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Research of microbial quality and hygiene of Koumiss koji (assets) of Mongolia

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Abstract - The objective of this paper was to study the microbial quality characteristics and diversity of Koumiss koji (fermented dairy product from mare's milk) in four different regions of Mongolia, which provides data for a comprehensive understanding of the composition of acid Koumiss koji (assets) and lays a foundation for the development and utilization of strain resources in traditional fermented dairy products. Using High-throughput sequencing of the V3–V4 regions of the 16S rRNA gene, we determined the community composition of bacteria from 12 samples of Koumiss koji (assets) sampled from four different areas of Mongolia: Bulgan province, Arkhangai province, Uvurkhangai province, and Tuv province. Alpha diversity analysis showed that there were significant differences in the bacterial diversity of Koumiss koji (assets) from four different regions ($P < 0.05$). Bacterial diversity was most important in samples from the Tuv province, while species richness was highest in samples from the Bulgan province. Bacterial community composition analysis revealed 16 bacterial species in the 12 samples, of which Firmicutes and Proteobacteria were predominant in all four regions. There were 117 bacterial genera, of which Lactobacillus was predominant in all four regions. The results demonstrate that bacterial diversity in Koumiss koji (assets), the traditional starter cultures for Koumiss, from the four different regions of Mongolia, is related to geographical location.

Keywords - Koumiss koji (assets), Bacteria, Microbial diversity, High-throughput sequencing

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1. INTRODUCTION

Koumiss, also known as sour mare's milk, tsegee, airag, Koumiss, etc., contains many probiotics, with lactic acid bacteria and yeast content being the most abundant, and is a healthy beverage [1]. In Mongolia, the fermentation and production of Koumiss have a history of hundreds of years. It is popular in Central Asia, Mongolia, Xinjiang, and Inner Mongolia due to its unique taste, therapeutic and health benefits, and high levels of nutrients [2]. The popular people of Mongolia like to drink mare's milk and Koumiss (fermented dairy products), but they rarely know their importance. Koumiss is rich in nutrients, such as vitamins, amino acids, proteins, lactose, minerals, etc., and a small amount of ethanol (alcohol), ultimately forming a healthy and delicious drink that can treat certain diseases. Koumiss is also very rich in unsaturated fatty acids, including oleic acid. Oleic acid prevents atherosclerosis, increases insulin release, reduces glucose release from the body, improves the immune system, and protects against some types of cancer [3].

Koumiss is usually brewed using traditional methods in summer and autumn. It is one of the most essential daily foods for Mongolian people in summer and a high-quality product with health care effects. Herds of horses are generally less unsusceptible to tuberculosis, brucellosis, and other livestock diseases, so mare's milk is relatively clean and pure [4].

Koumiss is very popular in Mongolia, and the provinces of Arkhangai, Uvurkhangai, Bulgan, Tuv, and Dundgobi are famous and rich in high-quality Koumiss. The composition of the microbiota in the Koumiss koji (assets) plays a crucial role in the quality of the final product. Fermented Koumiss for several years in a row, the Khukhuur (made from cowhide) has well absorbed the nutrients of the Koumiss; therefore, special funds are not required. The inheritors of the "Use of the Khukhuur fermentation Koumiss" are in the fermented provinces of Arkhangai, Bulgan, Uvurkhangai, Tuv, and Dundgobi. These regions' peoples use traditional fermentation methods, which have been recognized in the area and the region.

In this study, samples of Koumiss koji (assets) from four major koumiss-producing areas (Arkhangai province Khotont sum, Bulgan province Mogod sum, Uvurkhangai province Khujirt sum, Tuv province Batsumber) in Mongolia were collected, and high-throughput sequencing technology was used to determine the bacterial community composition and structure. These data are essential for further investigation of the microbial resources in Mongolia's traditional fermented dairy products and for the commercial production of Koumiss koji (assets).

The content of nutrients in Koumiss and microbial composition, acidity, and its medical effect directly depend on the design, cleanliness of fermentation vessel, fermentation time, fermentation temperature, storage container material, etc., [5].

2. THEORETICAL BACKGROUND

The pure culture and symbiotic microorganisms of Mongolian Koumiss koji (assets) are stored in the national gene bank and can be used when needed. People in Mongolian's Bulgan, Arkhangai, Uvurkhangai, Tuv, Dundgovi, and other province regions are accustomed to making Koumiss koji (assets) using traditional methods. Baldorj. R [6] isolated microorganisms from Koumiss koji (assets) in the above areas and mainly studied the diversity and physiological and

biochemical characteristics of *Lactobacillus, Escherichia coli, Streptococcus,* alcohol-oxidizing lactose, fermenting and non-fermenting yeasts. Professor Baldorj. R pointed out in his research that the microorganisms of Koumiss koji (assets) comprise *L. Bulgaricus, L. Casei,* and *Lactococcus lactis subsp. Cremoris,* lactic acid bacteria, decomposed or undecomposed yeast, propionic acid, and acetic acid bacteria [7].

Mongolian scientists Indra. R and Baldorj. R [8] researched Koumiss's components, fermentation technology, microorganisms, and nutritional aspects. The results show that using natural resources as fermentation primers to make Koumiss koji (assets) is superior to the processing technology of pure culture and symbiotic microorganisms. This is related to many biotechnological factors, such as the buffer solvent of Koumiss and the microbial compatibility of Koumiss koji (assets) substance. This result provides an essential basis for individual producers and production companies of milk and dairy products to select raw materials of koji (assets) [9].

2.1 DEFINITIONS OF THE KOUMISS KOJI (ASSETS)

The use of starter cultures has long been a custom of Mongolians. The indigenous starter culture used to make traditional fermented dairy products is Koumiss koji (assets). Since ancient times, a conventional method for fermentation of Koumiss has been utilizing a khukhuur (made from cowhide), and next year a leather bag in which Koumiss from the previous year is dried. When fresh mare’s milk becomes available in the next year, it is added to the made cowhide leather bag, and fermentation can proceed. Alternatively, freshly collected mare’s milk is poured into a fermentation tank, and then an appropriate amount of Koumiss koji (assets) is introduced to it, and fermentation is allowed to proceed. Studies have shown that Koumiss koji (assets) contains a complex community of microorganisms that strongly influences the resulting koumiss's final microbial community and quality [10, 11].

Until today, there have been no reports on the microbial diversity of the Koumiss koji (assets) used in Koumiss production in different regions of Mongolia. As the main production area for koumiss, it is especially important to analyze the bacterial diversity of Koumiss koji (assets) from Mongolia. Traditional bacterial culture methods are slow and laborious, and it is easy to ignore the total biodiversity because some uncultured species are present in the sample. For this reason, the analysis of microbial diversity using traditional methods is often limited. In recent years, developing second-generation sequencing techniques, such as the Illumina platform, has provided a convenient way to analyze the microbial composition in various samples. The low cost of high-throughput sequencing means it is now possible to detect microorganisms that are difficult to culture and low in abundance [12].

2.2 OVERVIEW OF THE SURVEY RESEARCH ON MONGOLIAN KOUMISS KOJI (ASSETS)

For more than 100 years, from 1881 to 1981, many experts such as Bil (Биль), Sorokin (Сорокин), and Grigiriev (Григириев) et al. made contributions to the research. Finally, they concluded that the microorganisms of Koumiss, cow's milk fat-free fermented milk, and koji (assets) microorganisms of fermented milk drinks have common characteristics [13].

Tsion (Цион 1948), Bogdanova (Богданова 1962), Korolev (Королев 1966), Kvasnikov (Квасников 1975), and other experts and professors studied the structure and morphology of

lactobacillus and colonies in Mongolian traditional Koumiss koji by physiological and biochemical analysis. Judging from the research conclusions of these multiple scholars, they identified one of the dominant strains as *Lactobacillus bulgaricus Mon-75* [14].

Since 2009, Mongolian scientists have been collaborating with Chinese and Russian scientists to identify further the DNA structure of the lactic acid bacteria in Koumiss koji and determine the characteristics of the microorganisms and probiotics. In 2009, Z. H. Sun and W. J. Liu collected samples of dairy products, yogurt, and Koumiss made by herders using traditional methods in my country's Ulaanbaatar city, Uvurkhangai, Govi-Altai province, and other regions, the dominant strains of lactic acid bacteria contained in them were determined. Research results show that the dominant strains in Koumiss koji are *Lactobacillus helveticus*, and the dominant strains in Koumiss are *Lactobacillus helveticus* and *Lactobacillus fermentum* [15].

3. MATERIALS OF METHODS

3.1 THE KOUMISS KOJI (ASSETS) SAMPLES AND MAIN CULTURE MEDIUM

They were collected from 12 herders' houses, including Arkhangai province Khotont sum, Bulgan province Mogod sum, Uvurkhangai province Khujirt sum, and Tuv province Batsumber sum in Mongolia. Koumiss koji (assets) fermented using the traditional method is a basis for Koumiss. It was transported to the School of Food Science and Engineering Laboratory, Inner Mongolia Agricultural University, through cryogenic preservation for analysis and research. The collection, sample codes, and grouping of samples of Koumiss koji (assets) are shown in Table 1.

Table 1. Sample code and grouping of Koumiss Koji

Sampling provinces name	Sample code	Group name
Bulgan province	BEG, BEG1, BEG2	B group
Arkhangai province	HHA, HHA1, HHA2	H group
Uvurkhangai province	QHA, QHA1, QHA2	Q group
Tuv province	ZY, ZY1, ZY2	Z group

Also, nutrient medium MRS agar and MRS broth, Skim milk medium, YPD (YEPD) agar and YPD (YEPD) broth, Bengal red medium extract, bacteriological peptone, glucose, and agar were used.

3.2 MICROBIOLOGICAL LIMITS OF MONGOLIAN KOUMISS KOJI (ASSETS)

According to the detection method of "Fermented Milk" (GB 19302-2010), the coliform bacteria (*Escherichia coli*), *Staphylococcus aureus*, *Salmonella*, Yeast, mold (Mucedine), and other microbial limits. The "Fermented Milk" (GB 19302-2010) uses the method to limit the quantity of Microorganism of Koumiss koji (assets), as shown in Table 2.

Table 2. Limit the quantity of Microorganism

Project	Sampling scheme [a] and limit (if not specified, expressed in CFU/g or CFU/mL)				Inspection method
	n	C	m	M	
Escherichia coli	5	2	1	5	GB 4789.3 Plate counting method
Staphylococcus aureus	5	0	0/25g (mL)	—	GB 4789.10 Qualitative test
Salmonella	5	0	0/25g (mL)	—	GB 4789.4
Yeast ≤			100		GB 4789.15
Mucedine ≤			30		

Note: The analysis and processing of [a] sample shall be carried out following GB 4789.1 and GB 4789.18. "—" means it cannot be detected.

The number of lactic acid bacteria in Koumiss koji (assets) samples collected from different regions was determined according to the Lactobacillus Test of Food Microbiology (GB 4789.35-2016). The Quantity of Lactic Acid Bacteria of Koumiss koji (assets) uses the identification method of Lactobacillus Test of Food Microbiology (GB 4789.35-2016) is shown in Table 3.

Table 3. Quantity of Lactic Acid Bacteria

Project	limit the quantity [CFU/g(mL)]	Inspection method
Lactic acid bacteria quantity [a] ≥	1×10^6	GB 4789.35

Note: [a] there is no requirement for the count of lactic acid bacteria for products that have been heat-treated after souring.

3.3 STUDY ON THE MICROBIAL DIVERSITY IN MONGOLIAN KOUMISS KOJI (ASSETS)

3.3.1 EXTRACTION OF TOTAL DNA FROM KOUMISS KOJI (ASSETS)

Cell isolation and total DNA extraction: 2 mL of the Koumiss koji (assets) samples were taken and placed in a 50 mL centrifuge tube, denoted as tube 1. 10 mL phosphate buffer saline (PBS) was added to the line and oscillated for 5 min to form a bacterial suspension, and then centrifuged at 5000 r/min for 5 min. Transfer the supernatant to a new 50 mL centrifuge tube, tube 2. Add 10 mL PBS into tube 1, shake for 1 min, centrifuge for 5000 r/min, and then transfer the supernatant into tube 2. Repeat the steps to add PBS to tube 1 until the supernatant transferred to tube 2 becomes clear; Centrifuge tube 2 (5000 r/min, 5 min) to take the precipitation, that is, the microbial flora in the Koumiss koji (assets). Fast DNA SPIN Kit extracted DNA for Soil. The extracted genomic DNA was detected by 1% agarose gel electrophoresis.

3.3.2 PCR AMPLIFICATION AND SEQUENCING OF TARGET GENE

Take an appropriate amount of sample in a centrifuge tube and dilute the sample with sterile water to 1 ng/μL. Using the diluted genomic deoxyribonucleic acid as a template, the V3-V4 region of the 16S rRNA gene of bacteria and the ITS genome of fungi were amplified by PCR according to the selection of sequencing region.

The V3-V4 region of bacteria was amplified using a universal primer (338F/806R), whose name and sequence were:

338F	ACTCCTACGGGAGGCAGCAC
806R	GGACTACHVGGGTWTCTAAT

A primer (ITS1F/ITS1R) was used to amplify the fungal ITS sequence. The name and line of the primer were:

ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'
ITS1R	5'-GCTGCGTTCT-TCATCGATGC-3'

PCR amplification conditions: pre-denaturation at 98°C for 3 minutes, denaturation at 98°C for 45 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 45 seconds, 35 cycles of reaction at 72°C and extension for 8 minutes, and storage at -20°C.

3.3.3 DATA ANALYSIS AND CHART DRAWING

MiSeq sequencing obtains paired-end sequence data. First, the paired reads are spliced (merged) into one sequence based on the overlap relationship between PE reads. At the same time, the quality of the reads and the splicing effect are quality-controlled and filtered. Based on the start and end of the sequence, The end barcode and primer sequences are used to distinguish samples to obtain adequate arrangements, and the sequence direction is corrected to obtain optimized data. Clustering operations divide lines into many groups according to their similarities. One group is an OUTs. All arrangements are divided into OTUs according to different similarity levels. Usually, biological information is performed on OTUs at the 97% similarity level. Scientific, statistical analysis, using the Rank-Abundance curve to indicate species abundance and species evenness, using the RDP classifier Bayesian algorithm to perform taxonomic analysis on OTUs representative sequences with a 97% similarity level, and classifying them at each classification level (such as domain, boundary, phylum, class, order, family, genus, species, OTUs, etc.) to count the community composition of each sample. The MOTHUR was used to calculate the Alpha diversity index under different random models, and R language tools were used to make dilution curves and Venn diagrams. The curves were constructed using each sample's microbial Alpha diversity index at different sequencing depths. This reflects the microbial diversity of each instance at different sequencing quantities and counts the number of standard and unique species in multiple groups or samples. QIIME calculated the Beta diversity distance matrix for hierarchical clustering analysis, used the UPGMA algorithm to construct a tree structure, and used the Kruskal-Wallis rank sum test to analyze significant differences between groups.

4. RESULT AND ANALYSIS

4.1 MICROBIOLOGICAL LIMITS OF MONGOLIAN KOUMISS KOJI (ASSETS)

The limits of microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, Yeast, and Mucedine are determined according to the detection method of "Fermented Milk" (GB 19302-2010). The results of the Microorganism indexes of Koumiss Koji (assets) are shown in Table 4.

From Table 4, it can be seen that the number of viable yeasts in 12 samples of Koumiss koji (assets) distiller's yeast from four different regions ranged from (5.6-6.5) × 10^4 to (5.7-6.7) × 10^6 CFU/mL. This result is consistent with most reports agree.

Mu et al. [16] detected a viable yeast count of 10^5-10^7 CFU/mL in Koumiss. Ni Huijuan [17] found that the number of yeasts in traditional fermented dairy products in Xinjiang and Qinghai areas of China was 10^4-10^6 CFU/mL. However, no mold was detected in the samples of Koumiss koji (assets) from four different regions. The reason may be that the total acidity of Koumiss koji (assets) is high and unsuitable for mold growth and reproduction.

According to research reports, although the dominant bacterial groups in Koumiss are lactic acid bacteria and yeast, it should not be ignored that Koumiss also contains a small number of spoilage bacteria and harmful microorganisms, such as *Klebsiella pneumoniae*, *Salmonella* from chicken, *Escherichia hermannii*, *Escherichia coli*, *Shigella dysenteriae* [18]. Pollution of harmful bacteria may be related to climate, pasture and home environment, processing conditions, and human introduction. However, coliform and *Staphylococcus aureus* were not detected in the samples from four different regions.

Table 4. Microorganism indexes of Koumiss Koji

Project	Sample ID			
	B group	H group	Q group	Z group
Escherichia coli 0/25g (mL)	-	-	-	-
Staphylococcus aureus 0/25g (mL)	-	-	-	-
Salmonella 0/25g (mL)	-	-	-	-
Yeast (CFU/mL) ≤	(5.7-6.7)×10^6	(5.6-6.5)×10^4	(5.7-7.1)×10^5	(5.3-6.7)×10^5
Mucedine (CFU/mL) ≤	-	-	-	-

Note: "-" is not detected

According to the "Food Microbiology Test - Lactic Acid Bacteria Test" (GB 4789.35-2016), the lactic acid bacteria quantity of Koumiss koji (assets) samples from different regions was determined. The results of the Lactic Acid Bacteria quantity of Koumiss Koji (assets) are shown in Table 5.

It can be seen from Table 5 that the quantity of viable lactic acid bacteria in 12 samples of Koumiss koji (assets) from four different sampling points ranged from (2.9-5.6) × 10^6 to (1.5-2.6) × 10^7 CFU/mL. This result is consistent with most reports. Watanabe et al. [19] studied traditional fermented Koumiss and found that the quantity of viable lactic acid bacteria and yeast in Koumiss was about 10^7 CFU/mL. Ishii et al. [20] determined the microorganisms of Koumiss in three areas of nomadic nationality in

Mongolia, and the count of lactic acid bacteria ranged from 1.26×10^7 CFU/mL to 7.94×10^8 CFU/mL. Although Koumiss inhibits harmful bacteria at a particular fermentation stage and reaches a specific pH value, some probiotics in Koumiss can also inhibit harmful bacteria. Still, the count of lactic acid bacteria is about one logarithm higher than that of yeast. The composition of microorganisms in Koumiss is affected by various factors such as region, natural environment, herdsman's families, production methods, seasons, fermentation periods, etc.

Table 5. Lactic Acid Bacteria quantity of Koumiss Koji (assets)

Sample ID	B group	H group	Q group	Z group
Quantity of lactic acid bacteria (CFU/mL)	$(1.5\text{-}2.6)\times10^7$	$(2.9\text{-}5.6)\times10^6$	$(8.8\text{-}9.7)\times10^6$	$(9.6\text{-}9.9)\times10^6$

4.2 STUDY ON THE MICROBIAL DIVERSITY IN MONGOLIAN KOUMISS KOJI (ASSETS)

4.2.1 SEQUENCING OF 16S rRNA V3-V4 REGION OF BACTERIA IN KOUMISS KOJI (ASSETS)

By using high-throughput sequencing technology, the 16S rRNA V3-V4 regions of bacteria in 12 samples of Koumiss koji (assets) prepared by traditional methods collected from Bulgan province Mogod sum, Arkhangai province Khotont sum, Uvurkhangai province Khujirt sum, and Tuv province Batsumber sum in Mongolia were sequenced as shown in Figure 1.

As can be seen from Figure 1, a total of 465 OTUs were obtained from 12 samples of Koumiss koji (assets) from four different regions and 154 OTUs were obtained from the B group, H group contains 99 OTUs, Q group 99 OTUs, Z group has 113 OTUs.

The OTUs number of group B bacteria was the highest, indicating the most abundant species. In contrast, the OTUs number of group Z bacteria was relatively low, indicating the rather large bacteria species. The remaining OUTs of group H and group Q were the same at 99, indicating little difference in richness and high uniformity in this region.

The number of optimized sequences obtained from 12 samples from four different regions was 577699, and the average length was 447bp. All arrangements were classified into OTUs with 97% similarity and were copolymerized into 135 OTUs, totaling 465 OTUs. The results were as follows: 1 domain, 1 kingdom, 16 phyla, 31 classes, 56 orders, 74 families, 98 genera, 117 species.

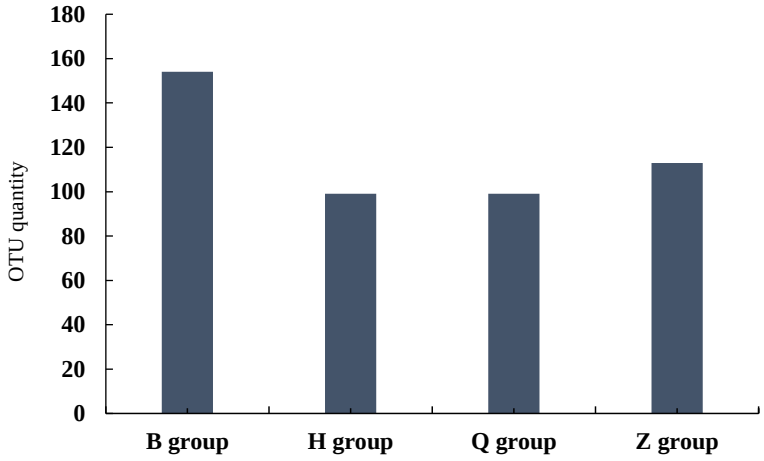

Fig. 1 Quantity and Distribution of OTUs in Bacterium

4.2.2 ABUNDANCE ANALYSIS OF BACTERIA IN KOUMISS KOJI (ASSETS)

The results of bacterial abundance analysis in 12 samples of Koumiss koji (assets) from four regions in Mongolia are shown in Figure 2. According to the abundance grade curve of bacteria in Figure 2, it can be seen that the sample curve span of groups Z, Q, and H is small. The curve's slope is significant, indicating that the abundance difference among OTUs is substantial, the uniformity is low, the bacterial composition is relatively simple, and the dominant strains are apparent. The curve span of the BEG1 region (indicating grade red curve) in group B is extensive, meaning that the abundance and uniformity among OTUs are high, and the dominant strains are not apparent.

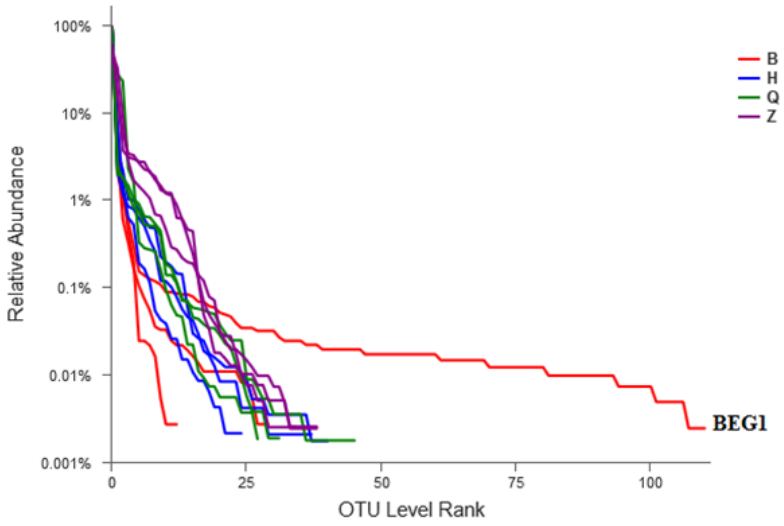

Fig. 2 Fungal Abundance Grade Curve of Koumiss Koji

4.2.3 ANALYSIS OF ALPHA DIVERSITY OF THE BACTERIAL COMMUNITY IN KOUMISS KOJI (ASSETS)

The results of the Alpha diversity analysis of the bacterial community in Koumiss koji (assets) are shown in Table 6. As can be seen from Table 6, the Shannon value of bacteria in 12 samples from four different sampling points ranged from 0.271063 to 1.620478. The ACE index values ranged from 15.39158 to 111.801944. Chao index values range from 14.5 to 111.857143. Coverage values ranged from 0.99985 to 0.999982, and Simpson values ranged from 0.327756 to 0.90097, indicating rich bacterial species. Moreover, it can be seen from the Shannon curve in Figure 3 that the number of OTUs tended to flatten out when it reached 1000. This indicates that the sequencing depth can satisfy the samples' microbial diversity study.

Table 6. Diversity Analysis of Alpha Bacterium

Sample ID	Alpha diversity				
	Shannon index	ACE index	Chao index	Coverage	Simpson
B group	0.36±0.10^{a}	52.75±51.73^{b}	52.62±52.00^{c}	1.00±0.00^{d}	0.87±0.03^{e}
H group	0.49±0.09^{a}	39.96±10.80^{b}	37.56±9.22^{c}	1.00±0.00^{d}	0.82±0.03^{e}
Q group	0.81±0.43^{a}	39.44±14.18^{b}	37.73±12.95^{c}	1.00±0.00^{d}	0.65±0.28^{e}
Z group	1.56±0.08^{a}	42.86±0.41^{b}	42.83±3.33^{c}	1.00±0.00^{d}	0.36±0.02^{e}

Note: Average ±SD; Significant differences between letters (P<0.05).

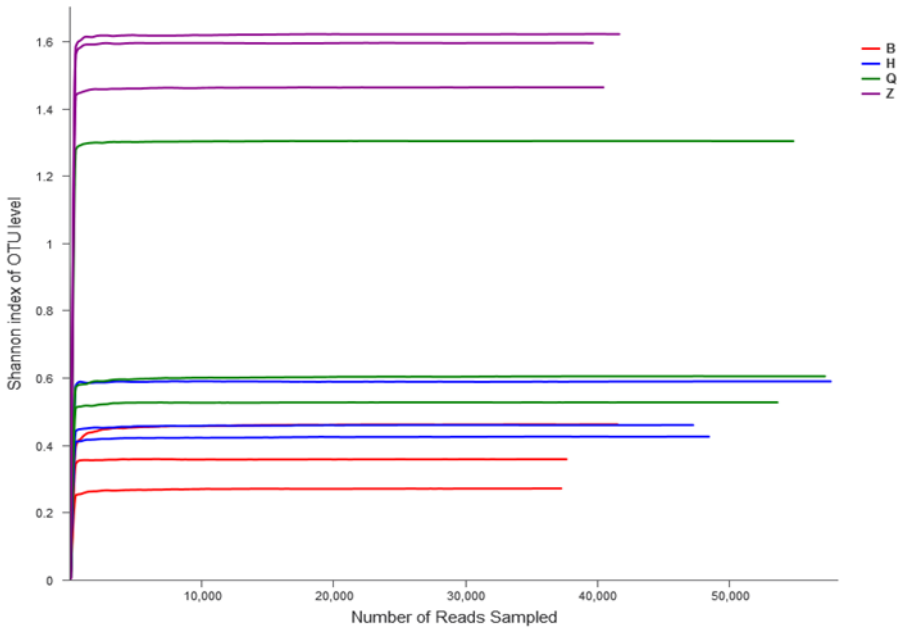

Fig. 3 Shannon dilution curve of Bacterium

4.2.4 ANALYSIS OF BACTERIAL COMMUNITY COMPOSITION KOUMISS KOJI (ASSETS)

Venn diagrams can count the number of standard and unique species in multiple groups or samples. They can more intuitively express the similarity and overlap of the species number composition of environmental samples. Different colors represent different groups, overlapping parts represent species common to multiple groups, non-overlapping parts represent species unique to that group, and numbers represent the number of corresponding species. The results of the Venn diagram analysis of the bacterial community composition in the Koumiss koji (assets) are shown in Figure 4. As can be seen from Figure 4, there are 3 OTUs unique to group Q, 2 OTUs unique to group H, 67 OTUs unique to group B, 1 OTUs unique to group Z, and 27 OTUs shared by the four regions.

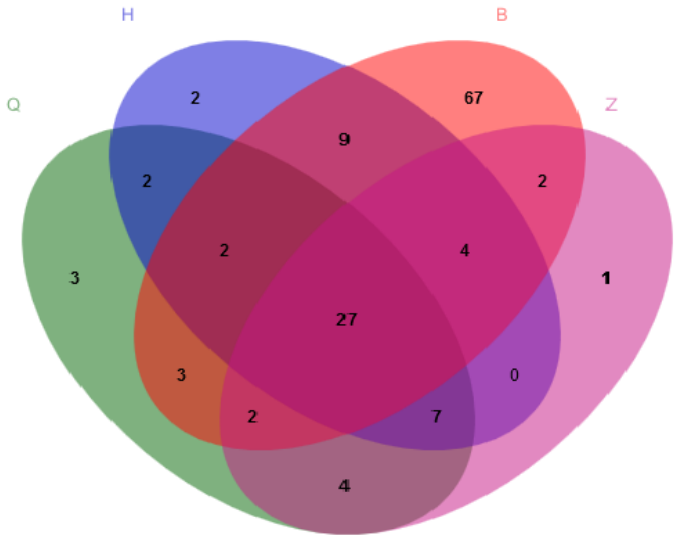

Fig. 4 OTUs sample distribution Venn of Bacterium

4.2.5 ANALYSIS OF BACTERIAL DIFFERENCES BETWEEN DIFFERENT REGIONS

The analysis results of the difference of bacteria at the genus level in the samples of Koumiss koji (assets) from different regions of Mongolia are shown in Figure 5. As can be seen from Figure 5, the significant difference test between groups uses strict statistical methods based on the obtained community abundance data to conduct hypothesis testing on species between different groups (or samples) of microbial communities to evaluate the significance of species abundance differences. Level to obtain significant differences between groups (or models).

Kruskal-Wallis rank sum test showed that the bacterial diversity of the from the four regions Koumiss koji (assets) samples was in *Lactobacillaceae*, *Streptococcaceae*, *Enterobacteriaceae*, and *Lactobacillaceae*. There was a significant difference between *Leuconostocaceae* and *Bifidobacteriaceae* ($0.01<P\leq0.05$).

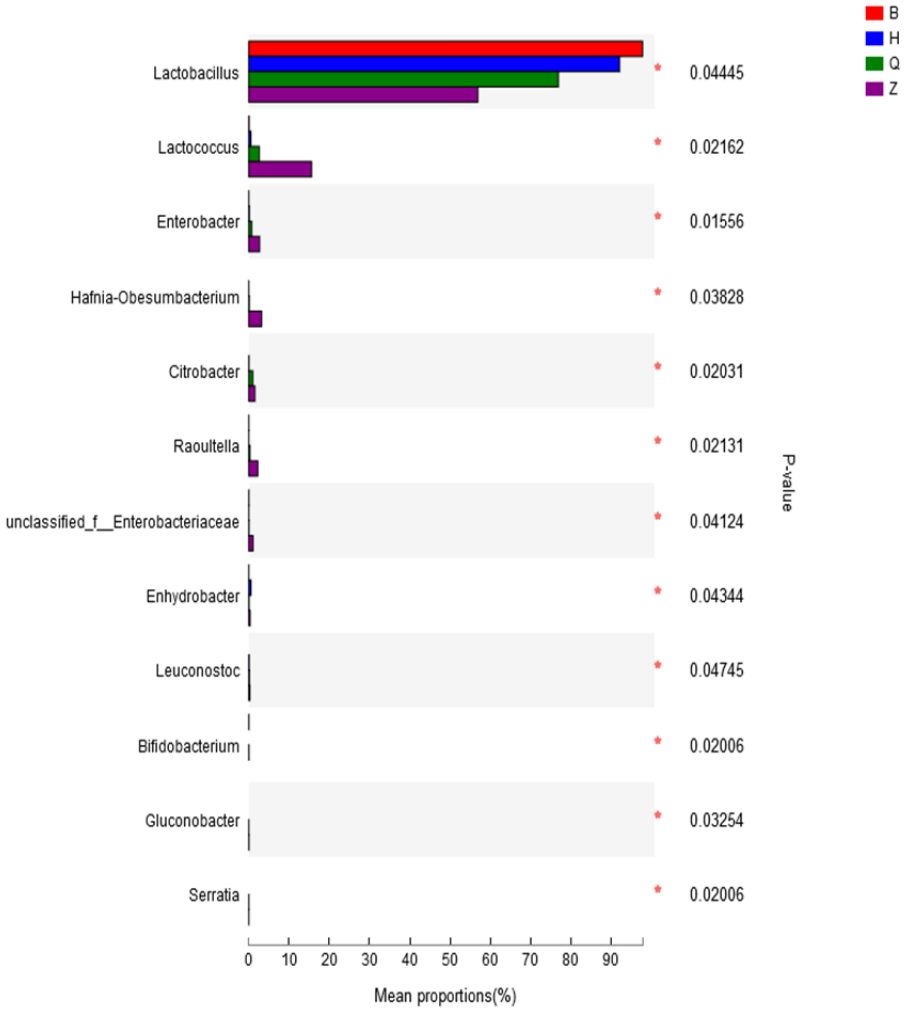

Fig. 5 Kruskal-Wallis Rank Sum Test at Classification Level of Bacteria Genus

Note: The vertical axis indicates the species name under a taxonomic level, the column length corresponding to the species tells the average relative abundance of the species in various groups, and different colors indicate different groups. The rightmost is the P value, * represents $0.01<P\leq0.05$.

4.2.6 SEQUENCING OF ITS REGION OF FUNGI IN KOUMISS KOJI (ASSETS)

The ITS region sequencing results of 12 samples of Koumiss koji (assets) are shown in Figure 6. As can be seen from Figure 6, a total of 278 OTUs were obtained from 12 samples of Koumiss koji (assets) from four regions, and there were 48 OTUs in group B, group H had 117 OTUs, and group Q had 81 OTUs, group Z had 32. The OTUs number of group H fungi was the highest, indicating the most abundant fungal species, while the OTUs number of group Z fungi was the lowest, indicating the relatively large fungal species. The number of OTUs in the remaining two groups was the most similar, with 48 and 81, respectively, indicating little difference in richness and high uniformity in this region. A total of 798,972 optimized sequences were obtained from 12 Koumiss koji (assets) samples from four different regions, with an average length of 275 bp. All arrangements were classified into 121 OTUs with 97% similarity, and the total OTUs was 278. The results were as follows: 1 domain, 1 kingdom, 5 phyla, 13 classes, 33 orders, 53 families, 73 genera, 88 species.

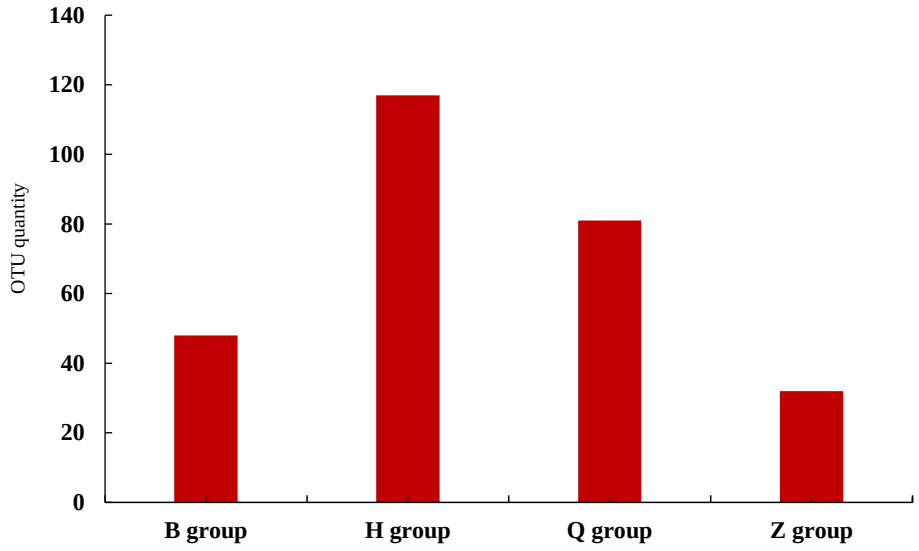

Fig. 6 Quantity and Distribution of OTUs in Fungal

4.2.7 ABUNDANCE ANALYSIS OF FUNGI IN KOUMISS KOJI (ASSETS)

The results of fungal abundance analysis in 12 samples of Koumiss koji (assets) from four regions of Mongolia are shown in Figure 7. According to the fungal abundance grade curve in Figure 7, it can be seen that the sample curves of group Z, group B, and group Q have a small span and almost steep decline, indicating that the abundance difference among OTUs is significant, the uniformity is low, the composition of bacteria is relatively simple, and the dominant strains are apparent, while the curve of HHA1 region of group H (indicating the grade curve blue) is wide and flat, and the decline is slow. The results showed that the abundance and uniformity of OTUs were high, and the dominant strains were not evident. The sample distribution of groups Z, B, and Q was concentrated, and 2 of the 3 samples in group H were clustered together, consistent with the overall OTUs sample distribution diagram.

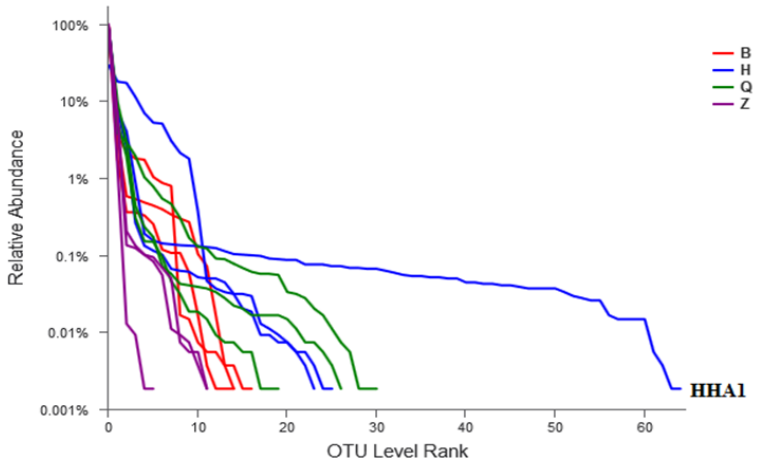

Fig. 7 Fungal Abundance Grade Curve of Koumiss Koji

4.2.8 ANALYSIS OF ALPHA DIVERSITY OF THE FUNGAL COMMUNITY IN KOUMISS KOJI (ASSETS)

The results of the Alpha diversity analysis of the fungi community in Koumiss koji (assets) are shown in Table 7. As can be seen from Table 7, the Shannon value of fungi in 12 samples from four different sampling points ranged from 0.206857 to 2.002307. ACE index values ranged from 12 to 65.945381, The Chao index value from 12 to 65.5, Simpson values ranged from 0.170506 to 0.973886, and Coverage values of 0.999943 to 1 indicate abundant fungal species. From the Shannon curve in Figure 8, it can be seen that the number of OTUs tends to flatten out when it reaches 1000, which indicates that the depth of this sequencing can satisfy the study of microbial diversity in the sample.

Table 7. Diversity Analysis of Alpha Fungal

Sample ID	Alpha Diversity				
	Shannon index	ACE index	Chao index	Coverage	Simpson
B group	0.39±0.19^{a}	22.07±6.18^{b}	17.33±2.08^{c}	1.00±0.00^{d}	0.86±0.08^{e}
H group	1.09±0.81^{a}	40.17±22.36^{b}	40.00±22.12^{c}	1.00±0.00^{d}	0.56±0.34^{e}
Q group	0.53±0.14^{a}	28.77±5.94^{b}	28.28±6.42^{c}	1.00±0.00^{d}	0.79±0.05^{e}
Z group	0.21±0.12^{a}	17.30±8.40^{b}	12.67±1.15^{c}	1.00±0.00^{d}	0.91±0.06^{e}

Note: Average ±SD; Significant differences between letters (P<0.05).

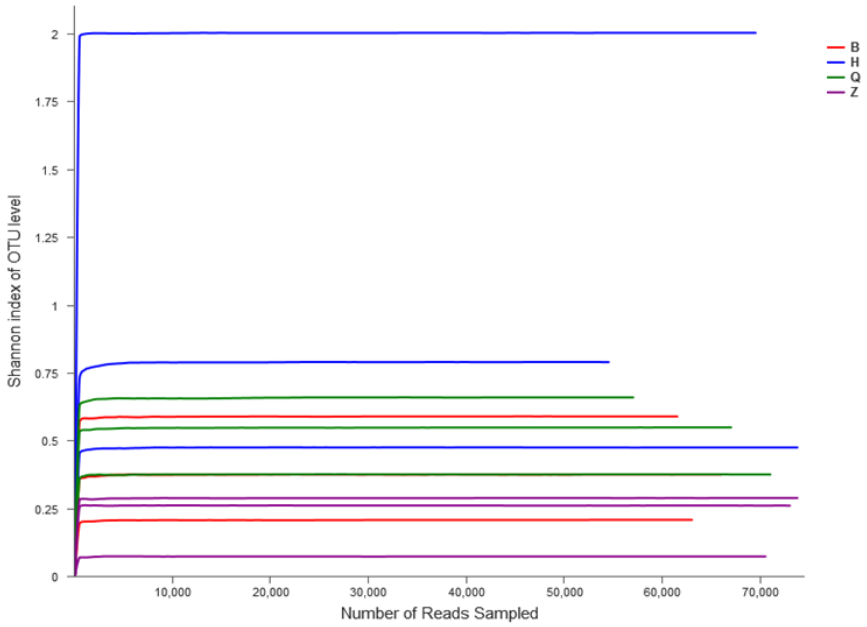

Fig. 8 Shannon dilution curve of Fungal

4.2.9 ANALYSIS OF COMMUNITY COMPOSITION OF THE FUNGI SAMPLES IN KOUMISS KOJI (ASSETS)

Venn diagram analysis results of community composition of fungi samples in Koumiss koji (assets) are shown in Figure 9. As can be seen from Figure 9, there are 22 unique OTUs in group Q, 46 unique OTUs in group H, 7 unique OTUs in both groups B, and 6 OTUs in group Z are common in the four regions, indicating that the strains in these four regions have low similarity and apparent differences.

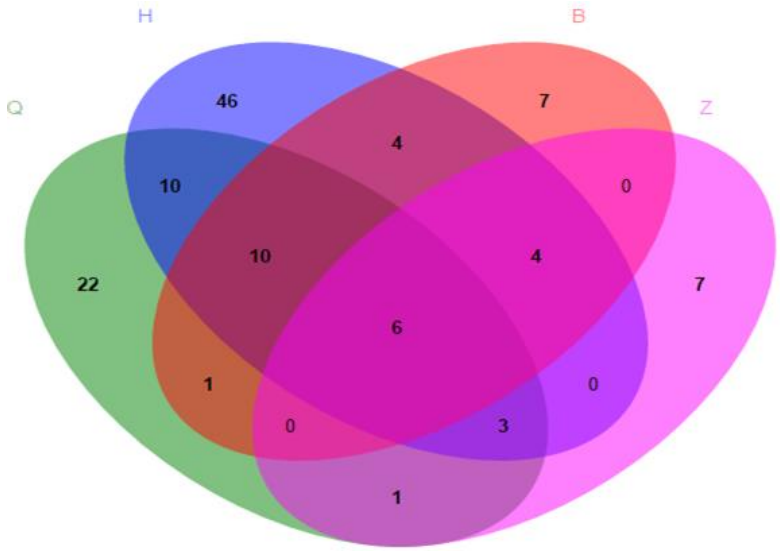

Fig. 9 OTUs sample distribution Venn of Fungal

4.2.10 ANALYSIS OF FUNGAL DIFFERENCES AMONG DIFFERENT REGIONS

The difference in the analysis results of fungi at the genus level in samples of Koumiss koji (assets) from different regions of Mongolia are shown in Figure 10. It can be seen from Figure 10 that at the genus classification level, the fungal diversity of the Koumiss koji samples from the four regions is in the genera *Kluyveromyces*, *Dekkera*, *Kazachstania*, and *Penicillium*. There is a significant difference in the genus *Penicillium* ($0.01<P\leq0.05$).

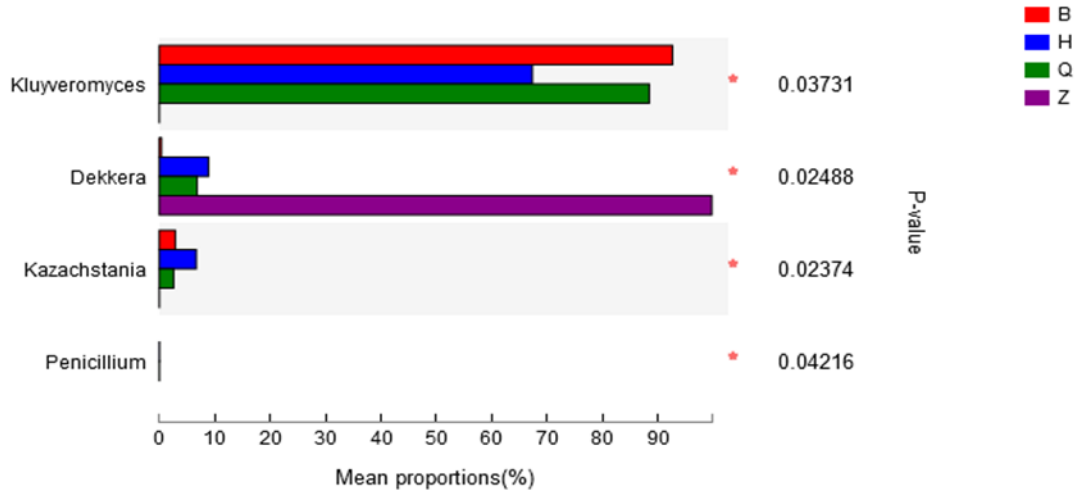

Fig. 10 Kruskal-Wallis Rank Sum Test at Classification Level of Fungus Genus

Note: The vertical axis indicates the species name under a taxonomic level, the column length corresponding to the species tells the average relative abundance of the species in various groups, and different colors indicate different groups. The rightmost is the P value, * represents 0.01<P≤0.05.

The difference in the analysis results of fungi at the family level in samples of Koumiss koji (assets) from different regions of Mongolia are shown in Figure 11. The results of the Kruskal-Wallis rank-sum test show that at the family classification level, the fungal diversity of the Koumiss koji (assets) samples from the four regions is significant in Saccharomycetaceae, Pichiaceae, and Trichocomaceae. There were substantial differences in Trichocomaceae (0.01<P≤0.05).

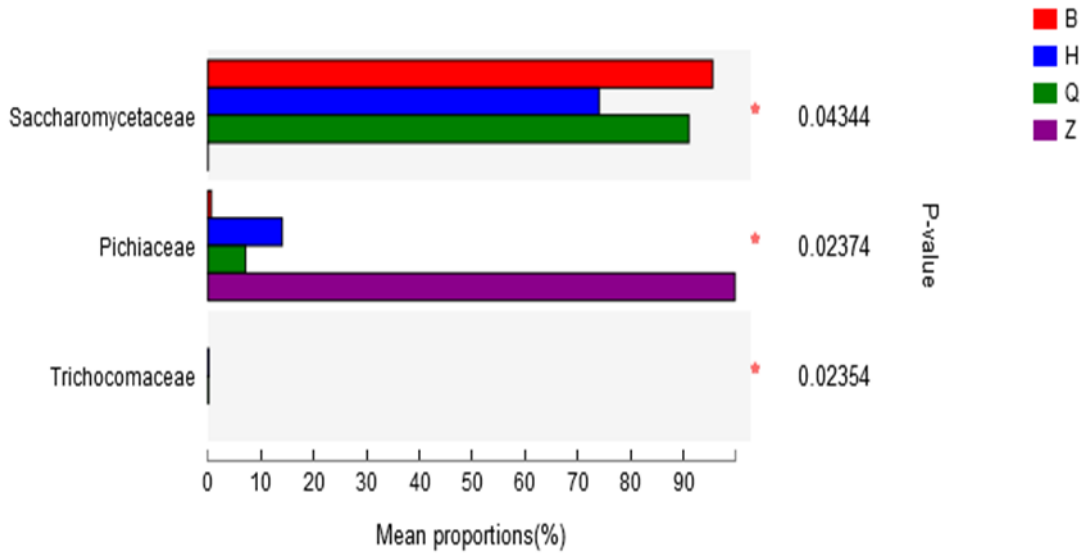

Fig. 11 Kruskal-Wallis Rank Sum Test at Family Classification Level of Fungal

Note: The vertical axis indicates the species name under a taxonomic level, the column length corresponding to the species tells the average relative abundance of the species in various groups, and different colors indicate different groups. The rightmost is the P value, * represents 0.01<P≤0.05.

5. CONCLUSION

Koumiss is a rich, nutritious, fermented dairy product with medical properties. The composition of the microbiota in the Koumiss koji (assets) plays a crucial role in the quality of the final product. The results demonstrate bacterial diversity in Koumiss koji (assets), the traditional starter cultures for Koumiss, from different regions of Mongolia, is related to geographical location. In summary, the Koumiss Koji (assets) in four different regions show microbial diversity, and this experiment has a clearer understanding of traditional fermented dairy products in Mongolia, which provides reference significance for future research.

Based on the study of microbial quantity and microbial diversity of traditional fermented Koumiss koji (assets) in four different regions of Mongolia, the main contents and results are as follows: The number of lactic acid bacteria in the samples of Koumiss koji (assets) from four different regions is that the number of lactic acid bacteria in the samples of group B is between (1.5-2.6) × 10^7 CFU/mL; the number of lactic acid bacteria in the samples of group H is between (2.9-5.6) × 10^6 CFU/mL; the number of lactic acid bacteria in the samples of group Q is between (8.8-9.7) × 10^6 CFU/mL; the number of lactic acid bacteria in the samples of group Z is between (9.6-9.9) × 10^6 CFU/mL;

The number of yeasts in the Koumiss koji (assets) samples from four different regions was between group B (5.7-6.7) × 10^6 CFU/mL; group H (5.6-6.5) × 10^4 CFU/mL; group Q (5.7-7.1) × 10^5 CFU/mL; Z group (5.3-6.7) × 10^5 CFU/mL. Generally speaking, the total number of lactic acid bacteria is higher than that of yeast.

The following conclusions were drawn using high-throughput sequencing technology data analysis: The number of optimized sequences obtained from 12 samples was 577699, with an average length of 447bp and a total of 465 OTUs. A total of 1 domain, 1 kingdom, 16 phyla, 31 classes, 56 orders, 74 families, 98 genera, and 117 species were detected. The number of optimized sequences obtained by fungi was 798972, with an average length of 275bp and 278 OTUs. Detected: 1 domain, 1 kingdom, 5 phyla, 13 classes, 33 orders, 53 families, 73 genera, and 88 species.

The research found that bacterial diversity differs in the four regions, and the microbial communities and compositions of the four areas differ. Among them, the abundance of group B is relatively high, and the bacterial diversity is high. The inter-group difference results showed that at the family classification level, the bacterial diversity of the Koumiss koji (assets) samples from the four regions was in the *Lactobacilliaceae*, *Streptococcus*, *Enterobacteriaceae*, *Leuconostocaceae*, and *Bifidobacteriaceae* ($0.01<P\leq0.05$); The bacterial genera with high abundance in the samples were there is a significant difference between *Lactobacillus*, *Lactococcus*, *Enterobacter*, *Hafnia-Obesumbacterium*, *Raoultella*, *Citrobacter*, *unclassified for Enterobacteriaceae*, *Enhydrobacter*, *Leuconostoc*, *Bifidobacterium*, *Gluconobacter*, and *Serratia* ($0.01<P\leq0.05$).

Among the fungal diversity, the abundance of group H is higher, and the fungal diversity is good. The inter-group difference results of fungi showed that the fungal diversity of the Koumiss koji (assets) samples from the four regions at the family classification differences level were significantly higher in *Saccharomycetaceae*, *Pichiaceae*, and *Trichocomaceae* ($0.01<P\leq0.05$); The fungal diversity there is a significant difference in Koumiss koji (assets) samples from the four regions at the genus classification level in the genera Kluyveromyces, Dekkera, Kazachstania, and Penicillium ($0.01<P\leq0.05$).

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