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Bacterial community succession and metabolite changes during the fermentation of koumiss, a traditional Mongolian fermented beverage

Wurihan [a, b], Liansheng Bao [c], Hasigaowa [c], Xiuping Bao [c], Yujie Dai [a], Shiru Jia [a, *]

[a] College of Biotechnology, Tianjin University of Science and Technology, Tianjin, 300457, China
[b] College of Life Science, Inner Mongolia University for Nationalities, Tongliao, 028000, China
[c] Rehabilitation Care Hospital of Inner Mongolia University for Nationalities Affiliated Hospital, Tongliao, 028000, China

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ABSTRACT

The microbial population in koumiss, a traditional Mongolian fermented beverage, plays a vital role in its nutritional value, beneficial functions, and flavour. We used the Illumina MiSeq platform to investigate the successive bacterial communities produced during koumiss fermentation; dynamic changes in chemical composition of koumiss were also analysed. The correlation between bacterial species and major metabolites was then evaluated. The dominant bacterial species identified during the process of koumiss fermentation were *Lactobacillus helveticus*, *Streptococcus parauberis* (phylum Firmicutes), and *Acetobacter pasteurianus* (phylum Proteobacteria). Lactose content decreased during fermentation, whereas lactic acid, acetic acid, and butyric acid content increased, resulting in decreased pH. The changes in lactose, lactic acid, butyric acid, and pH were mainly correlated with the presence of the bacterial genera *Acetobacter*, *Leuconostoc*, *Pediococcus*, and *Bacteroides*. Information from these results could advance our understanding of koumiss fermentation, and also help improve the safety, flavour, and therapeutic applications of koumiss.

1. Introduction

Dietary therapy has played an essential role in Mongolian medicine since ancient times, and koumiss is the oldest known drink used for this purpose (Hasisurong, Amuguleng, & Manglai, 2003). It is made by the fermentation of fresh mare milk by microorganisms such as lactic acid bacteria and yeast. Mongolian techniques for preparing and using koumiss for disease treatment have been known since the reign of the Yuan Dynasty in the 14th Century, both in Mongolia and abroad. The "Yin Shan Zheng Yao," edited by the 14th Century doctor and nutritionist Hu Sihui, outlines the uses of koumiss in dietary therapy (Sa Renqi Muge, 2016). The book "The Secret History of the Mongols" describes the pre-Yuan Dynasty use of koumiss for treating haemorrhages and fainting spells (Sa Renqi Muge, 2016). Modern medical research has revealed the beneficial effects of koumiss in treating various deficiencies and cardiovascular and respiratory diseases, strengthening the body, overcoming a stroke, as well as its therapeutic effects on the digestive tract (Abdel-Salam, Al-Dekheil, Babkr, Farahna, & Mousa, 2010; Montanari, Zambonelli, Grazia, Kamesheva, & Shigaeva, 1996). Today, "Koumiss medical centres" in Russia, Mongolia, and Inner Mongolia offer therapy or adjuvant therapy with koumiss for chronic diseases such as tuberculosis or disorders of the cardiovascular, digestive, and nervous systems (Sun et al., 2005).

The therapeutic effects of koumiss are closely related to its chemical and bacterial composition. The chemical profile of koumiss depends not only on the composition of the raw milk, but also on the bacterial community. Therefore, microbes play an essential role in defining the nutritional value, beneficial functions, and flavour of koumiss. As koumiss is traditionally produced through natural fermentation in an open environment, it is rich in microbial resources; this also makes koumiss subject to variations in geographic and climatic conditions, as well as to changes in temperature and duration of fermentation. All these factors contribute to variations in chemical composition, bacterial community structure, and flavour of koumiss among different regions (Sun et al., 2005).

The dominant microbes in traditional koumiss are lactic acid bacteria and yeast, with the former being particularly relevant for attaining the characteristic flavour, texture, and acidity (Hao et al., 2010; Wu et al., 2009; Xu, 2012). Therefore, many studies have

* Corresponding author. Tel.: +86 13 352068043.
E-mail address: jiashiru@tust.edu.cn (S. Jia).

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focused on the identification of lactic acid bacteria in koumiss. However, most studies have relied on culture-dependent methods, which are inefficient for detecting unculturable species and for characterising populations of microorganisms that require selective enrichment.

The development of high-throughput sequencing (HTS) has promoted research on the microbial diversity of fermented foods. When applied to the analysis of microbial community structure, HTS compensates for the limitations of traditional taxonomic methods by allowing the study of unculturable microorganisms in the environment (Ercolini, 2013; Zhao, Mu, & Sun, 2018). HTS has recently been applied for investigating microbial diversity in traditional koumiss. Gesudu et al. (2016) used PacBio SMRT sequencing to study bacterial microbial diversity in koumiss samples collected from five herding families of the Xilingol League in Inner Mongolia. They found that the core Proteobacteria and Firmicutes phyla in koumiss were represented by four bacterial species, namely, *Lactobacillus helveticus*, *Lactobacillus kefiranofaciens*, *Lactobacillus gallinarum*, and *Acetobacter pasteurianus*.

Burenqiqige et al. (2016) used the 454 sequencing platform to analyse the bacterial diversity of koumiss samples collected from the Xilingol League, confirming that *Lactobacillus* and *Lactococcus* were the dominant bacterial genera. Thus, the dominant bacterial species in koumiss differ between different regions.

In this study, we used the Illumina MiSeq platform to investigate bacterial community succession during the fermentation of koumiss from the Horqin area of Inner Mongolia. We also analysed dynamic changes in chemical composition. Moreover, because metabolites can reflect collective phenotypic features of microbial communities and can be detected more easily than the latter, we used the results to evaluate the correlation between bacterial groups and major metabolites. Thus, our findings could be useful for promoting food safety, improving flavour, and informing clinical applications of koumiss from the Horqin area, as well as for facilitating its standardised industrial production.

2. Materials and methods

2.1. Koumiss fermentation

Koumiss fermentation was carried out in Horqin, Inner Mongolia following an original koumiss-making procedure: fresh mare milk was poured into urns, mixed with a starter culture (a small aliquot of koumiss from a previous fermentation) at a 3:1 ratio, and beaten or stirred with a wooden stick every 2 h for 30 min to ensure even mixing and rapid fermentation. The entire process lasted for 96 h, during which temperature was maintained at 24–26 °C. Triplicate biological samples of koumiss were collected every 12 h and sealed into sampling tubes. These were cooled in liquid nitrogen and then transferred immediately to the laboratory, where they were stored at −80 °C for subsequent experiments.

2.2. Chemical composition analysis

2.2.1. Determination of lactose, galactose, and glucose content

Frozen koumiss samples were thawed at 25 °C and then shaken to ensure homogenisation. An aliquot of 0.4 g was mixed homogenously in 1.2 mL of ultrapure water, and this solution was sonicated for 3 min, treated at 105 °C for 15 min, and then centrifuged at 4500× *g* at 4 °C for 10 min. The supernatant was filtered through a 0.22-μm membrane and injected into an Agilent 1260 liquid chromatography system (Bio-Rad HPX-87H ion exchange column, 300 mm × 7.8 mm; Agilent, Santa Clara, CA, USA) for detection with a refractive index detector. The mobile phase was 5 mM H_2SO_4, the flow rate was 0.6 mL min^{-1}, the column temperature was 60 °C, the injection volume was 20 μL, and the stop time was 18 min.

2.2.2. Determination of pH during koumiss fermentation

The pH of each sample was measured with a pH meter (PHS-3C pH Meter, Shanghai INESA Instrument Co., Ltd., Shanghai, China).

2.2.3. Determination of organic acid content

Frozen koumiss samples were thawed at room temperature. An aliquot of 1.0 g was turbulently mixed with 3 mL of 1 M HCl, and the resulting mixture was centrifuged at 12,000× *g* for 15 min. The supernatant was passed through a 0.22-μm filter. The filtrate was analysed by liquid chromatography, as described in Section 2.2.1. The stop time was 25 min.

2.2.4. Determination of free amino acid content

Frozen koumiss samples were thawed at room temperature. An aliquot of 3.0 g was weighed, diluted with 2% salicylic acid 100 times, and analysed with an amino acid analyser (S-433D; Sykam GmbH, Kleinostheim, Germany). The flow rate was 0.46 mL min^{-1}, the column temperature was 130 °C, the injection volume was 50 μL, the reactor temperature was 58 °C, and the detection wavelengths were 440 nm and 570 nm.

2.2.5. Determination of fatty acids

2.2.5.1. Fat extraction. A frozen sample was thawed at room temperature. A 1-mL aliquot was added to 1.5 mL chloroform-methanol solution (2:1 v/v) for 3 h for extraction at 25 °C, followed by the addition of 0.9% NaCl. The mixture was allowed to stratify, after which the lower chloroform layer was collected and concentrated using nitrogen blow-down evaporation.

2.2.5.2. Methyl esterification of fatty acids. The fat extract was treated with 2 mL *n*-hexane, and the resulting mixture was homogenised by shaking. Next, 2 mL 14% boron trifluoride-methanol-complex solution was added, followed by heating at 50 °C in a water bath for 5 min. Saturated NaCl was added to bring the total volume to 10 mL, and the supernatant was collected by centrifugation and passed through a 0.45-μm filter membrane for subsequent analyses.

2.2.5.3. Gas chromatography. Detection was performed using an Agilent 7890A gas chromatography system (HP-innowax column, 30 m × 0.32 mm × 0.5 μm; Agilent, Santa Clara, CA) equipped with a hydrogen flame ionisation detector. The carrier gas was N_2, which was pumped at constant pressure and a linear velocity of 38.4 cm s^{-1}; the inlet temperature was 270 °C, the injection volume was 1 μL, and the split ratio was 20:1. The initial column oven temperature was held at 50 °C for 2 min, followed by a 25 °C min^{-1} temperature ramp to 175 °C and a 3.5 °C min^{-1} temperature ramp to 230 °C, after which the temperature was maintained at 230 °C for 5 min. The detector was operated at 280 °C, with H_2 (40 mL min^{-1}) and air (450 mL min^{-1}) used as detector gases; the flow rate of the makeup gas was 30 mL min^{-1}.

2.3. Analysis of bacterial diversity

2.3.1. Total DNA extraction

Each koumiss sample was fully thawed at room temperature. Genomic DNA was extracted using the FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's instructions. The quantity and quality of the DNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), with the following parameters:

concentration ≥ 20 ng μL^{-1}, total amount ≥ 500 ng, and $OD_{260/280}$ = 1.8–2.0.

2.3.2. Target region amplification

PCR amplification was performed in triplicate using high-fidelity enzymes (TopTaq DNA Polymerase Kit, Transgen Biotech, Beijing, China) with the extracted genomic DNA as the template. Primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′), which are specific to the 16S rDNA V3-V4 region, were used.

2.3.3. Mixing and purification of PCR products

The PCR products were separated on a 1% agarose gel; three parallel amplification products from the same sample were mixed, and an equal volume of Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA) was added to the resulting mixture for nucleic acid purification.

2.3.4. Addition of specific tag sequences to each sample DNA sequence

Specific tag sequences were added to the sequence ends in each sample library using index sequencing primers through a high-fidelity, 8-cycle PCR. The PCR products were detected on a 1% agarose gel and purified using Agencourt AMPure XP beads to obtain the original library for each sample.

2.3.5. Quantification and mixing of libraries

The purified amplicons were preliminarily quantified on 1% agarose gels, based on which, each indexed sample library was diluted. The diluted samples were then accurately quantified by Qubit fluorometry (Thermo Fisher Scientific). Based on sequencing throughput requirements, sample libraries were pooled in appropriate proportions and then subjected to MiSeq 2 × 250 bp paired-end sequencing and bioinformatic analyses. Sequencing and bioinformatic analyses were performed by Shanghai Genesky Bio-Tech Co., Ltd, Shanghai, China.

2.3.6. Bioinformatic analysis

To obtain high-quality sequencing data and improve the accuracy and reliability of the bioinformatic analyses, raw data were first assembled and filtered to produce clean reads with high quality and confidence. The clean reads were then subjected to operational taxonomic unit (OTU) clustering at a similarity threshold of 97%, and the representative sequence of each OTU was aligned to the GreenGenes 16S rDNA database using mothur software (https://www.mothur.org/) to complete species-level annotation of OTUs. The bacterial community structure was derived from statistical analyses of OTU abundance, alpha diversity, and species abundance at each classification level.

The association between genera and environmental factors was calculated using Spearman correlations.

2.4. Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM, Armonk, NY, USA). Differences with a P value of <0.05 were regarded as statistically significant.

3. Results

3.1. Changes in metabolites

As shown in Fig. 1A, lactose, glucose, and galactose content changed during koumiss fermentation. The lactose content continuously decreased, reaching a minimum of 0.016 ± 0.010 g 100 g^{-1} after 96 h. No glucose was detected at the onset (0 h) of fermentation; glucose content then rose to a maximum of 0.010 ± 0.003 g 100 g^{-1} at 12 h, followed by a constant decline until 48 h, after which no glucose was detected. The galactose content increased significantly between 0 and 12 h (P < 0.01), followed by an equally significant decrease between 12 and 36 h (P < 0.01). The galactose content increased again between 36 and 84 h, after which it decreased significantly, reaching its lowest value of 0.014 ± 0.001 g 100 g^{-1} at 96 h.

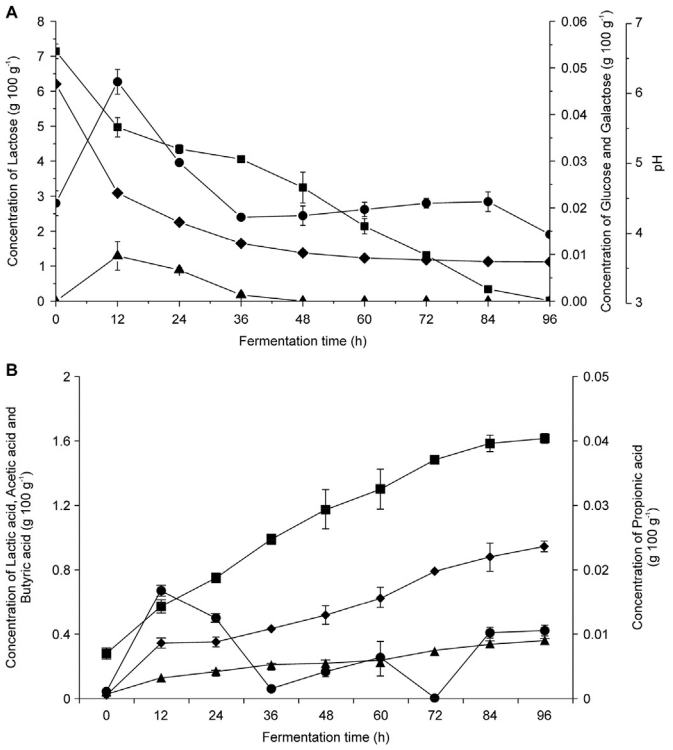

Fig. 1. Changes in (A) lactose (■), glucose (▲), galactose (●), and pH (◆) and (B) the organic acids lactic acid (■), acetic acid (▲), butyric acid (◆) and propionic acid (●) during koumiss fermentation. Data are presented as mean ± standard deviations (n = 3).

During koumiss fermentation, the pH decreased from 6.13 at 0 h to 3.59 at 84 h and stabilised thereafter (Fig. 1A). Statistical analysis showed that the pH decreased at a very significant rate between 0 and 48 h (P < 0.01).

As shown in Fig. 1B, the content of lactic acid, acetic acid, and butyric acid increased throughout the fermentation process, with lactic acid exhibiting the highest increase (0.278 ± 0.028 g 100 g^{-1} at 0 h to 1.619 ± 0.027 g 100 g^{-1} at 96 h). Butyric acid showed the second most significant increase in content, going from 0.023 ± 0.001 g 100 g^{-1} to 0.947 ± 0.024 g 100 g^{-1}; whereas acetic acid showed the smallest increase, rising from 0.040 ± 0.005 g 100 g^{-1} to 0.369 ± 0.006 g 100 g^{-1}. Propionic acid content remained low throughout the fermentation, with mild fluctuations.

A total of 14 amino acids were detected in this experiment (Supplementary material Table S1), with Glu being the most abundant, followed by His (Fig. 2A). Asp, Thr, Ser, Glu, Ala, and His decreased significantly between 0 and 12 h (P < 0.05), after which they gradually increased, peaking at 84 h. Val and Ile decreased significantly between 0 and 24 h (P < 0.05), followed by a gradual increase until the end of fermentation. Cys increased steadily throughout the fermentation period, reaching a maximum value of 15.60 ± 0.08 mg 100 mL^{-1} at 96 h. Gly content dropped from

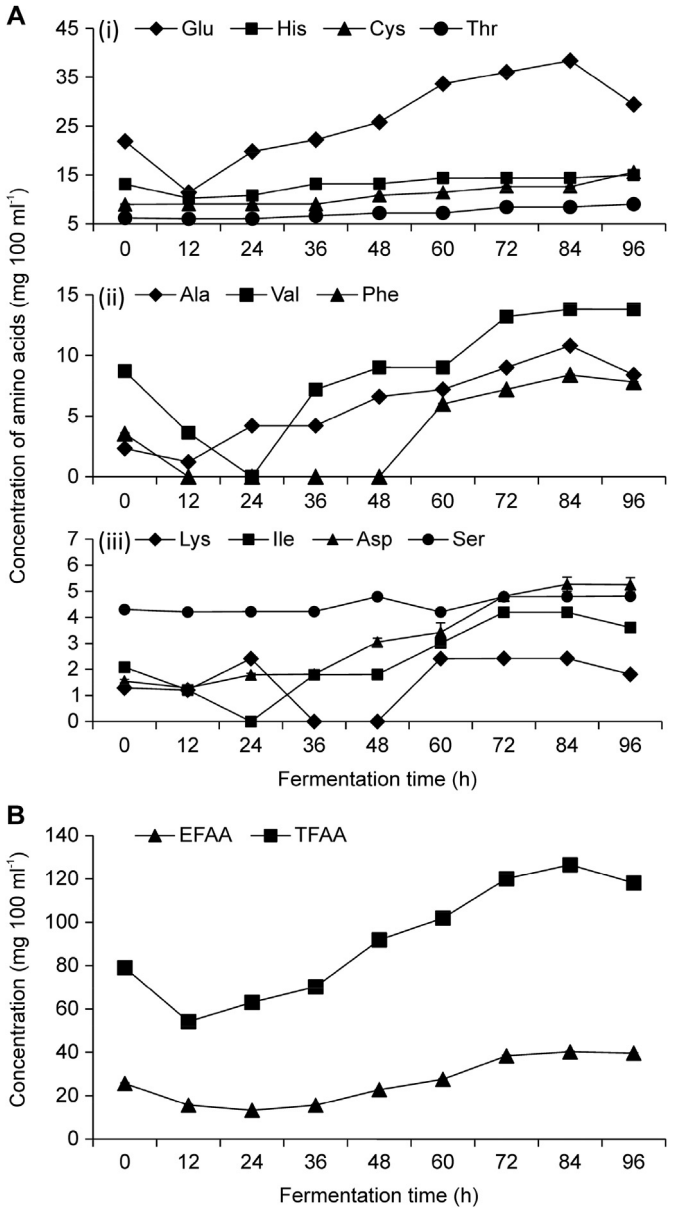

Fig. 2. Changes in amino acid content during koumiss fermentation: A(i), Glu (◆), His (■), Cys (▲), Thr (●); A(ii), Ala (◆), Val (■), Phe (▲); A(iii), Lys (◆), Ile (■), Asp (▲), Ser (●); B, total (■) and essential (▲) free amino acids. Data are presented as mean ± standard deviations (n = 3).

1.27 ± 0.04 mg 100 mL^{-1} to 0 mg 100 mL^{-1} between 0 and 4 h, becoming effectively undetectable. Tyr could be detected only at 48 h, when it amounted to 4.79 ± 0.10 mg 100 mL^{-1} but remained undetectable at other times. Statistical analyses of total free amino acid (TFAA) and essential free amino acid (EFAA) content (Fig. 2B) revealed a very significant decrease of TFAA content between 0 and 12 h ($P < 0.01$), followed by a gradual increase until it achieved a peak of 126.53 ± 0.92 mg 100 mL^{-1} at 84 h. EFAA content exhibited a very significant drop between 0 and 24 h, after which it mirrored TFAA, reaching a maximum of 40.25 ± 0.35 mg 100 mL^{-1} at 84 h. Thus, TFAA and EFAA were highest after 84 h of koumiss fermentation.

A total of 23 fatty acids were detected during koumiss fermentation, including nine unsaturated fatty acids, of which four were monounsaturated, and five were polyunsaturated. The content of unsaturated fatty acids fluctuated throughout the fermentation process and peaked at 84 h, with values of 16.36 ± 3.93 mg L^{-1} for monounsaturated and 33.68 ± 10.05 mg L^{-1} for polyunsaturated fatty acids (Fig. 3A). The five polyunsaturated fatty acids were linoleic acid (C18:2), α-linolenic acid (C18:3n3), γ-linolenic acid (C18:3n6), docosahexaenoic acid (C22:6n3), and eicosapentaenoic acid (C20:5n3); changes in their content are shown in Fig. 3B. Overall, the highest content of essential fatty acids was achieved after 84 h of koumiss fermentation.

3.2. Bacterial diversity

3.2.1. Alpha diversity analysis

The 27 koumiss samples studied produced a total of 2,459,036 high-quality clean reads of 16S rRNA sequences. Sequences with similarity higher than 97% were clustered into the same OTUs. After using de novo chimera detection to identify and remove chimeric sequences, we obtained 78 OTUs.

Rarefaction curves and Shannon–Wiener index curves were used to determine if the number of generated sequences was sufficient for bioinformatic analyses. The samples' rarefaction curves did not reach a plateau, whereas the Shannon–Wiener index curves did (Supplementary material Fig. S1). This discrepancy indicated that, as the amount of sequencing data increased, new OTUs would be generated and new phylotypes might be discovered, even though microbial diversity of the samples remained constant. Thus, the amount of sequencing data was sufficient to obtain the maximum microbial information and for subsequent analyses.

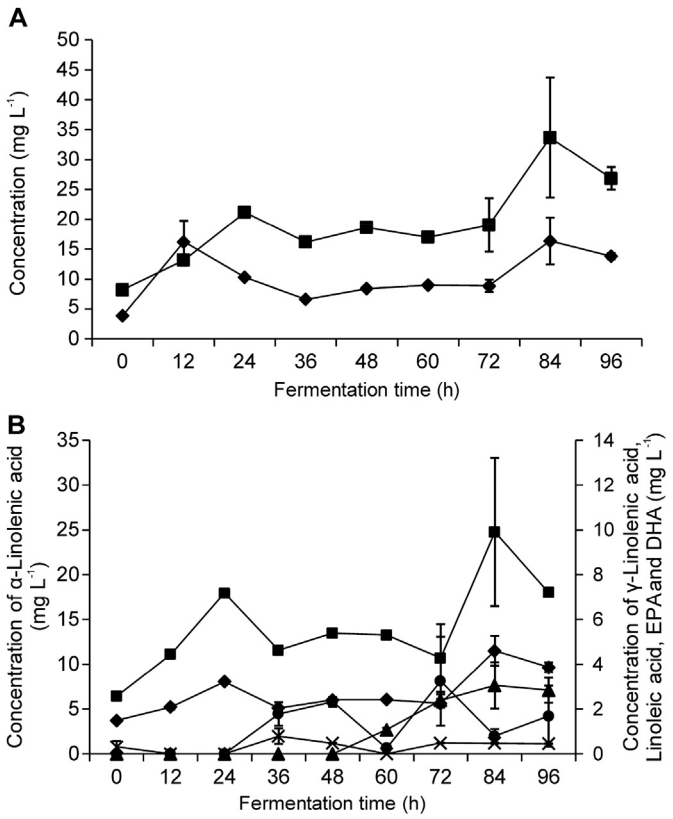

Fig. 3. Changes in (A) total monounsaturated (◆) and total polyunsaturated (■) fatty acids and (B) individual polyunsaturated fatty acids [■, α-linolenic acid (C18:3n3); ◆, linoleic acid (C18:2); ▲, γ-linolenic acid (C18:3n6); ×, eicosapentaenoic acid (EPA, C20:5); ●, docosahexaenoic acid (DHA, C22:6)] during koumiss fermentation. Data are presented as mean ± standard deviations (n = 3).

Dynamic changes to alpha-diversity indices such as the Chao1, ACE, Shannon, and Simpson indices during koumiss fermentation showed that bacterial richness of koumiss did not change significantly during the fermentation process, although bacterial diversity varied significantly, indicating substantial changes in relative abundance of some bacterial species (Supplementary material Fig. S2).

3.2.2. Structure and succession of the bacterial community

Representative sequences from all OTUs were compared with a database of sequences to complete their taxonomic annotation. For OTU annotation, mothur software was used to identify the species with the highest similarity to the OTU sequence, with a confidence level of more than 80%. The lowest taxonomic level of each OTU from the 27 koumiss samples included four phyla, eight classes, 17 orders, 32 families, 43 genera, and 48 species (Supplementary material Dataset S1).

The four phyla were Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes, with Firmicutes and Proteobacteria showing a relative abundance of more than 90% throughout the fermentation process (Fig. 4A). At 0 h, the koumiss sample also contained Actinobacteria with a relative abundance of 10.84%, but their abundance decreased sharply as fermentation progressed, dropping below 1% at 12 h and remaining low until the end of fermentation.

Bacterial genera whose relative abundance during fermentation was consistently above 1% were plotted in a histogram (Fig. 4B). At 0 h, these genera included *Lactobacillus* (23.21%), *Streptococcus* (8.81%), *Rothia* (10.83%), *Lactococcus* (3.00%), *Leuconostoc* (2.25%), and *Enhydrobacter* (1.81%). As fermentation proceeded, the relative abundance of *Acetobacter* gradually increased, whereas those of *Rothia*, *Lactococcus*, *Leuconostoc*, and *Enhydrobacter* decreased, dropping below 1% after 36 h; this resulted in the absolute dominance of *Lactobacillus*, *Streptococcus*, and *Acetobacter*, with a cumulative relative abundance of more than 90%. Notably, pathogens such as *Escherichia*, *Shigella*, and *Staphylococcus aureus* were detected at 0 h, but their relative abundance was only 0.07% and gradually decreased to below 0.01% as fermentation progressed.

Further identification at the species level revealed that the most abundant bacteria were *L. helveticus*, *Streptococcus parauberis*, and *A. pasteurianus* (Fig. 4C). The relative abundance of *L. helveticus* increased between 0 and 36 h, reaching a maximum of 53.18% at 36 h, followed by a decrease and final stabilisation after 72 h. *S. parauberis* increased to some extent between 0 and 12 h, but dropped to 30.78% between 12 and 36 h and remained constant after that. The relative abundance of *A. pasteurianus* increased gradually throughout the fermentation process, reaching a maximum of 16.57% at 96 h. Besides *L. helveticus*, other *Lactobacillus* probiotics such as *Lactobacillus kefiri*, *Lactobacillus acetotolerans*, *Lactobacillus brevis*, *Lactobacillus casei* str. Zhang, and *Lactobacillus plantarum* WCFS1 were detected in the samples (data not shown).

To better understand the changes in bacterial community structure at different time points during koumiss fermentation, the weighted UniFrac principal coordinate analysis (weighted UniFrac PCoA) and the unweighted pair group method with arithmetic mean (UPGMA) were used. As shown in Fig. 5, the first and second principal components of the weighted UniFrac PCoA accounted for 76.68% and 18.17% of the overall variation, respectively. Samples at 0 and 12 h showed some clustering with each other and appeared distant from those at other time points, indicating that bacterial community structure at these time points differed significantly from that at other time points. Samples at 24, 36, 48, and 60 h exhibited some clustering with one another, as did the samples at 72, 84, and 96 h. These observations were consistent with the results of cluster analysis. Thus, the fermentation of koumiss could be divided into three stages: (i) initial fermentation stage, 0–12 h; (ii) median fermentation stage, 12–60 h; and (iii) late fermentation stage, 60–96 h.

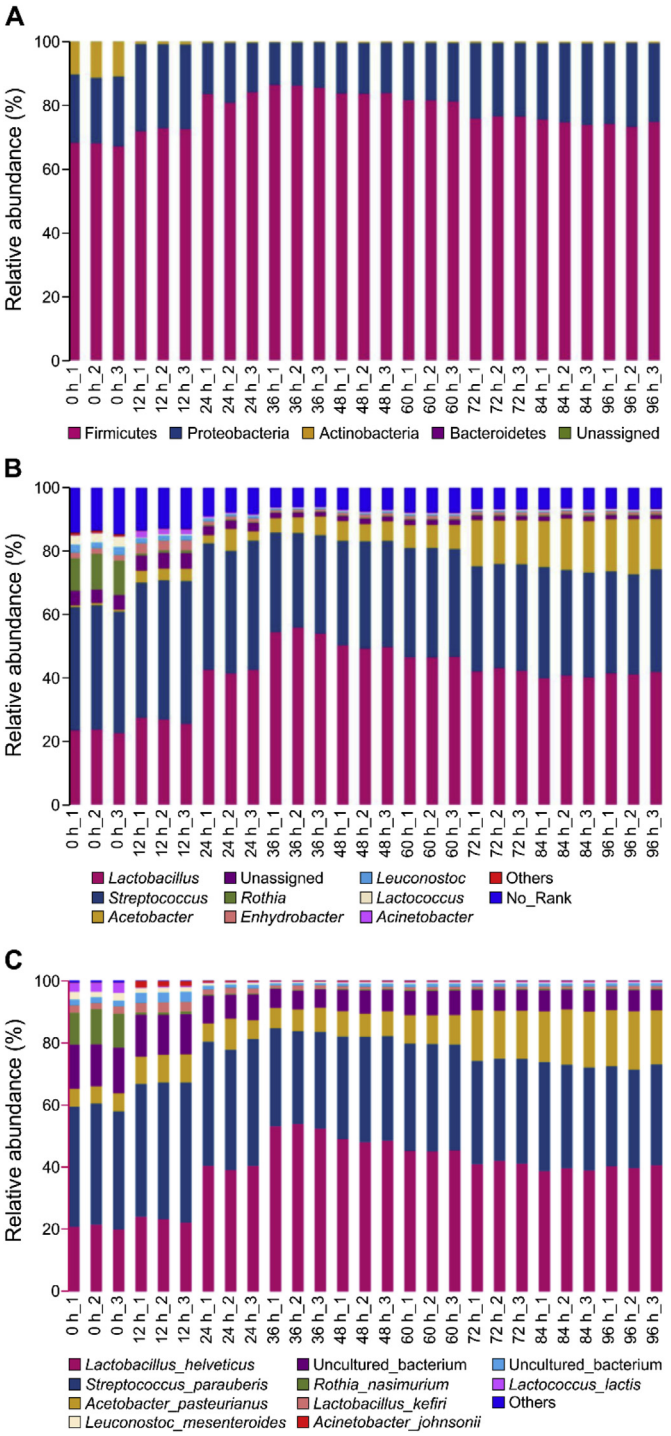

Fig. 4. Bacterial succession at the phylum (A), genus (B), and species (C) levels during different time points in the koumiss fermentation process.

3.3. Correlation between bacterial community and metabolites

Spearman's correlation coefficient (r) between bacterial community structure (at the genus level) and the pH and major metabolites (lactose, glucose, galactose, lactic acid, butyric acid, acetic acid, propionic acid, free amino acids, and fatty acids) was

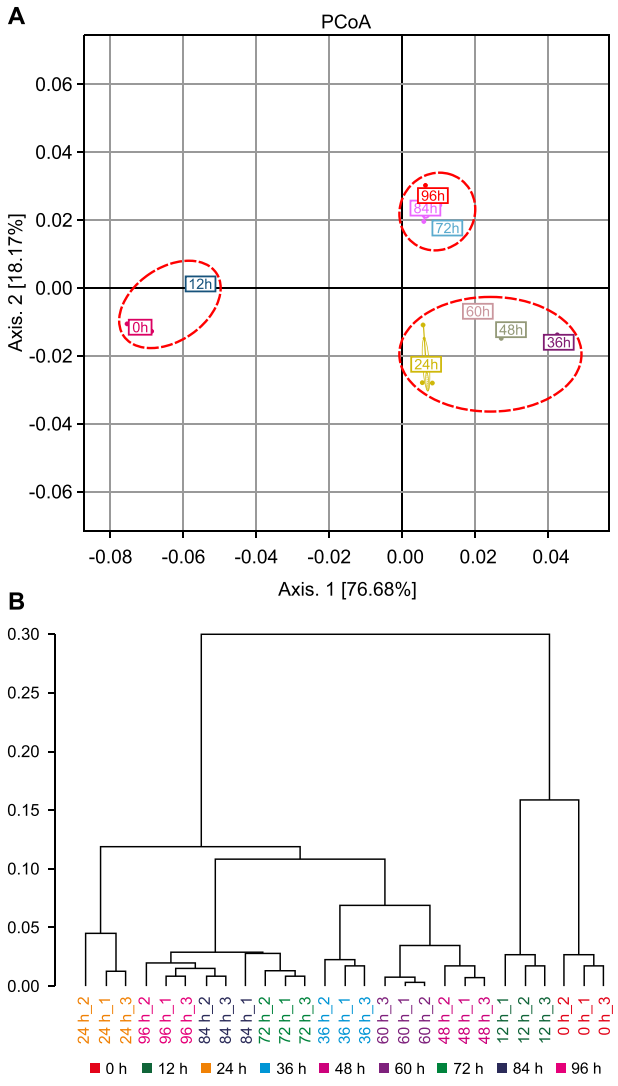

Fig. 5. Results of (A) weighted UniFrac principal coordinate analysis (PCoA), and (B) unweighted pair group method with arithmetic mean (UPGMA) analysis of bacterial communities at different time points during koumiss fermentation.

calculated (Supplementary material Table S2). We plotted a heatmap and a rose picture based on these calculations. As shown in Fig. 6, there was a very significant correlation between the pH of koumiss and the genera *Streptococcus*, *Enhydrobacter*, *Leuconostoc*, *Pediococcus*, *Ralstonia*, *Acetobacter*, *Bacteroides*, and *Paracoccus* ($P < 0.01$), with positive correlation to the first five and negative correlation to the last three; among them, positive correlation with *Leuconostoc* was the strongest, whereas that with *Acetobacter* was the most negative. Additionally, the pH correlated positively with the genera *Weissella*, *Pseudomonas*, and *Haemophilus* ($P < 0.05$), but negatively with the genus *Brevundimonas* ($P < 0.05$).

As shown in Fig. 6, the dominant genus *Lactobacillus* exhibited a significant positive correlation with Tyr and a significant negative correlation with Gly ($P < 0.05$). *Lactobacillus* correlated significantly also with galactose ($P < 0.01$). *Streptococcus* correlated positively with lactose, glucose, galactose, lactic acid, butyric acid, acetic acid, propionic acid, 11 amino acids (Glu, His, Val, Cys, Thr, Ala, Asp, Leu, Ser, Ile, and Gly), and three saturated fatty acids (C8:0, C10:0, and C23:0). *Acetobacter* correlated very significantly or significantly with lactose, glucose, galactose, lactic acid, butyric acid, acetic acid, 12 free amino acids (Glu, His, Val, Cys, Thr, Ala, Asp, Lys, Phe, Ser, Ile, and Gly), and eight fatty acids (C8:0, C10:0, C12:0, C14:0, C16:0, C16:1, C18:1, and C18:3n3). Lactose, lactic acid, and butyric acid correlated negatively with pH, but these two groups of variables were consistent with each other in terms of their correlation with bacterial genera.

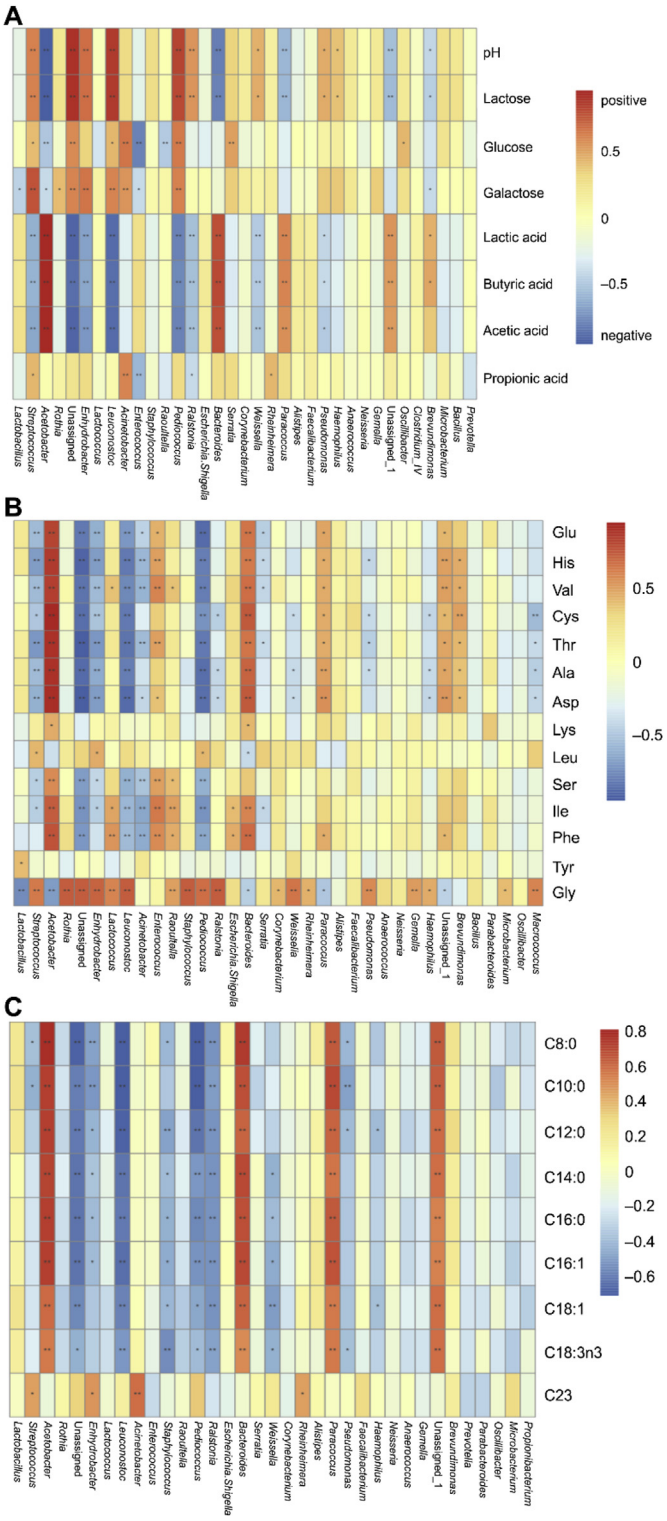

Fig. 6. Heatmap of Spearman correlation analysis between bacterial genera and metabolites: A, sugars and organic acids; B, free amino acids; C, fatty acids. Asterisks indicate significance of correlation: *, $P < 0.05$; **, $P < 0.01$.

4. Discussion

Microorganisms play a vital role in the preparation of fermented foods, and changes in their composition will directly affect the nutritional profiles and flavour of the product. This is especially true for medicinal foods such as koumiss, whose benefits in treating or relieving diseases may be affected by variations in bacterial content. To the best of our knowledge, the present study is the first to use Illumina MiSeq second-generation HTS technology to investigate bacterial community succession during koumiss fermentation and analyse its correlation with changes in major metabolites.

Metabolites play an essential role in determining the taste and flavour of fermented foods. For example, sugar is related to sweetness, acetic and lactic acids contribute to acidity, and amino acids enhance flavour, with some of them being involved in the formation of alcohol-, aldehyde-, acid-, and ester-related aromatic ingredients (Corral, Leitner, Siegmund, & Flores, 2016; Li et al., 2017; Smid & Kleerebezem, 2014). Here, we found that lactose content decreased during koumiss fermentation, while lactic acid, acetic acid, and butyric acid increased; this increase was particularly marked in the case of lactic acid. These results were consistent with the findings of Burenqiqige et al. (2016) and Yin, Wu, Qian, and Guan (2002). Lactic acid plays a key role in the production of fermented milk, not only because it is the main factor to lower the pH, but also for its contribution to the rich flavour and aroma of yoghurt (Datta & Henry, 2006). The amount of butyric acid in koumiss samples was second only to that of lactic acid.

Due to the increases in lactic acid and butyric acid, the pH of koumiss gradually decreased. Correlation analysis revealed that these changes in organic acids and pH correlated significantly with 11 bacterial genera, particularly *Acetobacter*, *Bacteroides*, *Leuconostoc*, and *Pediococcus*. This implies that lactose in mare milk is degraded mainly by *Acetobacter* and *Bacteroides*. Many studies have found that lactose is degraded to a certain extent during the fermentation and storage of fermented milk. Therefore, it is of great significance to study the influence of different bacteria species on lactose change to control the degradation of lactose and further improve the degradation rate of lactose.

A large number of organic acids are produced during the degradation of lactose by these bacteria, with lactic acid being the most relevant and thus responsible for lowering the pH. As pH correlated significantly with many important bacterial genera (Fig. 6), it could be used as a quality indicator during koumiss fermentation.

Amino acids in yoghurt are beneficial for intestinal health and can improve immune function. In particular, free amino acids can be readily absorbed and efficiently utilised by the body (Meydani & Ha, 2000). Dynamic changes in free amino acids observed in this study revealed that the content of 14 free amino acids decreased significantly between 0 and 12 h, after which it gradually increased and peaked at 84 h. Beshkova, Simova, Frengova, Simov, and Adilov (1998) had shown that the content of free amino acids increased during yoghurt fermentation. We found six essential amino acids in our koumiss samples, namely Val, Lys, Ile, Leu, Phe, and Thr. Their total amounts first decreased and then increased during the fermentation process, reaching a maximum value at 84 h. Thus, in terms of total free amino acids and essential amino acids, the koumiss samples had the highest nutritional value at 84 h.

Polyunsaturated fatty acids have many beneficial physiological effects such as regulating blood lipids, improving blood circulation, and preventing atherosclerosis and thrombosis (Schacky, 2006). Five polyunsaturated fatty acids were detected in the koumiss samples, namely linoleic acid (C18:2), α-linolenic acid (C18:3n3), γ-linolenic acid (C18:3n6), docosahexaenoic acid (C22:6n3), and eicosapentaenoic acid (C20:5n3). Although the amounts of these polyunsaturated fatty acids varied during the fermentation process, their combined amount peaked at 84 h (Fig. 3). Therefore, in terms of total unsaturated fatty acid content, koumiss was ideal for consumption after 84 h of fermentation.

HTS revealed that the major bacterial species present during koumiss fermentation were *L. helveticus* and *S. parauberis* (phylum Firmicutes) and *A. pasteurianus* (phylum Proteobacteria). Their cumulative relative abundance was above 90%; *L. helveticus* was the dominant species, particularly in the middle and late stages of fermentation. These results were consistent with those of Gesudu et al. (2016) and Sun et al. (2010).

A growing body of evidence from interventional studies and clinical trials has revealed that *L. helveticus* strains possess several health-promoting properties (Taverniti & Guglielmetti, 2012) preventing gastrointestinal infections (Vinderola, Matar, & Perdigón, 2007; Wine, Gareau, Johnson-Henry, & Sherman, 2009), enhancing protection against pathogens (Gareau, Wine, Reardon, & Sherman, 2010), regulating the composition of intestinal microbiota (Cavallini et al., 2011; Frece et al., 2009), and modulating host immune responses (Frece et al., 2009; LeBlanc, Fliss, & Matar, 2004). The angiotensin-I-converting enzyme (ACE) increases blood pressure by converting angiotensin-I to the vasoconstrictor angiotensin-II and inactivates the vasodilator bradykinin (Campbell, 1987). Yamamoto, Akino, and Takano (1994) found that milk fermented by *L. helveticus* showed higher ACE-inhibitory activity and antihypertensive activity than milk fermented by other species. *L. helveticus* was the dominant bacterial species in this study, indicating that koumiss may have protective and therapeutic functions.

Owing to recent developments in molecular technology, *S. parauberis* has been detected in many foods such as chicken and ham that have been subjected to modified atmosphere packaging, vacuum-packed frozen seafood products, and Tunisian seafood and meat products (Fernández-No et al., 2012; Koort, Coenye, Vandamme, & Björkroth, 2006). An, Adachi, and Ogawa (2004) detected *S. parauberis* in koumiss from Inner Mongolia's Xilingol League. Gesudu et al. (2016) employed HTS to determine the microbial community structure of koumiss samples from five different sampling sites in Inner Mongolia's Xilingol League. They found that *S. parauberis* occurred in samples collected from site D, which was located far away from the city of Xilinhaote, and site B, which was near the city. Li et al. (2017) found that the bacterial genus *Streptococcus* correlated negatively with the content of lactate, acetate, and most amino acids during doubanjiang-meju fermentation, which is consistent with our results.

A. pasteurianus is an important microorganism in the fermentation of table vinegar and fruit vinegar (Haruta et al., 2006; Wu, Gullo, Chen, & Giudici, 2010); it has also been detected in traditionally fermented koumiss (Gesudu et al., 2016). In this study, the relative abundance of *A. pasteurianus* increased as fermentation time increased. *Acetobacter* correlated positively with lactic acid, butyric acid, acetic acid, and most amino acids and fatty acids. Sainz, Mas, and Torija (2017) had reported that amino acids could enhance the growth of *Acetobacter*. Yin et al. (2017) investigated the effect of amino acids on metabolism and acid stress resistance of *A. pasteurianus* and found that cell growth and culturable cell counts increased by 0.51- and 0.72-fold, respectively, after the addition of Glu. This is believed to result primarily from the enhanced generation of pentose phosphates and NADPH for the synthesis of nucleic acids, fatty acids, and glutathione, as well as due to increased synthesis of unsaturated fatty acid and better lipid transport.

5. Conclusions

In summary, our results showed that lactose decreased throughout koumiss fermentation, whereas lactic acid, acetic acid, and butyric acid levels gradually increased. The degradation of lactose to lactic acid lowered the pH, which correlated with the presence of 11 bacterial genera, particularly *Acetobacter*, *Leuconostoc*, and *Pediococcus*. The contents of total free amino acids, essential amino acids, and total unsaturated fatty acids peaked at 84 h. These results could contribute to the standardisation of industrial production of koumiss from the Horqin area. They could also improve its safety, flavour, and applications for dietary therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.06.013.

References

Abdel-Salam, A., Al-Dekheil, A., Babkr, A., Farahna, M., & Mousa, H. (2010). High fiber probiotic fermented mare's milk reduces the toxic effects of mercury in rats. *North American Journal of Medical Sciences, 2*, 569–575.

An, Y., Adachi, Y., & Ogawa, Y. (2004). Classification of lactic acid bacteria isolated from chigee and mare milk collected in Inner Mongolia. *Animal Science Journal, 75*, 245–252.

Beshkova, D. M., Simova, E. D., Frengova, G. I., Simov, Z. I., & Adilov, E. F. (1998). Production of amino acids by yogurt bacteria. *Biotechnology Progress, 14*, 963–965.

Burenqiqige, J., Gao, Y., Bao, Y., Ren, X., Wei, R., Sarula, B., et al. (2016). Analysis of physicochemical parameters and bacteria community structure in Koumiss's fermentation process. *Science and Technology of Food Industry, 37*, 118–121 (In Chinese).

Campbell, D. J. (1987). Circulating and tissue angiotensin systems. *Journal of Clinical Investigation, 79*, 1–6.

Cavallini, D. C., Suzuki, J. Y., Abdalla, D. S., Vendramini, R. C., Pauly-Silveira, N. D., Roselino, M. N., et al. (2011). Influence of a probiotic soy product on fecal microbiota and its association with cardiovascular risk factors in an animal model. *Lipids in Health and Disease, 10*, 126.

Corral, S., Leitner, E., Siegmund, B., & Flores, M. (2016). Determination of sulfur and nitrogen compounds during the processing of dry fermented sausages and their relation to amino acid generation. *Food Chemistry, 190*, 657–664.

Datta, R., & Henry, M. (2006). Lactic acid: Recent advances in products, processes and technologies-a review. *Journal of Chemical Technology and Biotechnology, 81*, 1119–1129.

Ercolini, D. (2013). High-throughput sequencing and metagenomics: Moving forward in the culture-independent analysis of food microbial ecology. *Applied and Environmental Microbiology, 79*, 3148–3155.

Fernández-No, I. C., Böhme, K., Calo-Mata, P., Cañas, B., Gallardo, J. M., & Barros-Velázquez, J. (2012). Isolation and characterization of *Streptococcus parauberis* from vacuum-packaging refrigerated seafood products. *Food Microbiology, 30*, 91–97.

Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Beganović, J., Leboš, A., et al. (2009). Synbiotic effect of *Lactobacillus helveticus* M92 and prebiotics on the intestinal microflora and immune system of mice. *Journal of Dairy Research, 76*, 98–104.

Gareau, M. G., Wine, E., Reardon, C., & Sherman, P. M. (2010). Probiotics prevent death caused by *Citrobacter rodentium* infection in neonatal mice. *The Journal of Infectious Diseases, 201*, 81–91.

Gesudu, Q., Zheng, Y., Xi, X., Hou, Q. C., Xu, H., Huang, W., et al. (2016). Investigating bacterial population structure and dynamics in traditional koumiss from Inner Mongolia using single molecule real-time sequencing. *Journal of Dairy Science, 99*, 7852–7863.

Hao, Y., Zhao, L., Zhang, H., Zhai, Z., Huang, Y., Liu, X., et al. (2010). Identification of the bacterial biodiversity in koumiss by denaturing gradient gel electrophoresis and species-specific polymerase chain reaction. *Journal of Dairy Science, 93*, 1926–1933.

Haruta, S., Ueno, S., Egawa, I., Hashiguchi, K., Fujii, A., Nagano, M., et al. (2006). Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. *International Journal of Food Microbiology, 109*, 79–87.

Hasisurong, A., Amuguleng, H., & Manglai, D. (2003). Koumiss and its value in medicine. *Zhongguo Zhong Yao Za Zhi, 28*, 11–14 (In Chinese).

Koort, J., Coenye, T., Vandamme, P., & Björkroth, J. (2006). *Streptococcus parauberis* associated with modified atmosphere packaged broiler meat products and air samples from a poultry meat processing plant. *International Journal of Food Microbiology, 106*, 318–323.

LeBlanc, J., Fliss, I., & Matar, C. (2004). Induction of a humoral immune response following an *Escherichia coli* O157:H7 infection with an immunomodulatory peptidic fraction derived from *Lactobacillus helveticus*-fermented milk. *Clinical and Vaccine Immunology, 11*, 1171–1181.

Li, Z., Rui, J., Li, X., Li, J., Dong, L., Huang, Q., et al. (2017). Bacterial community succession and metabolite changes during doubanjiang-meju fermentation, a Chinese traditional fermented broad bean (*Vicia faba* L.) paste. *Food Chemistry, 218*, 534–542.

Meydani, S. N., & Ha, W.-K. (2000). Immunologic effects of yogurt. *American Journal of Clinical Nutrition, 71*, 861–872.

Montanari, G., Zambonelli, C., Grazia, L., Kamesheva, G. K., & Shigaeva, M. K. (1996). *Saccharomyces unisporus* as the principal alcoholic fermentation microorganism of traditional koumiss. *Journal of Dairy Research, 63*, 327–331.

Muge, S. R. (2016). Mongolian doctors' understanding of sour horse milk. *World Latest Medicine Information (Electronic Version), 16*, 169–170 (In Chinese).

Sainz, F., Mas, A., & Torija, M. J. (2017). Effect of ammonium and amino acids on the growth of selected strains of *Gluconobacter* and *Acetobacter*. *International Journal of Food Microbiology, 242*, 45–52.

Schacky, C. V. (2006). A review of omega-3 ethyl esters for cardiovascular prevention and treatment of increased blood triglyceride levels. *Vascular Health and Risk Management, 2*, 251–262.

Smid, E. J., & Kleerebezem, M. (2014). Production of aroma compounds in lactic fermentations. *Food Science and Technology, 5*, 313–326.

Sun, Z., Liu, W., Zhang, J., Yu, J., Zhang, W., Cai, C., et al. (2010). Identification and characterization of the dominant lactobacilli isolated from koumiss in China. *Journal of General and Applied Microbiology, 56*, 257–265.

Sun, T., Menghe, B., Wang, J., Chen, Y., Zhao, D., & Zhang, H. (2005). Analysis of chemical composition and microorganism flora of traditionally home-made koumiss in Xinjiang. *China Dairy Industry, 33*, 9–13.

Taverniti, V., & Guglielmetti, S. (2012). Health-promoting properties of *Lactobacillus helveticus*. *Frontiers in Microbiology, 3*. Article 392.

Vinderola, G., Matar, C., & Perdigón, G. (2007). Milk fermentation products of *L. helveticus* R389 activate calcineurin as a signal to promote gut mucosal immunity. *BMC Immunology, 8*. Article 19.

Wine, E., Gareau, M. G., Johnson-Henry, K., & Sherman, P. M. (2009). Strain-specific probiotic (*Lactobacillus helveticus*) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells. *FEMS Microbiology Letters, 300*, 146–152.

Wu, J., Gullo, M., Chen, F., & Giudici, P. (2010). Diversity of *Acetobacter pasteurianus* strains isolated from solid-state fermentation of cereal vinegars. *Current Microbiology, 60*, 280–286.

Wu, R., Wang, L., Wang, J., Li, H., Menghe, B., Wu, J., et al. (2009). Isolation and preliminary probiotic selection of lactobacilli from koumiss in inner Mongolia. *Journal of Basic Microbiology, 49*, 318–326.

Xu, S. (2012). The dietary therapy and medical value of mare milk and koumiss. *Journal of Medicine and Pharmacy of Chinese Minorities, 2*, 21–22.

Yamamoto, N., Akino, A., & Takano, T. (1994). Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. *Bioscience Biotechnology and Biochemistry, 58*, 776–778.

Yin, W., Wu, N., Qian, J., & Guan, H. (2002). Study on biological activity dynamic of kumys from Xilinguole. *Journal of Inner Mongolia Agricultural University, 23*, 9–16.

Yin, H., Zhang, R., Xia, M., Bai, X., Mou, J., Zheng, Y., et al. (2017). Effect of aspartic acid and glutamate on metabolism and acid stress resistance of *Acetobacter pasteurianus*. *Microbial Cell Factories, 16*, 109.

Zhao, Z., Mu, T., & Sun, H. (2018). Microbial characterization of five Chinese traditional sourdoughs by high-throughput sequencing and their impact on the quality of potato steamed bread. *Food Chemistry, 274*, 710–717.