling completeness using a probability calculation with randomly selected amplicon sequences, n is the number of OTU, and N is the total number of sequences.

Figure 1. Relative abundance of bacterial sequence read in each tarag sample collected in China and Mongolia.

for the principal components analysis and unweighted pair group method with arithmetic mean clustering. Phylogenetic trees of *Lactobacillus* OTU representative sequences were constructed and drawn using MAGE 5.0 (Tamura et al., 2011) and Interactive Tree of Life (iTOL; Letunic and Bork, 2007) software, respectively.

Statistical Analysis

The Mann-Whitney and Kruskal-Wallis tests were used to evaluate the differences in the bacterial and fungal populations. Multivariate ANOVA (MANOVA) and clustering of microbiota of tarag based on Mahalanobis distances calculated using MANOVA were conducted in Matlab R2011b software (The MathWorks Inc., Natick, MA).

Nucleotide Sequence Accession Numbers

The sequence data reported in this paper have been deposited in the MG-RAST database (<http://metagenomics.anl.gov/>; accession no. 2912, 4509270–4509296).

RESULTS AND DISCUSSION

Richness and Diversity Analysis

After preprocessing, a total of 142,660 high-quality reads were obtained from the one-fourth plate using a 454 genome sequencer (454 Life Sciences, Branford, CT), corresponding to 54,986 for bacteria and 87,674 reads for fungi. Average read length was 155 bases for bacteria and 180 bases for fungi, and each individual sample was covered by an average of 3,234 ($\pm 1,140$) and 5,157 ($\pm 2,528$) bacterial and fungal reads, respectively. The average number of OTU for bacteria and fungi was 87 (± 34) and 168 (± 44) at 3% sequence dissimilarities, respectively (Table 1).

Although the individual rarefaction curves for the bacterial and fungal diversity for most samples plateaued with the current sequencing, they failed to reach the saturation phase (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2014-8360>). The Shannon diversity estimates of the samples did reach stable values (Supplemental Figure S2). These results indicate that although new phylotypes would be expected with ad-

ditional sequencing, most of the microbial diversity had already been captured (Wang et al., 2012).

In addition, the Good's coverage, an estimator of completeness of sampling, highlighted good overall sampling with levels of 94.5 to 99.2% and 90.3 to 99.4% for bacteria and fungi, respectively. These results indicated that although new phylotypes would be expected with additional sequencing, the majority of bacterial and fungal phylotypes present in tarag had already been captured (Jung et al., 2012).

We compared the microbial diversity, as estimated by the Shannon index, and the results showed that the index of bacteria (1.46 ± 1.23) was significantly lower than that of fungi (2.98 ± 0.43) in tarag collected in Mongolia ($P < 0.05$). Similarly, the observed number of bacterial OTU (87 ± 34) was significantly lower than that of fungi (168 ± 44 ; $P < 0.05$). However, the difference between the Shannon index of bacteria and fungi was not statistically significant ($P > 0.05$) in tarag collected in Inner Mongolia, Sichuan, and Gansu (China). These indicated more phylotypes of fungi than bacteria existing in tarag collected in Mongolia, but not samples from the studied Chinese provinces. In contrast, Wata-

nabe et al. (2008) and Sun et al. (2010b) reported that the number of isolated strains and concentration of lactic acid bacteria were significantly higher than yeasts in tarag, a traditionally fermented dairy product of Mongolia. This contradiction may be due to differences in the analytical goals and methods used.

Taxonomic Profiling of Bacterial Communities

The phylogenetic classification of bacterial sequences from tarag is summarized in Figure 1. The sequences were distributed among 4 bacterial phyla, namely *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. Of the 4 bacterial phyla, *Firmicutes* dominated in all tarag samples from different sampling locations, containing 213 OTU, corresponding to 86.40, 86.54, 88.64, and 66.83% of the reads from Mongolia, and Gansu, Sichuan, and Inner Mongolia provinces, respectively. The second dominated bacterial phylum, comprising 192 OTU, was *Proteobacteria*, representing an average of 17.15% ($\pm 9.63\%$) of the population. *Bacteroidetes* ($0.53 \pm 0.59\%$), *Actinobacteria* ($0.11 \pm 0.05\%$), and unclassified bacteria ($0.11 \pm 0.07\%$) were

Table 2. Percentage of bacterial sequence reads at the phylum and genus level in tarag samples from Mongolia and 3 provinces in China

Bacteria	Region			
	Mongolia (n = 5)	Gansu (n = 4)	Sichuan (n = 3)	Inner Mongolia (n = 5)
Phylum				
<i>Firmicutes</i>	86.40	86.54	88.64	66.83
<i>Proteobacteria</i>	13.01	13.19	10.87	31.51
<i>Bacteroidetes</i>	0.25	0.19	0.26	1.41
<i>Actinobacteria</i>	0.12	0.04	0.15	0.13
Uncultured	0.21	0.04	0.08	0.12
Genus				
<i>Lactobacillus</i>	67.22	52.51	49.91	43.70
<i>Streptococcus</i>	12.07	33.55	29.51	0.71
<i>Lactococcus</i>	3.66	0.17	2.26	21.86
<i>Leuconostoc</i>	1.92	0.02	1.60	0.16
<i>Acinetobacter</i>	1.13	0.75	3.09	1.31
<i>Enhydrobacter</i>	0.15	0.16	0.42	0.37
<i>Cloacibacterium</i>	0.15	0.09	0.18	1.09
<i>Acetobacter</i>	0.10	12.09	3.57	20.06
<i>Macrococcus</i>	0.05	0.17	1.52	0.02
<i>Chryseobacterium</i>	0.05	0.03	0.02	0.12
<i>Delftia</i>	0.03	0.01	0.03	0.12
<i>Escherichia</i>	0.02	0.05	0.06	0.04
<i>Rhizobium</i>	0.02	0.01	0.16	0.09
<i>Roseomonas</i>	0.02	0.01	0.03	0.06
<i>Kurthia</i>	0.01	0.02	3.49	0
<i>Enterobacter</i>	9.77	0	0.25	6.86
<i>Klebsiella</i>	1.37	0	0.01	0.13
<i>Bacillus</i>	1.30	0	0.02	0
<i>Citrobacter</i>	0.14	0	0.05	0.19
<i>Bifidobacterium</i>	0.10	0	0.01	0.04
Other	0.72	0.36	3.81	3.07

Table 3. Percentage of fungal sequence reads at the phylum and genus level in tarag samples from Mongolia and 3 provinces in China

Fungi	Region			
	Mongolia (n = 5)	Gansu (n = 4)	Sichuan (n = 3)	Inner Mongolia (n = 5)
Phylum				
Ascomycota	96.87	98.78	98.71	73.76
Basidiomycota	2.68	1.06	1.05	26.02
Mucoromycotina	0.01	0	0.07	0
Uncultured	0.44	0.18	0.17	0.22
Genus				
<i>Galactomyces</i>	15.72	58.63	75.52	57.74
<i>Saccharomyces</i>	60.40	19.66	19.23	0.43
<i>Trichosporon</i>	0.58	0.20	0.51	25.66
<i>Kluyveromyces</i>	9.97	10.4	0.01	0.44
<i>Pichia</i>	2.12	7.96	0.49	4.52
<i>Candida</i>	0.75	0.51	2.30	3.54
<i>Torulaspora</i>	4.72	0.19	0.38	0.01
<i>Debaryomyces</i>	0.41	0.99	0.57	0.04
<i>Cryptococcus</i>	0.63	0.14	0.11	0.07
<i>Rhodotorula</i>	0.46	0.04	0.20	0.13
<i>Guehomyces</i>	0.05	0.61	0.08	0.06
<i>Cladosporium</i>	0.14	0.33	0.10	0.04
<i>Kazachstania</i>	1.09	0.03	0	1.68
Other	2.96	0.31	0.50	5.64

the minor members of the domain bacteria in all tarag samples (Table 2). These results were in agreement with some previous studies that confirmed the presence of the 4 major bacterial phyla in the fermented foods, such as fermented seafood (Roh et al., 2010), pearl millet slurries (Humblot and Guyot, 2009), kefir (Dobson et al., 2011), alcoholic beverages (Jung et al., 2012), kochujang (Nam et al., 2012), and artisanal cheeses (Quigley et al., 2012), and *Firmicutes* was the predominant bacterial phylum. However, different results were reported in the previous studies described by Leite et al. (2012) and Illegheems et al. (2012), which only found 3 major phyla, including *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, in kefir and cocoa bean. The latter results indicated that *Firmicutes* was the predominant bacterial phylum in the environment of those fermented foods.

At the genus level, a total of 47 bacterial genera were identified and distributed among the 4 phyla, dominated by *Lactobacillus*. Further analysis revealed that 14 genera were found to be common in all tarag samples [from 4 different sampling locations (Mongolia, and Gansu, Sichuan, and Inner Mongolia provinces)]: *Lactobacillus* (67.22, 52.51, 49.91, and 43.70%), *Streptococcus* (12.07, 33.55, 29.51, and 0.71%), *Lactococcus* (3.66, 0.17, 2.26, and 21.86%), *Leuconostoc* (1.92, 0.02, 1.60, and 0.16%), *Acinetobacter* (1.13, 0.75, 3.09, and 1.31%), *Enhydrobacter* (0.15, 0.16, 0.42, and 0.37%), *Cloacibacterium* (0.15, 0.09, 0.18, and 1.09%), *Acetobacter* (0.10, 12.09, 3.57, and 20.06%), *Macroccoccus* (0.05, 0.17, 1.52, and

0.02%), *Chryseobacterium* (0.05, 0.03, 0.02, and 0.12%), *Delftia* (0.03, 0.01, 0.03, and 0.12%), *Escherichia* (0.02, 0.05, 0.06, and 0.04%), *Rhizobium* (0.02, 0.01, 0.16, and 0.09%), and *Roseomonas* (0.02, 0.01, 0.03, and 0.06%). It is well known that *Lactobacillus* and *Streptococcus* are indeed 2 widespread genera associated with tarag fermentation processes (Watanabe et al., 2008; Sun et al., 2010a, Bao et al., 2012b). As lactobacilli have been reported to have higher acid tolerance than streptococci and lactococci (Rogosa et al., 1951), the members of the genus of *Lactobacillus* may be supplied from old tarag that was used as the starter for fermentation. In some previous reports, some species of *Lactococcus* and *Leuconostoc* were isolated from raw milk (An et al., 2004; Bao et al., 2012a), which indicated that *Lactococcus* and *Leuconostoc* spp. could have been sourced from the raw milk. They were possibly able to grow during the early stage of fermentation, but could not survive in the final products because of their low acid tolerance (Miyamoto et al., 2010). Therefore, our result of the relatively low percentage of *Lactococcus* and *Leuconostoc* spp. compared with *Lactobacillus* and *Streptococcus* spp. could most likely be explained by the acid inhibition of the 2 former genera during the later stage of fermentation of tarag.

In the current study, 21 genera of *Proteobacteria* were detected in the different tarag samples, including *Acinetobacter*, *Klebsiella*, *Escherichia*, and *Salmonella*. This is the first report of the presence of these 4 genera in tarag. The dominating genus was *Acetobacter* (Table

3). It is known that *Acetobacter* spp. have great ability to oxidize ethanol to acetic acid because of their possession of the enzyme alcohol dehydrogenase (Yoo et al., 1994). Therefore, they can produce large amounts of acetic acid from the alcohol produced by the yeasts present in tarag. Escalante et al. (2008) previously reported that the proportion of *Proteobacteria*, such as *Acinetobacter* spp., *Enterobacter* spp., and *Escherichia* spp., gradually decreased throughout the fermentation process of Mexican alcoholic beverages. In our results, we speculated that these genera were contaminants from the raw milk and, consistent with Escalante et al. (2008), they were subsequently killed during the fermentation process.

For the genus of *Lactobacillus*, a total of 126 OTU were detected in all tarag samples, in which *Lactobacillus delbrueckii* ssp. *bulgaricus* was the predominant species (Figure 2). In all of the OTU, part 1 was identified as the *Lactobacillus kefiranofaciens* and *Lactobacillus helveticus* group, containing 78 OTU (5.63% of reads for *Lactobacillus*). The second part, comprising 91.72% (31 OTU) of the total reads for *Lactobacillus*, was identified as the *Lactobacillus delbrueckii* ssp. *bulgaricus* group. The third part, which contained 17 OTU (2.65% of reads for *Lactobacillus*), was assigned to the *Lactobacillus casei* and *Lactobacillus plantarum* group. This result was in agreement with a previous study in which *Lactobacillus delbrueckii* ssp. *bulgaricus* was found to be

Figure 2. Maximum likelihood tree of all representative *Lactobacillus* operational taxonomic units (OTU). Sequence labels of representative OTU generated from the current study are colored, whereas sequences retrieved from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) are designated with uncolored sequence labels. Each color dot represents an OTU. The color of dot distinguishes the different sampling sites, for which black, red, green, and blue signifies tarag sampled in Gansu (China), Mongolia, Inner Mongolia (China), and Sichuan (China), respectively. The size of dot indicates the abundance of sequence reads.

Figure 3. Relative abundance of fungal sequence read in each tarag sample collected in China and Mongolia.

the predominant species of *Lactobacillus* in tarag from Mongolia (Yu et al., 2011). However, different results were reported in some previous studies of Liu et al. (2012a) and Bao et al. (2012a,b), in which *Lactobacillus helveticus* was found to be the predominant species in tarag from Inner Mongolia, Sichuan, and Gansu provinces, respectively. The major reason for this difference could be ascribed to the different methods of analysis (Watanabe et al., 2008). In addition, we compared the content of reads for each OTU (Figure 2). The results indicated that the *Lactobacillus casei* and *Lactobacillus plantarum* group was more abundant in the tarag from Inner Mongolia, whereas the *Lactobacillus kefirano-faciens* and *Lactobacillus helveticus* group was barely present in the tarag from Mongolia. This leads to the conclusion that different geographic regions may be inhabited with different microbial diversities (Watanabe et al., 2008).

Taxonomic Profiling of the Fungal Community

The composition of the fungal communities in all tarag samples was simpler than that of the bacterial communities (Figure 3) and contained 2 phyla: Asco-

mycota and Basidiomycota, and 1 subphylum: Mucoromycotina. All of the tarag samples were predominated by the phylum Ascomycota, containing 475 OTU and constituting 92.03% ($\pm 12.21\%$) of the fungal populations with the minor members of Basidiomycota ($7.7 \pm 12.24\%$), Mucoromycotina ($0.02 \pm 0.03\%$), and uncultured fungi ($0.25 \pm 0.13\%$; Table 3). Similar findings of a predomination of the fungal phylum Ascomycota was reported in Korean alcoholic beverages (Jung et al., 2012), cocoa bean (Illegheims et al., 2012), and Chinese liquor (Li et al., 2011). These results indicate that the phylum Ascomycota is the primary fungi in traditional fermented foods.

Likewise, we also carried out an analysis of the fungal composition at the genus level, in which 43 fungal genera were identified. The results showed that the most prevailing fungal genus was *Galactomyces*, followed by *Saccharomyces*, *Trichosporon*, *Kluyveromyces*, *Pichia*, and *Candida*, which accounted for 51.9 ± 25.47 , 24.93 ± 25.29 , 6.74 ± 12.62 , 5.21 ± 5.76 , 3.77 ± 3.25 , and $1.78 \pm 1.42\%$ of all fungal populations, respectively. Although several species of *Galactomyces* seem to have been rarely reported (Smith et al., 1995), the genus *Galactomyces* was previously found to be the dominant

yeast in cheese (Eliskases-Lechner and Ginzinger, 1995; Prillinger et al., 1999). Our data also showed that this genus of fungi was present in high abundance in tarag. The fungi may play a role in lactic acid utilization, lipolysis, proteolysis and flavor development (Wyder et al., 1999). Other significantly present genera, including *Saccharomyces*, *Kluyveromyces*, *Pichia*, and *Can-*

dida, are commonly associated with the fermentation processes of tarag and cheese (Watanabe et al., 2008; Rahman et al., 2009; Miyamoto et al., 2010). They play important roles in processes such as hydrolyzing milk proteins and fat, assimilating lactic acid, and producing ethanol (Sudun et al., 2013). Furthermore, some fungal genera that were not yet reported to be associated with tarag fermentations were detected in the present study. For instance, *Trichosporon*, a genus of the phylum Basidiomycota, is common in raw milk, which, in a previous study, caused the formation of a surface film on the cottage cheese and led to spoilage (Welthagen and Viljoen, 1998). Our results showed that the percentage of *Trichosporon* (25.66%) was apparently higher in tarag in Inner Mongolia than the other 3 sampling sites. This could indicate poor hygiene and ineffective cleaning procedures, and, hence, reveal the need for improved sanitization procedures in tarag production in Inner Mongolia.

Comparison of Bacterial and Fungal Communities of Tarag

Multivariate statistical analyses were performed to compare the overall structure of microbiota of all samples. Principal components analysis based on the unweighted UniFrac metric revealed the bacterial microbial communities of the Sichuan and Gansu samples were distinct from those of the Inner Mongolia samples based on the principal components 1 and 2 (23.55 and 13.33% of variance, respectively), and also revealed that few of the samples in the Mongolian and Inner Mongolia groups overlapped (Figure 4). A MANOVA also indicated that the 4 groups were first separated as 2 clades (Figure 5), with a much smaller within-clade distance between Mongolian and Inner Mongolia samples compared with Sichuan samples. Principal components analysis also revealed that the fungal communities of Sichuan were distinct from those of Inner Mongolia and Mongolian (principal components 1 and 2 were 25.01 and 13.95%, respectively; Figure 4). This was confirmed by MANOVA, in which significant difference in fungal composition of tarag from Sichuan and Mongolian was detected (Figure 5). Although a few samples overlapped (Figure 4), a MANOVA using the principal components based on the unweighted UniFrac metric further indicated that the difference between the 2 groups was significant ($P = 0.046$; Figure 5). Thus, we may conclude that the microbial flora in different samples was stratified by geographic region. The tarag samples used in this study were produced by similar traditionally preserved methods. Therefore, most likely, the microbial structural differences between samples of various regions can be attributed to geographic

Figure 4. Principal components analysis (PCoA) of the bacterial (A) and fungal (B) diversity in tarag from different sampling sites. The PCoA plots were generated using unweighted UniFrac distances (Lozupone and Knight, 2005) for the operational taxonomic units from samples of different regions. The green, blue, and black symbols represent samples from Inner Mongolia, Sichuan, and Gansu in China, respectively, and the red symbol represents Mongolian tarag.

Figure 5. Clustering of gut microbiota based on distances between different groups calculated with multivariate ANOVA of the first 14 principal components of bacterial (A) and fungal (B) diversity data. The Mahalanobis distances between group means are shown. * $P < 0.05$. Color version available in the online PDF.

location and other environmental factors. Similarly, Watanabe et al. (2008) reported that the microbial communities of traditional fermented milk varied with the sampling geographic region. Environmental factors, such as elevation, climate, temperature, oxygen level, atmospheric pressure, sunlight, and length of sunlight radiation, may play roles in modulating the microbiome of fermented dairy products (Sun et al., 2010a).

CONCLUSIONS

This is the first report on the bacterial and fungal communities of Chinese and Mongolian tarag based on a high-throughput pyrosequencing metagenomic approach. To our knowledge, we provided the most thorough description and analysis on the bacterial and fungal communities in tarag sampled in Mongolia and 3 Chinese provinces. Finally, our results strongly suggest that the diversity of microbial flora in different samples was stratified by geographic region.

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