A snapshot study of the microbial community dynamics in naturally fermented cow’s milk

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Abstract
Natural fermentation of milk is a prerequisite in the production of traditional dairy products and is considered a bioresource of fermentative microorganisms and probiotics. To understand the microbial dynamics during distinct fermentative phases, the roles of different microbes, and the relationship between bacteria and fungi, microbial community dynamics was investigated by culture-dependent and culture-independent approaches. Natural, static fermentation of milk induces the formation of the underlying curds and the superficial sour cream (Zuohe in the Mongolian language). From an overall perspective, viable LAB increased remarkably. Yeast showed an initial increase in their abundance (from 0 hr to 24 hr), which was followed by a decrease, and mold was detected at the later stages of fermentation (after 68 hr). The observed trends in microbiota variation suggest an antagonistic interaction between bacteria (LAB) and fungi (yeast and mold). The beneficial bacterial and fungal genus and species (e.g., Lactococcus, Streptococcus, Leuconostoc, Dipodascus, Lactococcus lacti, Dipodascus ausraliensis) are gradually increased in concentration, and the potentially detrimental microbial genus and species (e.g., Acinetobacter, Pseudomonas, Fusarium, Aspergillus, Mortierella, Acinetobacter johnsonii, Fusarium solani) decrease during the decline of bacterial and fungi diversity from natural fermentation. The study of microbial community dynamics could make a great contribution to understand the mechanism of natural fermentation of milk and the formation of curds and Zuohe, and to discover the potentially fermentative microbes for industrial starter cultures.

KEYWORDS
bacteria, fungi, microbial dynamics, natural fermentation

1 | INTRODUCTION

Milk natural fermentation is an ancient and traditional process that uses raw milk to ferment spontaneously. Traditional dairy products from natural fermentation of milk have always played relevant roles in the nutrition of nomad communities worldwide. Milk natural fermentation was originally developed by nomad Mongolian families, which usually placed the fermentation container into a cold storage room (below 20°C), to stimulate flavor production. Flavor diversity in naturally fermented milk results
from increased metabolite production by fermentative bacteria and fungi.

Several naturally fermented milk products derived from livestock of different countries have been investigated in terms of their production process (Guo, Xu, et al., 2019; Yamei et al., 2019), physicochemical composition (Bornaz et al., 2010; Guo, Xu, et al., 2019; Guo, Ya, et al., 2019; Yamei et al., 2019), microbial community (Akabanda et al., 2013; Gao et al., 2017; Gesudu et al., 2016; Guo, Ya, et al., 2019; Liu et al., 2015; Mathara et al., 2004; Nahidul-Islam et al., 2018; Oki et al., 2014; Shangpliang et al., 2017, 2018; Sun et al., 2014; Takeda et al., 2011; Yamei et al., 2019; Yao et al., 2017; Yu et al., 2011), and health benefits of such microorganisms (Karami et al., 2017; Takeda et al., 2011; Wang et al., 2016, 2018; Yi et al., 2016), such as traditional dairy products in China (Gao et al., 2017; Gesudu et al., 2016; Guo, Ya, et al., 2019; Sun et al., 2014; Wang et al., 2016, 2018; Yamei et al., 2019; Yao et al., 2017; Yi et al., 2016), Mongolia (Oki et al., 2014; Sun et al., 2014; Takeda et al., 2011; Yao et al., 2017; Yu et al., 2011), Russia (Liu et al., 2015), Iran (Karami et al., 2017), India (Shangpliang et al., 2018), Bangladesh (Nahidul-Islam et al., 2018), Tunisia (Bornaz et al., 2010), Bhutan (Shangpliang et al., 2017), Kenya (Mathara et al., 2004), and Ghana (Akabanda et al., 2013) (Table S1). However, few studies addressed the completely natural static fermentation, the intermediate process for the production of cheese and Zuohe (traditional sour cream). The sour cream moves upwards
### TABLE 2 | Bacterial diversity indices of 16S rRNA sequencing of the samples from the continuous fermentation process

| Time  | Reads  | OTUs | Chao   | Shannon | Simpson | Good's coverage |
|-------|--------|------|--------|---------|---------|-----------------|
| 0 hr  | 92,188 | 876  | 1,150  | 6.08    | 0.96    | 0.9972          |
| 8 hr  | 92,327 | 873  | 1,150  | 4.57    | 0.89    | 0.9965          |
| 16 hr | 88,513 | 830  | 1,193  | 4.12    | 0.86    | 0.9961          |
| 24 hr | 86,996 | 701  | 1,080  | 3.97    | 0.87    | 0.9966          |
| 32 hr | 85,005 | 685  | 1,042  | 4.23    | 0.89    | 0.9967          |
| 36 hr | 81,765 | 570  | 1,169  | 3.97    | 0.89    | 0.9961          |
| 40 hr | 83,742 | 625  | 1,100  | 3.98    | 0.89    | 0.9962          |
| 44 hr | 89,021 | 758  | 1,151  | 4.09    | 0.88    | 0.9963          |
| 48 hr | 86,974 | 605  | 1,147  | 3.93    | 0.88    | 0.9961          |
| 52 hr | 86,837 | 611  | 1,016  | 4.02    | 0.88    | 0.9968          |
| 56 hr | 87,323 | 526  | 967    | 3.52    | 0.81    | 0.9968          |
| 60 hr | 88,950 | 662  | 1,001  | 3.51    | 0.81    | 0.9968          |
| 64 hr | 95,528 | 610  | 1,112  | 3.52    | 0.80    | 0.9967          |
| 68 hr | 94,235 | 651  | 1,161  | 3.58    | 0.81    | 0.9968          |
| 72 hr | 83,684 | 2,905 | 2,941  | 7.57    | 0.96    | 0.9968          |
| 76 hr | 97,999 | 667  | 1,209  | 3.34    | 0.78    | 0.9966          |
| 80 hr | 94,440 | 855  | 1,312  | 4.72    | 0.88    | 0.9968          |
| 84 hr | 88,560 | 643  | 1,033  | 3.99    | 0.85    | 0.9969          |
| 88 hr | 87,692 | 589  | 940    | 3.31    | 0.77    | 0.9970          |
| 92 hr | 93,675 | 517  | 883    | 3.48    | 0.82    | 0.9974          |
| 96 hr | 86,015 | 572  | 916    | 3.61    | 0.84    | 0.9972          |
| Z1    | 85,607 | 650  | 992    | 4.93    | 0.92    | 0.9973          |
| Z2    | 91,116 | 565  | 873    | 4.28    | 0.90    | 0.9973          |
| Z3    | 84,827 | 515  | 793    | 4.05    | 0.89    | 0.9973          |

**FIGURE 3** Relative abundance of bacterial sequences (genus level) in the samples from the continuous fermentation process (a). The representative genera change during the continuous three stages of fermentation (b and c). The three consecutive sampling time points can be classified as a fermentative stage, namely as S1 to S7, and the superficial sour cream (Z1, Z2, and Z3) were collected at 88 hr, 92 hr, and 96 hr.
during natural fermentation of milk to form Zuohe, which is regarded as a Mongolian nutritional dairy product. Furthermore, previous research has been mostly focused on the microbial community of the final products from natural fermentation (Akaibanda et al., 2013; Gao et al., 2017; Guo, Ya, et al., 2019; Liu et al., 2015; Mathara et al., 2004; Nahidul- Islam et al., 2018; Oki et al., 2014; Shangpliang et al., 2017, 2018; Sun et al., 2014; Takeda et al., 2011; Yamei et al., 2019; Yao et al., 2017; Yu et al., 2011), neglecting the dynamics of the microbial community during natural fermentation. In this study, the microbial community, including LAB, yeast, and mold, during the process of natural fermentation of cow's milk were investigated by using culture-dependent methods and high-throughput amplicon sequencing including 16S rRNA and internal transcribed spacer (ITS) to understand the detailed fermentation process, the roles of distinct fermentative microorganisms, and the interplay between bacteria and fungi.

### TABLE 3  Bacterial diversity indices of 16S rRNA sequencing in the representative samples of fermentation

| Time   | Reads      | OTUs       | Chao      | Shannon | Simpson | Good's coverage |
|--------|------------|------------|-----------|---------|---------|-----------------|
| 0 hr   | 91,009 ± 2,163 | 876 ± 23   | 1,230 ± 31 | 5.06 ± 0.95 | 0.908 ± 0.044 | 0.9965 ± 0.0005 |
| 36 hr  | 88,032 ± 5,806 | 675 ± 38   | 1,175 ± 12 | 4.17 ± 0.04 | 0.902 ± 0.001 | 0.9961 ± 0.0003 |
| 44 hr  | 86,802 ± 3,976 | 794 ± 86   | 1,187 ± 84 | 4.14 ± 0.01 | 0.884 ± 0.012 | 0.9960 ± 0.0001 |
| 52 hr  | 85,756 ± 4,636 | 739 ± 83   | 1,118 ± 51 | 4.25 ± 0.38 | 0.886 ± 0.017 | 0.9964 ± 0.0002 |
| 68 hr  | 90,167 ± 4,471 | 685 ± 17   | 1,182 ± 62 | 3.32 ± 0.27 | 0.781 ± 0.027 | 0.9963 ± 0.0002 |
| 80 hr  | 91,884 ± 2,888 | 762 ± 138  | 1,229 ± 168 | 4.20 ± 0.55 | 0.859 ± 0.027 | 0.9966 ± 0.0002 |
| 88 hr  | 92,473 ± 4,263 | 684 ± 91   | 1,099 ± 99 | 3.62 ± 0.38 | 0.795 ± 0.021 | 0.9968 ± 0.0003 |
| 96 hr  | 86,276 ± 1,976 | 669 ± 100  | 1,037 ± 107 | 3.62 ± 0.03 | 0.834 ± 0.001 | 0.9966 ± 0.0003 |
| Z1     | 90,565 ± 5,338 | 703 ± 36   | 1,136 ± 106 | 4.48 ± 0.44 | 0.901 ± 0.013 | 0.9966 ± 0.0006 |
| Z3     | 86,235 ± 2,042 | 599 ± 19   | 999 ± 70   | 4.04 ± 0.07 | 0.890 ± 0.006 | 0.9968 ± 0.0002 |

2  | MATERIALS AND METHODS

2.1  | Construction of the Natural Fermentation Model and Quantification of LAB, Yeast, and Mold

To simulate natural milk fermentation, 100 kg of raw milk of Holstein from Xilingol prairie was used to ferment at low temperature (6.3 to 16.7°C) (Figure 1). We hypothesized that the fermentation of large amounts of raw milk could stabilize microorganism dynamics, which makes the results more representative. Large-scale, low-temperature natural fermentation was established for investigating bacterial and fungal community dynamics in details, which helps us further understand the changes and relationships of LAB, yeast, and mold in the process of natural fermentation. The protein, fat, and lactose content of raw cow's milk was 3.27%, 3.88%, and 4.92%, and the acidity and pH were 15°T and 6.85. We maintained the temperature of fermentation below 20°C to extend the period of natural fermentation, allowing a detailed study of microbial dynamics during the process. We clearly found that the viable count of LAB reached its highest value after 92-hr fermentation, and the pH fell to 4.8 which induced to the curding of milk. Based on these reasons, the 96-hr fermentation time was determined to be the end of natural fermentation in this snapshot study. The sampling intervals range from 8 hr to 4 hr (0 hr, 8 hr, 16 hr, 24 hr, 32 hr, 36 hr, 40 hr, 44 hr, 48 hr, 52 hr, 56 hr, 60 hr, 64 hr, 68 hr, 72 hr, 76 hr, 80 hr, 84 hr, 88 hr, 92 hr, 96 hr), and the underlying curds and superficial Zuohe (traditional sour cream) were collected to investigate bacterial and fungi community dynamics by using culture-dependent methods and high-throughput amplicon sequencing (16S rRNA and ITS). LAB (China National food safety standard, 2016a) were quantified using Man Rogosa Sharp (MRS) for 72 hr at 36°C according to national food safety standards in China. The yeast and mold (China National food safety standard, 2016b) were enumerated using Rose Bengal Agar for 5 d at 28°C.

2.2  | 16S rRNA and ITS sequencing, bioinformatics, and statistical analysis

Microbial DNA was extracted using E.Z.N.A stool DNA kit (Omega Bio-Tek, Norcross, US). The 16S rRNA V3-V4 region was amplified with the following primers: 341F 5'- CCTACGGGNGGCWGCAG -3' and 806R 5'- GGACTACHVGGGTATCTAAT -3'. The ITS sequence was amplified with the following primers: ITS3-KYO2F 5'- GATGAAGAAGCAGYRAA -3' and ITS4R 5'- TCCTCGCTATTGTATGG -3'. The PCR consisted of 5 μl of 10 X KOD buffer, 1 μl of KOD polymerase, 5 μl of 2.5 mM dNTPs, 1.5 μl of each primer (5 μM), and 100 ng of microbial DNA. The thermal program of the reaction was set up as follows: 1 x (95°C for 2 min), 27 x (98°C for 10 s, 62°C for 30 s, 68°C for 30 s), 1 x (68°C for 10 min). The amplicon was quantified and subjected to paired-end sequencing (2 x 250) by the Illumina MiSeq platform (Illumina, San Diego, CA). High-quality clean reads were obtained by removing reads with more than 10% of unknown nucleotides and less than 80% of bases with quality (Q-value) > 20. The final effective reads were acquired by removing chimeric tags and were clustered into the operational taxonomic units (OTU) of ≥97% similarity using the UPARSE pipeline.
(Edgar, 2013). The OTU was classified into organisms by the Naïve Bayesian Model using RDP classifier (Wang et al., 2007) based on SILVA database for 16S rRNA sequencing (Pruesse et al., 2007) and UNITE database for ITS sequencing (Koljalg et al., 2005).

3 | RESULTS AND DISCUSSION

3.1 | Viability changes in LAB, yeast, and mold during natural fermentation of milk

The total number of LAB and yeast in raw milk at the beginning of the fermentation process were 6.22 log cfu/ml and 4.16 log cfu/ml, respectively. In contrast, we did not detect any mold at this stage of the process. Given the small changes in the microbial counts, as well as in the pH, in early stages of fermentation, samples were collected every 8 hr followed by every 4 hr in middle and later stages. As shown in Figure 2 and Table 1, total LAB number significantly increased from 0 hr (6.22 log cfu/ml) to 32 hr (9.57 log cfu/ml) to 96 hr (12.29 log cfu/ml). Total yeast number rapidly increased after 24 hr (6.80 log cfu/ml), then gradually decreased until 96 hr (4.56 log cfu/ml). After 68 hr of fermentation, we detected the presence of mold (2.7 log cfu/ml), which gradually increased until 96 hr (4.56 log cfu/ml). After 68 hr of fermentation, we observed floating sour cream (pH 4.82). We collected the superficial sour cream (Z1, Z2, and Z3) as well as underlying curds at 86 hr, 92 hr, and 96 hr, respectively. The average number of viable LAB, yeast, and mold in the Zuohe was 12.18 ± 0.50 log cfu/ml, 6.05 ± 0.30 log cfu/ml and 5.41 ± 0.24 log cfu/ml, respectively (Figure 2 and Table 1). We did not observe differences in the LAB count nor in the temperature between the underlying curds and superficial Zuohe (p > .05), whereas the number of viable yeast and mold in the curds was significantly lower than in the Zuohe (p < .01 and .05, respectively). Concerning the pH, we observed a significantly higher value in the curds compared with Zuohe (p < .05). We hypothesize the lower pH results from the exposure of the superficial sour cream to environmental fungi, as well as to the increased yeast number.

The LAB counts in the traditional fermented dairy products from Mongolia, Chigee (naturally fermented mare’s milk from Inner Mongolia), the traditional Bangladeshi fermented milk products (dahi), and kefir grains varied from 3.41 to 9.03 log cfu/ml (Yu et al., 2011), 5.32 to 8.56 log cfu/ml (Guo, Xu, et al., 2019), 6.6 to 8.4 log cfu/g (Nahidul-Islam et al., 2018), and 4.81 to 8.93 log cfu/g (Witthuhn et al., 2004), respectively. Overall, LAB counts in our fermented samples (the curds: 11.38 ± 1.37 log cfu/ml; Zuohe: 12.18 ± 0.50 log cfu/ml) were higher than in any of the above fermented products tests. In addition, the viable yeast count ranged from 2.41 to 6.98 log cfu/ml in Chigee (Guo, Xu, et al., 2019), 2.8 to 7.8 log CFU/g in the traditional Bangladeshi fermented milk.
products (Nahidul-Islam et al., 2018), and 5.18 to 8.57 log cfu/g in kefir grains (Witthuhn et al., 2004). In contrast, yeast counts in our naturally fermented products (the curds: 4.15 ± 0.38 cfu/ml; Zuohe: 6.05 ± 0.30 cfu/ml) were generally lower. We reasoned that the large-scale, low temperature, and static natural fermentation could contribute to LAB proliferation, which in turn might inhibit the yeast growth.

LAB and yeast often coexist in the process of natural fermentation, such as traditional dairy products (Akabanda et al., 2013; Guo, Xu, et al., 2019; Guo, Ya, et al., 2019; Liu et al., 2015; Nahidul-Islam et al., 2018; Sun et al., 2014; Witthuhn et al., 2004; Yamei et al., 2019), sourdoughs (Lhomme et al., 2015), traditional Chinese liquor (Li et al., 2013), traditional Korean alcoholic beverages (Jung et al., 2012), Chinese horse bean-chili-paste (Lu et al., 2020), fermented Pu-erh tea (Zhang et al., 2016), and Chinese Sichuan Paocai (Xiao et al., 2018). Although natural fermentation of milk, predominantly resulting from lactic fermentation, the invariable co-occurrence of LAB and yeast suggests that an interplay between these two might occur, which contributes to the characteristics of dairy products. Yeast play an important role in the production of carbon dioxide and ethanol in naturally fermented dairy products, such as kefir and koumiss. However, the interaction between LAB and yeast has not been extensively studied. Such limitation can be overcome by the study of distinct strains occurring in naturally fermented products (Carbonetto et al., 2020; Mendoza et al., 2010; Sieuwerts et al., 2018; Sudun et al., 2013). The stimulation or inhibition of growth of one, or both, of the cocultured strains does not totally explain the potential interactions between LAB and yeast community in natural fermentation. In this study, despite the gradual increase in LAB as a whole, the dynamics of this process included four significant rise and fall stages (L1, L2, L3, and L4) (Figure 2). More interestingly, the corresponding stages of yeast (Y1, Y2, Y3, and Y4) mirrored the variation of LAB growth, which were similar to the distinct phases of mold growth (M4) (Figure 2). The above results indicate that the fermentative microbiota exhibited antagonist behavior, when comparing bacteria (LAB) to fungi (yeast and mold). We hypothesize that LAB and yeast in coculture may compete for nutrients or that they produce some metabolic substances that inhibit each other’s growth.

3.2 | Dynamic change of bacterial community during consecutive natural fermentation of milk

After removing low-quality and chimera reads, a total of 2,133,019 bacterial reads (Average ± SD: 88,876 ± 4,210) were obtained, and the OTUs of 0 hr to Z3 are shown in Table 2. Chao1, Shannon, Simpson, and Good’s coverage were utilized to evaluate bacterial community enrichment and diversity, and shown in Table 2. These indexes demonstrated that the bacterial community was adequately
represented, and changes of OTUs and Shannon indicated that bacterial diversity declined during natural fermentation. In addition, there were no significant differences in bacterial diversity between the curds (88 hr, 92 hr, and 96 hr) and Zuohe (Z1, Z2, and Z3) \((p > .05)\).

The three consecutive sampling time points can be classified as a fermentative stage, namely as S1 to S7 (Figure 3). For the abundance of diverse bacterial phylum group, Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria represented 69.49 ± 15.37%, 17.42 ± 5.70%, 5.71 ± 3.33% and 2.72 ± 0.69%, respectively in the S1. Finally, Firmicutes increased to 67.88 ± 3.83% (S7), whereas Proteobacteria, Bacteroidetes, and Actinobacteria declined to 31.37 ± 3.91% (S7), 0.40 ± 0.17% (S7), and 0.08 ± 0.02% (S7), respectively. In the Zuohe, the abundances of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria were 51.49 ± 3.96%, 44.71 ± 3.54%, 0.75 ± 0.67%, and 0.52 ± 0.62%, respectively. Concerning the bacterial genus group (Figure 3a), Lactococcus, Acinetobacter, Pseudomonas, Streptococcus, Oseumbacterium, and Leuconostoc represented 7.56 ± 1.40%, 27.57 ± 7.50%, 23.66 ± 7.24%, 4.34 ± 2.32%, 0.74 ± 0.22%, and 0.06 ± 0.04%, respectively, in S1. In S7, Lactococcus and Leuconostoc increased to 59.17 ± 5.44% and 2.41 ± 0.80%, respectively (Figure 3b and c), and Acinetobacter and Pseudomonas declined to 4.00 ± 0.34% and 5.16 ± 0.80%, respectively (Figure 3b). The abundance of Streptococcus first decreased to 1.30 ± 0.13% (S2), increasing to 5.38 ± 1.43% during S7 (Figure 3c). In contrast, Oseumbacterium increased to 3.40 ± 0.35% during S3, then decreasing to 1.15 ± 0.18% at S7 (Figure 3c). In the Zuohe, the abundances of Lactococcus, Acinetobacter, Pseudomonas, Streptococcus, Oseumbacterium, and Leuconostoc were of 32.91 ± 2.20%, 5.80 ± 0.38%, 7.12 ± 0.18%, 9.98 ± 2.97%, 2.24 ± 0.33%, and 0.27 ± 0.04%, respectively (Figure 3a).

### 3.3 Bacterial community variations in the representative fermentation stage

To assess the bacterial dynamics during natural fermentation, some critical time points (0 hr, 36 hr, 44 hr, 52 hr, 68 hr, 80 hr, 88 hr, 96 hr, Z1 and Z3) were chosen to carry out three biological replicates. A total of 2,667,601 bacterial reads (Average ± SD: 88,920 ± 4,110) were obtained, and the OTUs are shown in Table 3. Chao1, Shannon, Simpson, and Good’s coverage were used to evaluate bacterial community enrichment and diversity, as shown in Table 3. The alpha indexes demonstrated that the bacterial diversity declined during natural fermentation \((p < .01)\), with higher bacterial diversity in the Zuohe than in the final curds (96 hr, \(p < .01\)).

For bacterial phylum group, the abundance of Firmicutes \((p < .01)\) increased during natural fermentation, whereas Proteobacteria \((p < .01)\), Bacteroidetes \((p < .05)\), and Actinobacteria \((p < .05)\)
declined from 0 hr to 96 hr (Table 4). In addition, the Zuohe exhibited higher abundance of Proteobacteria than the curds, and less Firmicutes (p < .01) (Table 4). The results are in line with previous investigations, showing the four major bacterial phyla present in traditionally fermented dairy products (Gesudu et al., 2016; Guo, Ya, et al., 2019; Sun et al., 2014; Yamei et al., 2019). Moreover, although we observed a predominance of the Proteobacteria in early fermentation stages, its abundance decreased concomitantly with the increase in Firmicutes, as described previously in traditionally fermented foods, such as in traditional Korean salted seafood (Lee et al., 2014), traditional Chinese fish sauce (Du et al., 2019), and traditional Indian food idli (Mandhania et al., 2019). Concerning bacterial genus group (Figure 4 and Table 4), Lactococcus and Leuconostoc increased significantly (p < .01), whereas Acinetobacter and Pseudomonas declined significantly (p < .01). Streptococcus increase, after an initial decrease in abundance (Figure 4c and Table 4)

| Time  | Reads   | OTUs  | Chao  | Shannon | Simpson | Good’s coverage |
|-------|---------|-------|-------|---------|---------|-----------------|
| 0 hr  | 77,396 ± 8,822 | 272 ± 51 | 240 ± 43 | 4.86 ± 0.72 | 0.93 ± 0.03 | 0.9991 ± 0.0005 |
| 36 hr | 72,668 ± 9,947  | 259 ± 8  | 232 ± 7  | 3.60 ± 0.37 | 0.81 ± 0.06 | 0.9987 ± 0.0002 |
| 44 hr | 67,488 ± 12,306 | 297 ± 38 | 250 ± 42 | 4.80 ± 0.31 | 0.94 ± 0.01 | 0.9988 ± 0.0001 |
| 52 hr | 85,059 ± 6,122  | 272 ± 12 | 211 ± 7  | 5.31 ± 0.23 | 0.959 ± 0.003 | 0.9994 ± 0.0002 |
| 68 hr | 72,919 ± 9,129  | 231 ± 36 | 155 ± 23 | 2.75 ± 0.96 | 0.59 ± 0.19 | 0.9985 ± 0.0008 |
| Z1    | 83,372 ± 127     | 103 ± 4  | 290 ± 59 | 1.34 ± 0.13 | 0.29 ± 0.03 | 0.9992 ± 0.0001 |
| Z2    | 82,948 ± 97      | 96 ± 2   | 133 ± 50 | 0.90 ± 0.58 | 0.18 ± 0.13 | 0.9994 ± 0.0005 |
| Z3    | 91,210 ± 60      | 64 ± 2   | 165 ± 28 | 0.98 ± 0.59 | 0.22 ± 0.15 | 0.9996 ± 0.0002 |

TABLE 6 Fungal diversity indices of ITS sequencing in the representative samples of fermentation

| Time  | Reads   | OTUs  | Chao  | Shannon | Simpson | Good’s coverage |
|-------|---------|-------|-------|---------|---------|-----------------|
| 0 hr  | 77,641 ± 231 | 194 | 4.31 | 0.91 | 0.9992 |
| 8 hr  | 86,093 ± 320 | 289 | 5.64 | 0.96 | 0.9996 |
| 16 hr | 68,454 ± 240 | 246 | 4.56 | 0.93 | 0.9985 |
| 24 hr | 53,424 ± 243 | 220 | 4.07 | 0.88 | 0.9983 |
| 32 hr | 78,491 ± 250 | 230 | 3.91 | 0.88 | 0.9991 |
| 36 hr | 81,583 ± 263 | 223 | 3.71 | 0.83 | 0.9987 |
| 40 hr | 72,463 ± 243 | 179 | 4.39 | 0.91 | 0.9993 |
| 44 hr | 56,190 ± 249 | 214 | 4.46 | 0.93 | 0.9990 |
| 48 hr | 71,502 ± 246 | 202 | 4.39 | 0.93 | 0.9992 |
| 52 hr | 91,306 ± 260 | 206 | 5.46 | 0.96 | 0.9996 |
| 56 hr | 74,516 ± 213 | 135 | 2.77 | 0.58 | 0.9993 |
| 60 hr | 68,181 ± 208 | 157 | 3.18 | 0.66 | 0.9981 |
| 64 hr | 75,622 ± 214 | 167 | 1.77 | 0.38 | 0.9986 |
| 68 hr | 81,639 ± 198 | 118 | 1.49 | 0.31 | 0.9995 |
| 72 hr | 73,423 ± 265 | 209 | 1.42 | 0.28 | 0.9984 |
| 76 hr | 73,777 ± 230 | 162 | 2.43 | 0.52 | 0.9989 |
| 80 hr | 77,634 ± 229 | 133 | 1.32 | 0.27 | 0.9993 |
| 84 hr | 83,745 ± 213 | 124 | 1.37 | 0.28 | 0.9994 |
| 88 hr | 92,600 ± 359 | 230 | 3.82 | 0.85 | 0.9993 |
| 92 hr | 70,286 ± 197 | 105 | 2.62 | 0.57 | 0.9991 |
| 96 hr | 90,660 ± 175 | 117 | 1.06 | 0.22 | 0.9997 |
| Z1    | 83,372 ± 127  | 103 | 0.43 | 0.10 | 0.9998 |
| Z2    | 82,948 ± 97    | 96  | 0.80 | 0.27 | 0.9997 |
| Z3    | 91,210 ± 60    | 64  | 0.20 | 0.04 | 0.9998 |
(p < .01), whereas Obesumbacterium initially increased, followed by a significant decrease (Figure 4c and Table 4) (p < .01). The abundances of Lactococcus and Leuconostoc were lower in the Zuohe than in the curds (p < .01). In contrast, Acinetobacter, Pseudomonas, Streptococcus, and Obesumbacterium were more present in the Zuohe than in the curds (p < .05) (Table 4). Regarding the bacterial species group, Lactococcus lactis showed a significant increase (p < .01), contrary to Acinetobacter johnsonii, which abundance declined significantly (p < .01) (Table 4). Furthermore, the amount of Lactococcus lactis in the Zuohe was lower than in the curds (p < .01), whereas Acinetobacter johnsonii was higher (p < .05) (Table 4).

Each of the previously mentioned bacteria as a key role during natural fermentation. Lactococcus strains are widely used for industrial production of fermented dairy products. Besides the capacity to extend the shelf-life of dairy products, increasing amounts of Lactococcus during milk natural fermentation are at the basis of the sour and fermentative fragrances (Casalta & Montel, 2008; Cavanagh et al., 2015; Song et al., 2017). Leuconostoc spp. are a major contributor to the production of aromatic compounds during dairy fermentations (Endo et al., 2020). Streptococcus thermophilus is a species of lactic acid bacteria which is essential for the manufacturing of many types of fermented dairy products (Harnett et al., 2020). Acinetobacter (Kämpfer, 2014), Pseudomonas (Dodd, 2014), and Obesumbacterium (Enterobacteriaceae family) (Patel et al., 2014) are regarded as spoilage microbes for food, bringing about concerns about human health.

Multivariate analysis was performed to compare the bacterial community structures from naturally fermented samples. As demonstrated in Figure 7a, PcoA, which uses species-level OTUs, showed significant differences among samples from different fermentation time points (ANOSIM, R = 0.82, p = .001), supporting the successional dynamics of bacteria. In addition, samples from different fermentation time points were largely separated in the Bray analysis (accounting for 60.16% and 19.33% of the total variance by the two principal components, respectively). In conclusion, the process of natural fermentation is accompanied by the growth of viable LAB and the decay of bacterial diversity. At the same time, dairy fermentative microorganisms gradually increase, whereas potential spoilage or pathogenic microbes decrease dramatically.

### 3.4 | Dynamic change of fungal community during consecutive natural fermentation of milk

After removal of the low-quality and chimera reads, a total of 1,856,760 fungal reads (Average ± SD: 77,365 ± 10,108) were obtained. OTUs of 0 hr to Z3 are shown in Table 5. Chao1, Shannon, Simpson, and Good’s coverage were used to evaluate fungal community enrichment and diversity, as shown in Table 5. The indexes demonstrated that the fungal community was adequately represented, with changes in OTUs and Shannon indicating that the fungal diversity declined during natural fermentation. In addition, there were no significant differences between the fungal diversity of the curds (88 hr, 92 hr, and 96 hr) and Zuohe (Z1, Z2, and Z3) (p > .05).

**TABLE 7** The abundance (% of diverse fungal phyllum, genus, and species changes during natural fermentation of milk

| Ascomycota                        | Basidiomycota                        | Dipodascus                         |
|-----------------------------------|--------------------------------------|-----------------------------------|
| **0 hr**                          | **20.23 ± 28.49**                    | **91.65 ± 4.08**                  |
| **1 hr**                          | **28.45 ± 15.45**                    | **98.71 ± 0.55**                  |
| **3 hr**                          | **35.36 ± 0.78**                     | **90.90 ± 4.08**                  |
| **6 hr**                          | **40.74 ± 5.45**                     | **98.71 ± 0.55**                  |
| **9 hr**                          | **47.71 ± 12.02**                    | **96.79 ± 0.55**                  |
| **12 hr**                         | **54.11 ± 3.91**                     | **99.37 ± 0.55**                  |
| **18 hr**                         | **61.85 ± 4.08**                     | **100.00 ± 0.01**                 |
| **24 hr**                         | **68.47 ± 5.45**                     | **100.00 ± 0.01**                 |
| **36 hr**                         | **75.03 ± 5.45**                     | **100.00 ± 0.01**                 |
| **48 hr**                         | **81.78 ± 12.18**                    | **100.00 ± 0.01**                 |
| **60 hr**                         | **88.61 ± 10.68**                    | **100.00 ± 0.01**                 |
| **72 hr**                         | **95.03 ± 10.68**                    | **100.00 ± 0.01**                 |
| **80 hr**                         | **101.20 ± 10.68**                   | **100.00 ± 0.01**                 |
| **86 hr**                         | **103.45 ± 10.68**                   | **100.00 ± 0.01**                 |
| **96 hr**                         | **107.97 ± 10.68**                   | **100.00 ± 0.01**                 |
| **108 hr**                        | **112.47 ± 10.68**                   | **100.00 ± 0.01**                 |
| **120 hr**                        | **116.97 ± 10.68**                   | **100.00 ± 0.01**                 |
| **144 hr**                        | **121.47 ± 10.68**                   | **100.00 ± 0.01**                 |
| **168 hr**                        | **125.97 ± 10.68**                   | **100.00 ± 0.01**                 |
| **192 hr**                        | **130.47 ± 10.68**                   | **100.00 ± 0.01**                 |
| **216 hr**                        | **134.97 ± 10.68**                   | **100.00 ± 0.01**                 |
| **240 hr**                        | **139.47 ± 10.68**                   | **100.00 ± 0.01**                 |
| **252 hr**                        | **143.97 ± 10.68**                   | **100.00 ± 0.01**                 |
| **276 hr**                        | **148.47 ± 10.68**                   | **100.00 ± 0.01**                 |
| **300 hr**                        | **152.97 ± 10.68**                   | **100.00 ± 0.01**                 |
| **324 hr**                        | **157.47 ± 10.68**                   | **100.00 ± 0.01**                 |
| **348 hr**                        | **161.97 ± 10.68**                   | **100.00 ± 0.01**                 |
The fungal sequencing reads were classified at the phylum and genus levels. At the phylum level, the phyla Ascomycota, Mortierellomycota, and Basidiomycota were detected at 81.78 ± 12.17%, 12.02 ± 11.35%, and 3.46 ± 2.87%, respectively, during S1. Finally, during S7, Ascomycota increased to 96.55 ± 0.67%, whereas Mortierellomycota and Basidiomycota declined to 0.08 ± 0.14% and 0.96 ± 0.94%, respectively. In the Zuohe, Ascomycota, Mortierellomycota, and Basidiomycota represented 98.40 ± 0.86%, 0, and 0.30 ± 0.10% of fungi community, respectively. Concerning the fungal genus group (Figure 5a), Dipodascus, Aspergillus, Fusarium, and Mortierella were detected at 0.01 ± 0.02%, 16.19 ± 16.13%, 15.65 ± 12.41%, and 12.02 ± 11.35%, respectively, during S1. At S7, Dipodascus increased to 54.03 ± 41.09% (Figure 5b), whereas Aspergillus, Fusarium, and Mortierella declined to 2.34 ± 1.60%, 2.51 ± 2.17%, and 0.08 ± 0.14%, respectively (Figure 5b). In the Zuohe, Dipodascus, Aspergillus, Fusarium, and Mortierella represented 96.90 ± 1.69%, 0.19 ± 0.11%, 0.04 ± 0.05%, and 0, respectively (Figure 5a).

3.5 | Fungal community variations in the representative fermentation stage

To assess fungal dynamics during natural fermentation, key fermentation time points (0 hr, 36 hr, 44 hr, 52 hr, 68 hr, 80 hr, 88 hr, 96 hr, Z1, and Z3) were chosen to carry out three biological replicates. A total of 2,386,130 fungal reads (Average ± SD: 79,538 ± 9,264) were obtained. OTUs are shown in Table 6. Chao1, Shannon, Simpson, and Good’s coverage were used to evaluate fungal community enrichment and diversity, as shown in Table 6. The alpha indexes showed that fungal diversity declined during natural fermentation (p < .01), with no significant differences in the fungal diversity between the curds (96 hr) and Zuohe (Z3) (p > .05). At the phylum level, the phyla Ascomycota (p < .01) increased during natural fermentation, whereas Mortierellomycota (p < .01) and Basidiomycota (p < .01) declined from 0 hr to 96 hr (Table 7). At the genus level (Figure 6 and Table 7), Dipodascus increased significantly (p < .01), and Aspergillus, Fusarium, and Mortierella showed an initial increase (0 hr to 44 hr) followed by a marked reduction (Figure 6b and Table 7) (p < .01). Concerning fungal species group, Dipodascus australiensis increased significantly (p < .01), which contrasts with Fusarium solani, which declined after an initial increase (from 0 hr to 36 hr, p < .01) (Table 7). We did not observe significant differences in terms of the abundances of fungal phylum, genus, and species between the curds and Zuohe (Table 7) (p > .05).

The yeast Dipodascus has been identified in naturally fermented dairy products from Inner Mongolia (Guo, Ya, et al., 2019; Yamei et al., 2019). Aspergillus, Fusarium, and Mortierella present potential threats to cattle and humans that might result from fungal abortion, mycotoxins, and aspergillosis (Davies et al., 2010; Foster, 2017; Rodrigues, 2016; Thrane, 2014). We reasoned that, in the context of dairy products, Aspergillus, Fusarium, and Mortierella behave as...
spoilage microorganisms. After the initial growth (0 hr to 44 hr), these three genera become feeble and gradually disappear. Raw milk is a favorable environment that supports their initial growth. However, growth is likely inhibited by the increasing amounts of LAB and, consequently, of increased lactic acid concentration. This might inhibit the growth of these fungi in the stages of metaphase and anaphase of natural fermentation. A reduction in potentially pathogenic molds throughout the natural fermentation meant that potential mycotoxins were produced in the natural fermentation of cow’s milk. As a result, further studies will be carried out for the detection and quantification of mycotoxins in the traditionally fermented milk.

Multivariate analysis was performed to compare the fungal community structures from samples during natural fermentation. As demonstrated in Figure 7b, PcoA, which uses species-level OTUs, showed significant differences among two groups of samples from the early stage (0 hr, 36 hr, 44 hr, and 52 hr) of the fermentation and the late (68 hr, 80 hr, 88 hr, 96 hr, Z1, and Z3) (ANOSIM, R = 0.6, p = .001). Still, the analysis showed overlapping between samples from the two groups. The results supported the successive changes of fungi, Dipodascus increased significantly after 52 hr of fermentation, whereas Aspergillus, Fusarium, and Mortierella decreased gradually during the early stage (0 hr, 36 hr, 44 hr, and 52 hr) of the fermentation. In addition, the values of samples from different fermentation time points were largely separated in the bray analysis (accounting for 26.49% and 19.14% of the total variance by the two principal components, respectively).

4 | CONCLUSIONS

Traditional dairy products derived from milk’s natural fermentation are worldwide used for their nutrient content, fermented flavor and long shelf-life, providing substantial benefits for human health. Still, the presence of potentially pathogenic microorganisms derived from natural fermentation, together with lack of proper sanitary conditions raised public concerns. In this study, we found that the beneficial bacteria and fungi (e.g., Lactococcus, Streptococcus, Leuconostoc, Dipodascus) are gradually increased in concentration and that potentially pathogenic microorganisms (e.g., Acinetobacter, Pseudomonas, Fusarium, Aspergillus, Mortierella) decrease during the process of natural fermentation. The results support the health beneficial properties of naturally fermented products and highlight the nomadic dairy culture for consumption of naturally fermented milk. Natural, static milk fermentation forms curds and Zuohe (traditional sour cream). Although there were no significant differences in the cultured LAB between the underlying curds and superficial Zuohe, the potentially detrimental bacteria (Acinetobacter, Pseudomonas, Fusarium) were significantly increased in the second, which results in an increase of its bacterial diversity. Furthermore, the Zuohe was significantly more enriched in yeast and mold than the curds. However, there were no significant differences in fungi diversity in terms of genus and species between the two types of fermented samples. Given that the Zuohe was at the surface, and thus exposed to environmental microorganisms, it was expected that the potentially detrimental bacteria, yeast, and mold in the air were also significantly more enriched in the Zuohe than in the underlying curds.

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CONFLICT OF INTEREST
All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Wei-Liang Xu: Data curation-Equal, Software-Equal, Validation-Equal, Writing-review & editing-Equal. Chun-Dong Li: Data curation-Equal, Methodology-Equal, Software-Equal, Validation-Equal. Yuan-Sheng Guo: Data curation-Equal, Software-Equal, Validation-Equal. Yi Zhang: Software-Equal, Validation-Equal. Mei Ya: Software-Equal, Validation-Equal. Liang Guo: Conceptualization-Lead, Funding acquisition-Lead, Investigation-Lead, Project administration-Lead, Writing-original draft-Lead, Writing-review & editing-Lead.

ETHICAL APPROVAL
This study does not involve any human or animal testing.

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