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ORIGINAL ARTICLE

Microbial composition and dynamic succession during the Daqu production process of Northern Jiang-flavored liquor in China

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Abstract

The microbial community structure and succession regularity of six key periods during high-temperature Daqu production were revealed using high-throughput sequencing to explore the factors affecting the flavor formation of Northern Jiang-flavored Baijiu technology. The results showed that among the six Daqu samples, the bacteria mainly included Firmicutes, Actinobacteriota, and Proteobacteria, of which Proteobacteria was the most dominant. The primary fungus was Ascomycota. At the genus level, the primary bacterial groups were *Lactobacillus*, *Weissella*, *Bacillus*, *Delftia*, *Achromobacter*, *Saccharopolyspora*, *Thermoactinomyces*, *Scopulibacillus*, *Pseudomonas,* and *Stenotrophomonas*. The main fungal groups in the Daqu were *Wickerhamomyces*, *Saccharomycopsis*, *Thermoascus,* and *Thermomyces*. During the initial stage of Daqu production, the dominant bacteria were *Lactobacillus* (20.07%) and *Weissella* (48.30%). As the fermentation temperature of the Daqu increased, *Achromobacter*, *Stenotrophomonas*, and *Delftia* became the dominant bacteria during the first Daqu flipping period, the second Daqu flipping period, and the dry-fire period. During these three periods, many bacteria were eliminated, decreasing the bacterial diversity, while a decline in temperature was evident during the Daqu exit period. After adapting to the high-temperature environment, the accumulation of *Saccharopolyspora* (22.07%), *Thermoactinomyces* (16.73%), *Scopulibacillus* (27.13%), *Kroppenstedtia* (9.03%), and *Bacillus* (6.97%) increased the bacterial diversity during the Daqu exit period. *Wickerhamomyces* (83.47%) represented the main dominant fungus during the initial production stage but were eliminated with increased temperature. Furthermore, a higher temperature increased the abundance of *Saccharomycopsis* and *Thermoascus*, while *Thermomyces* gradually accumulated in the D, E, and F samples. *Thermomyces* (79.90%) and *Thermoascus* (13.83%) became the dominant fungi during the Daqu exit period. In this study, high-throughput sequencing technology was used to reveal the microbial diversity during the high-temperature Daqu production process of Northern Jiang-flavored Baijiu. This provided a scientific basis for improving the production process of this product in the future. Therefore, understanding the formation of the flavor substances and the related microorganisms in Northern Jiang-flavored Baijiu can provide guidance for using them to manipulate the preparation process while implementing microbial control and improving the production procedures.

Keywords Northern Jiang-flavour Baijiu · Daqu · Microbial composition · Community diversity · High-throughput sequencing

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Introduction

Baijiu is one of the oldest distilled liquors in the world and is a national product of China. It has a history of 2000 years, dating back to the Western Han Dynasty (Liu and Sun 2018). Baijiu occupies a unique position in traditional Chinese culture and is exceedingly popular among consumers (Xie et al. 2020). Chinese Baijiu fermentation is a unique, complicated process that simultaneously utilizes saccharification and spontaneous fermentation (Jin et al. 2017). Jiang-flavored Baijiu represents one of the foremost flavored liquors in China (Wang et al. 2019a, b). It is unique due to its mellow body, outstanding Jiang-flavor, pleasant aroma, and lingering aftertaste. The unique flavor of Jiang-flavored Baijiu is related to the traditional brewing process, which mainly includes four steps: Daqu starter preparation, alcohol fermentation, solid distillation, and aging (Zhang et al. 2021).

Various factors, such as the geographical environment, brewing materials, saccharifying screwdrivers, and brewing containers, have resulted in the different flavors of Chinese Baijiu (Wei et al. 2020). The variation in regions and production processes leads to differences in the evolution of the microbial community structure during the fermentation process, ultimately modifying the Baijiu quality. Since Jiang-flavored Baijiu is mostly found in the hot and humid south, previous reports mainly focused on examining microorganisms in southern Jiang-flavored Baijiu Daqu. Due to the unique geographical location of Maotai Town, the Baijiu produced in other places does not exhibit the style characteristics of this region. Northern Jiang-flavored Baijiu does not display the same flavor properties as Maotai, instead exhibiting uniquely northern characteristics.

As the microbial carrier and saccharification starter for Northern Jiang-flavored Baijiu, the high-temperature Daqu provides rich microbial strains, unique Jiang-flavor components, and precursor substances for Baijiu production (Wang et al. 2017b). High-temperature Daqu fermentation is crucial during the production of Northern Jiang-flavored Baijiu, and its quality is directly related to the quality of the Baijiu (Yao et al. 2015; Yi et al. 2019). As a key factor in the preparation of Daqu, microorganisms can produce various biological enzymes, flavor components, and their precursors, playing an essential role in developing the unique flavor of the Baijiu (Huang et al. 2017; Wang et al. 2017b).

In this study, high-throughput sequencing technology was used to study the microbial composition, and its succession in Northern Jiang-flavored Baijiu brewed in high-temperature Daqu during six key periods. This research clarifies the development of Northern Jiang-flavored Baijiu, providing a scientific basis for microbial control during the fermentation process of this product. Furthermore, it is essential in improving the quality of Northern Jiang-flavored Baijiu and balancing the microbial metabolism.

Materials and methods

Sample collection

Daqu samples were collected from the high temperature Daqu fermentation room of Hebei Zhuojiu Group Co., Ltd., Hebei Province, China. The six samples were collected during six periods of the production process and included the Daqu entry period (sample A, June 28st, 2020), the first Daqu flipping period (sample B, July 3st, 2020), the second Daqu flipping period (sample C, July 7th, 2020), the dry-fire period (sample D, July 15st, 2020), the post-fire period (sample E, July 25st, 2020), and the Daqu exit period (sample F, August 20st, 2020). Sample A was collected at 25 ℃ during the entry period. Samples B and C were collected at 62 ℃ during the first Daqu flipping period and at 61℃ during the second Daqu flipping period. Samples D and E were collected at 61 ℃ during the dry-fire period and at 45 ℃ during the post-fire period. The Daqu exit period was sampled at 40 ℃. The six samples with three replicates for each were collected randomly from the four corners and the central point of Daqu bricks at different fermentation periods. The outer (0–3 cm) and central portions of each brick were mixed and ground with the weight of each sample approximately 1000 g. The samples were transferred to sterile bags, sealed and stored at 4 ℃ until processed.

DNA extraction

Using the Fast DNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) to extract the total DNA from each Daqu sample (2 g) according to the manufacturer's instructions.

Amplicon library preparation and sequencing

The V3–V4 regions on the 16S rRNA gene of the bacteria were amplified with forward primers 338F (5′-ACTCCT ACGGGAGGCAGCA-3′) and reverse primers 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Klindworth et al. 2013). The barcode of 8 bp was added to the 5′ end of the forward primer as the sample differentiation. The fungi were identified via polymerase chain reaction (PCR) amplification in the ITS1 region, and the primers were ITS1F (5′-CTT GGTCATTTAGAGGAAGTAA-3′) and ITS1R (5′-GCT GCGTTCTTCATCGATGC-3′) (Usyk et al. 2017). The forward primer was bar coded with a 6-base sample specific sequence to facilitate multiplexing of a sample set. The reaction volume was 50 µL, which comprised 27 µL ddH_2O,

2 µL (5 µM) each of the forward and reverse primers, 2.5 µL (10 ng) of template DNA, 5 µL (2.5 mM) of deoxynucleoside triphosphates, 10 µL of 5× FastPfu buffer, 0.5 µL of bovine serum albumin and 1 µL of TransStart FastPfu Polymerase (TransGen, Beijing, China). The bacteria were first denaturated at 95 ℃ for 5 min, and then the thermal cycle was carried out. Each thermal cycle was was denatured at 94 ℃ for 30 s, annealed at 55 ℃ for 35 s, and elongated at 72 ℃ for 30 s. At 30 cycles, the final extension step was performed at 72 ℃ for 8 min. In the same way, Furthermore, the predenaturation of the fungi occurred at 95 °C for 5 min, after which each thermal cycle was denaturated at 94 °C for 30 s, annealed at 54 °C for 40 s, and elongated at 72 °C for 30 s. After 30 cycles, the final extension step was performed at 72 °C for 8 min. The PCR product was purified using a gel recovery kit (Life Technology, USA), and Qubit 3.0 (Life Technology, USA) was used for accurate double-stranded DNA concentration quantification. The purified amplicons were pooled in equimolar concentrations, and library-specific sequencing adapters were added via a NEBNext Ultra (NEB#e7370S/L) assay, following the manufacturer's instructions. Dual index sequencing of the paired-end 250 bp reads were run on an Illumina HiSeq2500 instrument (Illumina, San Diego, CA, USA).

Sequence data pre-processing and analysis

The original data obtained were processed using next-generation sequencing (NGS) toolkit software to remove low-quality reads (parameters are default) (Patel and Jain 2012). Overlapping pairs were merged by FLASH software (Magoč and Salzberg 2011). Split_libraries_fastq.py in the QIIME software was used to separate the data according to the 6 bp barcode in the 5′ forward primer (Kuczynski et al. 2012). The clean labels were processed along the pipeline with MOTHUR software (Caporaso et al. 2010), and barcodes and primer sequences in labels were trimmed. The sequences were filtered and denoised according to the database RDP 16S rRNA reference db128 to remove chimeras and classified by bayesian methods (Cole et al. 2014). Non-16s sequences (e.g. unknown, archaea, chloroplasts, mitochondria and cyanobacteria) were removed.

The edited sequences were divided into operational taxonomic units (OTUs) using VSEARCH software at 97% distance (Rognes et al. 2016). The representative OTU sequences were selected according to the most abundant sequences in OTUs. The OTU table was rarefied to the minimum number of sequences across samples to directly compare the alpha and beta diversity values of each flora. Principal coordinate analysis (PCA) based on UniFrac distance matrix (to estimate similarity based on distance between samples) (Yue and Clayton 2005), and two-way multivariate analysis of variance (PERMANOVA) based on Bray–Curtis dissimilarity were used to assess the variances in microbial community composition using MOTHUR and the R package vegan (version 2.2) (Schloss et al. 2009). Linear discriminant analysis (LDA) effect size (LEfSe) was used to analyze the differences in the microbiota of the six Daqu samples (Kozik et al. 2017).

Results

Sequencing data quality control

The raw high-throughput sequencing data were submitted to the National Center for Biotechnology Information (NCBI) database under the BioProject number, PRJNA687340. A total of 3.38 M raw sequences were obtained after sequencing, while 3.29 available reads were acquired after the quality control process. After filtering out the nontarget fragments, a total of approximately 1.41 M bacterial 16S rDNA fragment sequences (ranging from 15,611 to 248,151) and 1.88 M fungi ITS DNA fragment sequences (ranging from 24,047 to 167,796) were obtained.

Supplementary Fig. 1 shows that the rarefaction bacterial and fungal curves approached the saturation plateau, suggesting that the depth of sequencing in this study was sufficient to represent the diversity of microorganisms in the samples and indicating that the selected sample was representative.

The microbial diversity in the six Daqu samples

The diversity indexes shown in Table 1 (including the number of OTUs, Ace, Chao, Shannon, and Simpson values) were used to evaluate the abundance and uniformity of the microbial Daqu community. The richness estimators (Chao and Ace) were consistent with the Shannon indices. The bacterial OTUs, Chao, and Ace were the most significant in sample E, followed by sample F. The Shannon index and Simpson index described species diversity. The higher the Shannon index, the higher the community diversity. The bacterial Shannon value was the highest in sample F and the lowest in sample B. No significant differences were evident in the Ace, OTU, and Chao values of the different fungal genotypes. The fungal Shannon, OTU, and Chao values of sample A were the highest, while the Simpson value was the lowest. Table 1 shows that samples E and F displayed the highest bacterial diversity, while sample A exhibited the highest fungal diversity.

The Venn diagram (Fig. 1) created based on the sample was used to investigate whether exclusively shared OTUs existed. At a 97% similarity level, the number of bacterial OTUs for samples A, B, C, D, E and F was 91, 106, 66, 57, 259, and 108, while the number of fungal OTUs was 60, 42,

Table 1 Species richness estimator and diversity index

	Group	Num of OTUs[1]	Ace	Simpson	Shannon	Chao[2]
Bacteria	A	58.67 ± 7.51^{b}	71.54 ± 16.37^{b}	0.29 ± 0.06^{c}	1.92 ± 0.25^{b}	67.71 ± 14.65^{bc}
	B	64.33 ± 4.04^{b}	114.46 ± 33.51^{b}	0.60 ± 0.01^{a}	0.98 ± 0.03^{d}	92.67 ± 12.21^{b}
	C	33.00 ± 3.46^{c}	86.91 ± 29.05^{b}	0.34 ± 0.001^{c}	1.20 ± 0.01^{c}	56.50 ± 16.35^{c}
	D	26.67 ± 3.79^{c}	78.26 ± 39.51^{b}	0.43 ± 0.01^{b}	1.00 ± 0.02^{cd}	51.67 ± 22.54^{c}
	E	145.67 ± 13.58^{a}	194.92 ± 13.79^{a}	0.28 ± 0.04^{c}	1.82 ± 0.07^{b}	180.31 ± 8.66^{a}
	F	67.00 ± 8.89^{b}	108.25 ± 14.30^{b}	0.16 ± 0.01^{d}	2.20 ± 0.06^{a}	91.85 ± 7.73^{b}
Fungi	A	26.67 ± 12.50^{a}	37.42 ± 23.02^{a}	0.37 ± 0.03^{c}	1.30 ± 0.17^{a}	34.83 ± 21.18^{a}
	B	18.33 ± 2.08^{a}	25.78 ± 4.83^{a}	0.81 ± 0.04^{a}	0.47 ± 0.06^{c}	21.83 ± 2.57^{a}
	C	22.33 ± 3.51^{a}	40.88 ± 20.17^{a}	0.60 ± 0.30^{abc}	0.89 ± 0.55^{abc}	29.98 ± 8.64^{a}
	D	23.67 ± 2.08^{a}	30.45 ± 5.08^{a}	0.85 ± 0.04^{a}	0.41 ± 0.11^{c}	27.08 ± 5.31^{a}
	E	19.33 ± 4.16^{a}	24.62 ± 9.10^{a}	0.44 ± 0.10^{bc}	1.11 ± 0.22^{ab}	21.67 ± 8.13^{a}
	F	19.67 ± 5.76^{a}	25.21 ± 9.90^{a}	0.66 ± 0.05^{ab}	0.73 ± 0.04^{bc}	26.33 ± 5.69^{a}

[1]Microbial abundance was observed based on the number of OTUs in each group

[2]Non-parametric statistical predictions of the total richness of OTUs; Values with superscript letters a, b, c, and d are significantly different across columns ($p < 0.05$)

52, 47, 42, and 42. The number of bacterial OTUs was the highest in sample E and the lowest in sample D. However, the number of fungal OTUs was the highest in sample A. Although eight bacterial OTUs and 17 fungal OTUs were found in all six samples, each sample displayed unique bacterial and fungal characteristics. Similarly, the independent bacterial OTUs in sample E and the independent fungal OTUs in sample A far exceeded those in the other samples. The proportion of unique bacterial OTUs in samples A, B, C, D, E, and F was 91.2%, 92.5%, 87.9%, 86.0%, 97.0%, and 92.6%, while that of the independent fungal OTUs was 71.7%, 59.5%, 67.3%, 63.8%, 59.5%, and 59.5%.

The microbial community structures and compositions in the six Daqu samples

The Mantel test calculation showed a strong correlation ($r = 0.9887$, $p < 0.001$) between the Bray–Curtis dissimilarity matrices (based on the bacterial genera-level classification and OTUs). Figure 2a shows that the distribution of phyla of the bacterial species in the six samples was notably regular. The main phylum in sample A was Firmicutes, accounting for 86.9%, followed by Proteobacteria. Furthermore, a small amount of Actinobacteriota was also present in sample A at 1.53%. The most dominant phylum in samples B, C, D, and E was Proteobacteria and a small amount of Actinobacteriota and Firmicutes. Two dominant phyla were present during the final period, namely Actinobacteriota and Firmicutes. Overall, the bacterial structure was optimized, and the bacterial flora gradually stabilized during the Daqu fermentation process. Proteobacteria denoted the most abundant phylum during the entire fermentation process, increasing initially, followed by a decline. The Proteobacteria only accounted for 11.5% in sample A and over 95% in samples B, C, D, and E, decreasing sharply to 1.6% during the final sampling period. Firmicutes represented the second most abundant phylum, and its proportions and dynamic succession in Daqu were the exact opposite than in Proteobacteria. Firmicutes accounted for 86.9% of the total prokaryotic sequences at the beginning of fermentation, decreasing sharply as the process progressed, accounting for only 0.1% in sample D. However, Firmicutes stabilized at around 69.4% in sample F, becoming the dominant phylum. Actinobacteriota represented the third most dominant phylum. Although negligible at the beginning, it increased as the fermentation progresses, eventually becoming the dominant flora. During fermentation, many low-abundance phyla were gradually eliminated, further increasing the abundance of the dominant bacteria. Figure 2b shows that the major constituents of the six samples experienced significant changes at the bacterial genus level. At the beginning of fermentation, *Weissella* (48.30%) and *Lactobacillus* (20.07%) were regarded as the predominant genera, with a relative abundance exceeding 20%. There was also a small amount of *Stenotrophomonas* (4.80%), *Lactococcus* (6.90%), *Delftia* (1.40%), *Achromobacter* (1.60%), and *Leuconostoc* (5.0%) in sample A. *Weissella* almost disappeared in samples B, C, and D, while *Stenotrophomonas, Achromobacter,* and *Delftia* increased to become the dominant genera. There were also small amounts of *Kroppenstedtia* (1.27%) and *Pseudomonas* (0.87%) in samples B, C, and D. As the fermentation progressed, the proportion of *Achromobacter* increased to 41.23% in sample E. Furthermore, in addition to the dominant *Achromobacter*, *Stenotrophomonas*, and *Delftia* genera, *Pseudomonas* and other genera also appeared. The bacterial species changed significantly during the final stage and the dominant genera of the previous period disappeared. *Saccharopolyspora* (22.07%), *Thermoactinomyces* (16.73%), *Scopulibacillus* (27.13%),

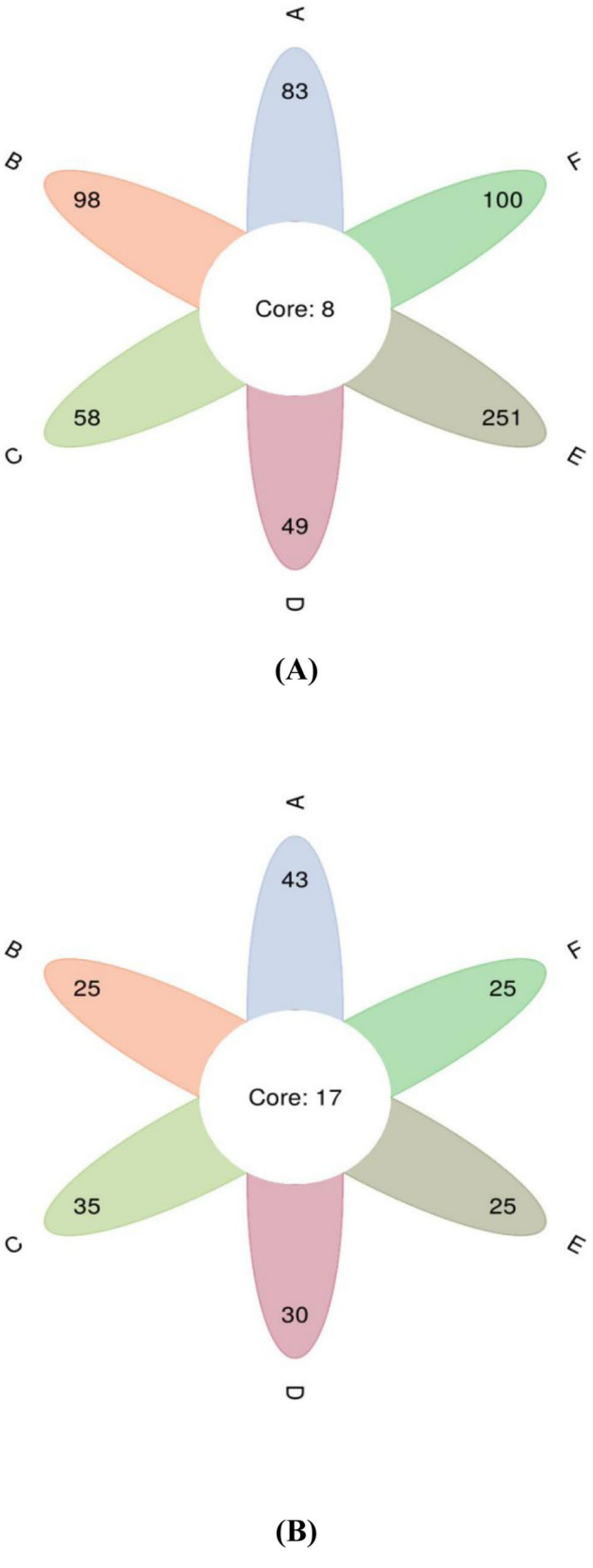


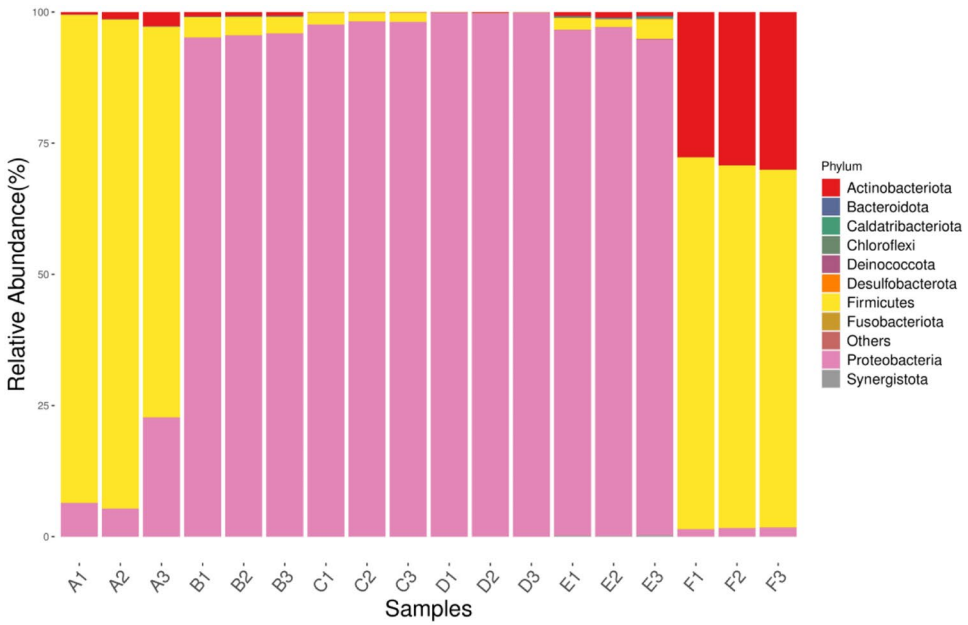
Fig. 1 A Venn diagram showing the shared and unique OTUs at 97% identity among the six Daqu samples, with OTU abundance < 1% removed. **a** A Venn diagram of the bacteria. **b** A Venn diagram of the fungi. Sample A was collected at Daqu entry; Sample B was collected at the first Daqu flipping; Sample C was collected at the second Daqu flipping; Sample D was collected at the Daqu dry-fire period; Sample E was collected at the Daqu post-fire period and Sample F was collected at Daqu exit. Core refers to the number of OTUs shared by the six samples in the bacterial and fungal Venn diagrams

Kroppenstedtia (9.03%), and *Bacillus* (6.97%) became the dominant genera. *Weissella* (5.20%) appeared again, and the bacterial diversity increased (Table 2).

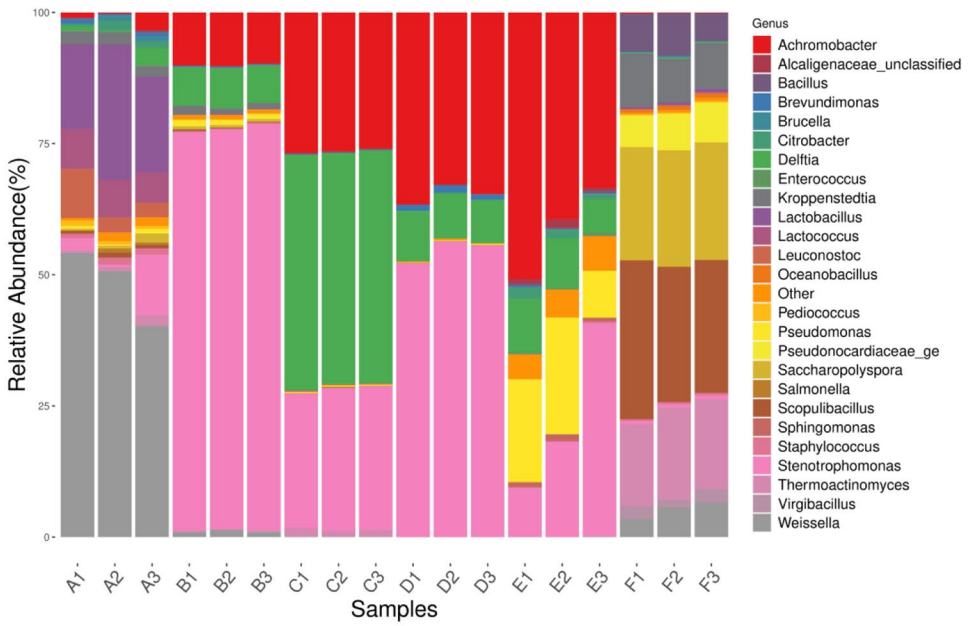
The taxonomic distribution of fungi in the six samples at the phylum level is shown in Fig. 3a. Compared with the prokaryotic communities, the diversity of the eukaryotic microbes was smaller. At the phylum level, the predominant eukaryotic community was Ascomycota during the entire fermentation process. In general, Ascomycota constituted more than 99.0% of the total abundance. Zygomycota only accounted for 0.9% in sample E, while a small number of Basidiomycota also appeared during the fermentation process. The eukaryotic community succession was investigated according to OTUs classified at the genus level (Fig. 4b). *Wickerhamomyces* (83.47%) dominated in sample A, while *Saccharomycopsis*, *Thermoascus, Thermomyces* accounted for less than 4%. *Debaryomyces* (2.53%) appeared in sample A but subsequently disappeared. However, *Saccharomycopsis* increased to 90.03% in sample B, subsequently showing a decreasing trend. Furthermore, small amounts of *Thermomyces*, *Thermoascus*, *Wickerhamomyces* were evident in sample B. *Thermomyces* first increased and then declined during the entire fermentation process, especially in sample D, accounting for 92.03%. Although a decline was evident in samples E and F, it still dominated in sample F (Table 2).

Principal coordinate analysis (PCA) showed significant differences between the microbial communities of the six Daqu samples (Fig. 4). Although the dominant bacteria (top 20) and fungal genera (top 12) were almost identical in each sample, significant differences were apparent in the microbial abundance among the six groups. No substantial differences were evident between the three replicates of each sample, indicating the reliability of the samples. The bidirectional PERMANOVA based on Bray Curtis similarity was used to identify the statistical differences between the microbial populations in these groups.

At the genus level, linear discriminant analysis effect size (LEfSe) analysis was used to determine the significant differences between the bacterial communities in the Daqu samples (Fig. 5). Overall, the Firmicutes phylum, the Bacilli class, the Leuconostocaceae family, and the *Weissella* genus were significantly higher in sample A than in other samples. The Xanthomonadaceae family and the *Stenotrophomonas* genus in sample B were higher during



(A)



(B)

Fig. 2 The distribution of the predominant bacteria in the six Daqu samples (three biological replicates in each group). **a** Results at the phylum level and **b** at the genus level (the 20 most abundant taxa were shown). Sample A was collected at Daqu entry; Sample B was collected at the first Daqu flipping; Sample C was collected at the second Daqu flipping; Sample D was collected at the Daqu dry-fire period; Sample E was collected at the Daqu post-fire period and Sample F was collected at Daqu exit

fermentation. Furthermore, the Burkholderiales order, the Comamonadaceae family, and the *Delftia* genus were significantly higher in sample C. The Proteobacteria phylum and the Gammaproteobacteria class were considerably higher in sample D than in other samples. The Alphaproteobacteria class, the Oligoflexales order, and the *Achromobacter* and *Pseudomonas* genera displayed higher abundance in sample E. Finally, at the family level, the relative abundance of Thermoactinomycetaceae, Sporolactobacillaceae, Pseudonocardiaceae, and Bacillaceae were significantly higher in sample F, as was the relative abundance of the Bacillales order and seven genera.

At the genus, LEfSe analysis was used to determine the significant differences in the fungal communities of the Daqu

Table 2 The predominant groups of Daqu bacteria in each sample

OTU ID	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)
Achromobacter	1.60	10.03	26.43	34.70	41.23	0.13
Saccharopolyspora	0.83	0.53	0.03	0.03	0.00	22.07
Bacillus	0.33	0.17	0.23	0.00	0.40	6.97
Pseudonocardia	0.20	0.13	0.00	0.00	0.00	6.73
Delftia	1.40	7.33	44.47	8.87	8.73	0.23
Kroppenstedtia	2.233	1.27	0.13	0.00	0.07	9.03
Thermoactinomyces	1.00	0.20	1.37	0.00	0.00	16.73
Weissella	48.30	1.07	0.10	0.00	0.00	5.20
Pseudomonas	0.37	0.87	0.13	0.13	16.93	0.13
Stenotrophomonas	4.80	76.57	26.73	54.73	22.73	0.67
Lactobacillus	20.07	0.07	0.00	0.00	0.00	0.47
Scopulibacillus	0.73	0.20	0.03	0.00	0.00	27.13
Thermomyces	3.73	4.00	14.67	92.03	58.13	79.90
Thermoascus	3.23	2.80	64.4	3.03	21.23	13.83
Wickerhamomyces	83.47	2.31	1.30	0.93	1.00	1.27
Saccharomycopsis	2.57	90.03	5.10	1.53	1.23	1.37
Debaryomyces	2.53	0	0	0	0	0.03

samples (Fig. 6). In general, the Eurotiales order in sample A was significantly higher than in other samples. In sample B, the Eurotiales order, the *Aspergillus* genus, and the Trichocomaceae family were higher during the fermentation process. In sample C, the Agaricomycetes class, the *Corynascus* genus, and the *Thermomyces* genus were significantly higher than in other samples. In sample D, *Thermoascus*, *Monascus*, *Rasamsonia,* and Eurotiomycetes_order_Incertae_sedis were substantially higher than other samples, while sample E displayed a higher content of the Saccharomycetales order. *Debaryomyces*, Saccharomycetales_family_Incertae_sedis, *Wickerhamomyces*, *Torulaspora* dominated in sample F.

Discussion

Baijiu is traditional Chinese distilled liquor (Pang et al. 2018; Zou et al. 2018a). As the primary source of microorganisms and enzymes during the Baijiu fermentation process, Daqu can determine the quality of the final Baijiu products (Zuo et al. 2020). The microbial species and community structures varied in differently flavored Baijiu, influencing the flavor formation of the Baijiu (Liu and Sun 2018). Northern Jiang-flavored Baijiu has a unique flavor, and high-temperature fermentation plays an essential role in this product. During the production of Northern Jiang-flavored Baijiu, the core microorganisms play a vital role in the characteristics of the flavor metabolites in the Baijiu. The thermophilic yeasts and molds in the Daqu were eliminated as the temperature increased, and the microbial community structure was mainly formed by high-temperature bacteria, affecting the taste of the Baijiu (Yi et al. 2019). This article analyzed the microbial communities in the Daqu samples from six sampling periods, ranging from the Daqu entry period to the Daqu exit period in Northern Jiang-flavored Baijiu, aiming to provide a scientific basis for examining the relationship between the microorganisms and the taste.

In 2019, Wang et al. studied the succession of microorganisms and the changes in metabolites during the fermentation of Chinese light-flavored Baijiu. The results showed that microorganisms produced from Daqu and the environment were dominant, such as *Bacillus*, *Lactobacillus*, *Pediococcus*, *Saccharomycopsis*, *Saccharomyces*, *Pichia*, *Wickerhamomyces*, and *Aspergillus* (Wang et al. 2018; Wang and Xu 2018; Huang et al. 2020). In 2019, Gan et al. analyzed the microbial community composition and functional characteristics of mature Maotai starter, revealing that the dominant bacteria were *Desmospora*, *Bacillus*, *Lactobacillus*, *Oceanobacillus*, *Saccharopolyspora*, and *Virgibacillus*. The dominant fungi were *Aspergillus*, *Rasamsonia*, and *Byssochlamys* (Gan et al. 2019). The high-throughput sequencing results showed that the main bacterial groups in sesame-flavored Daqu samples were *Kroppenstedtia*, *Lactobacillus*, *Weissella*, *Lentibacillus*, *Bacillus,* and *Saccharopolyspora*, while the bacterial community structure and diversity showed a dynamic succession during the Daqu production process (Xie et al. 2020). In 2015, Wang et al. studied the dynamic changes in the bacterial community during the fermentation of Maotai Baijiu and found that the *Weissella* and *Enterococcus* genera were more abundant during the production of the starter, dominating the lactic acid bacteria (LAB) community. However, during the pit-fermentation stage, the main genus of the LAB community changed to *Lactobacillus* (with a relative abundance of over 78.31%). Similarly,

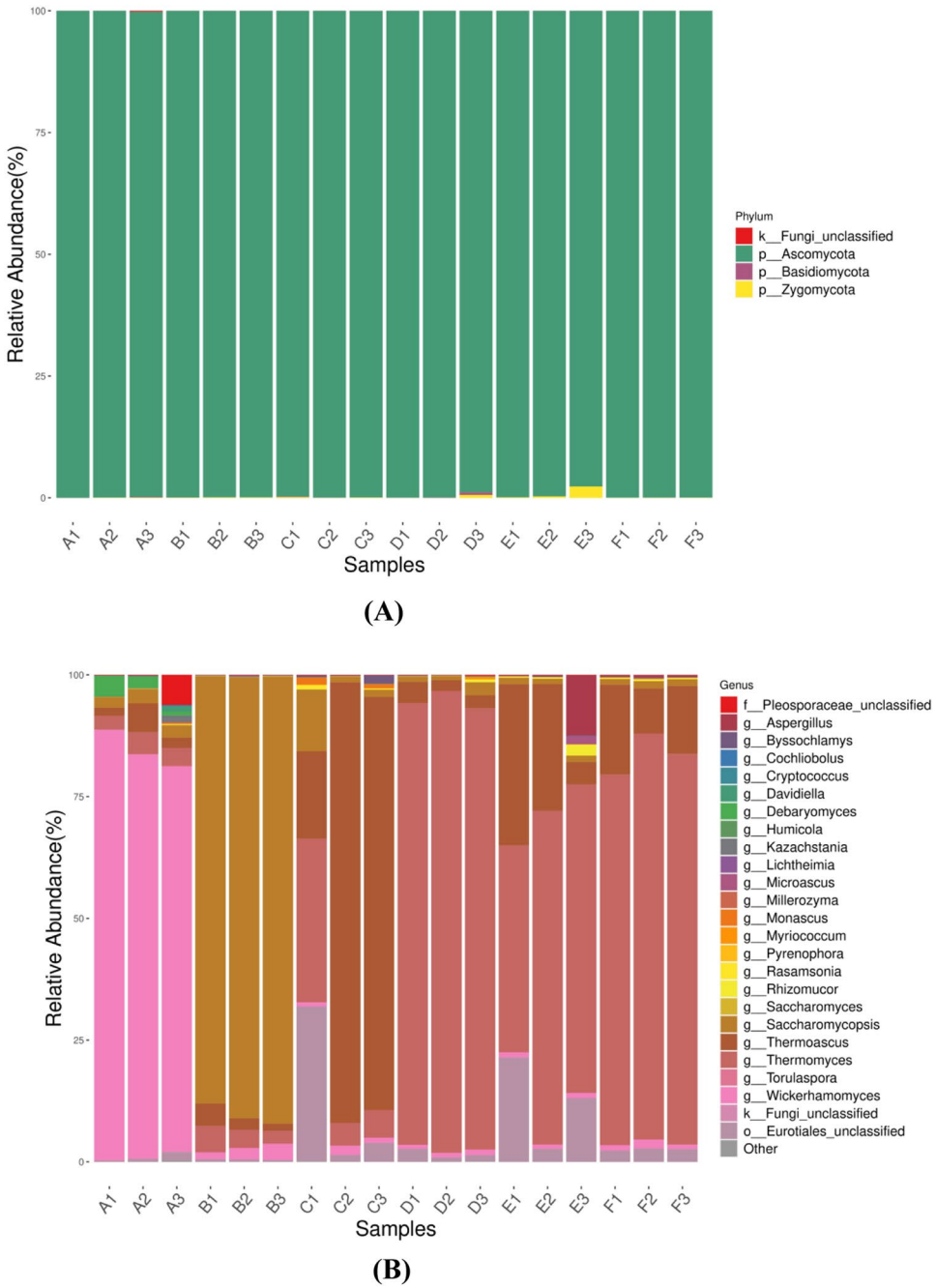


Fig. 3 The distribution of the predominant fungi in the six Daqu samples (three biological replicates in each group). **a** Results at the phylum level and **b** at the genus level (the 20 most abundant taxa were shown). Sample A was collected at Daqu entry; Sample B was collected at the first Daqu flipping; Sample C was collected at the second Daqu flipping; Sample D was collected at the Daqu dry-fire period; Sample E was collected at the Daqu post-fire period and Sample F was collected at Daqu exit

during the ripening stage of Daqu production, the *Bacillus* genus was most abundant in the Bacillaceae family. However, during the stack-fermentation stage, the main components of the Bacillaceae family were diversified, including *Bacillus*, *Lentibacillus,* and undefined bacterial genera within this family (Wang et al. 2015). In 2016, Wang et al. used nested PCR-denaturing gradient gel electrophoresis to analyze the distribution of typical microorganisms in Baijiu samples while performing quantitative PCR amplification of 16S rRNA and internal transcription spacer genes to estimate the abundance of microorganisms in the samples. The results showed that Daqu was the main bacterial source, followed by air, soil, and sorghum samples. Fermented grains displayed the highest concentration of bacteria, followed by Daqu and sorghum. The highest concentration of fungi was Daqu, followed by sorghum and air samples from outside the Baijiu producing area (Wang et al. 2016).

In 2019, Huang et al. used fortified Daqu to study the microbial community and flavor characteristics during Chinese strong-flavored liquor (CSFL) brewing. In 50% of the fortified Daqu, prokaryotes (*Bacillus*, *Lactobacillus*, and *Lactococcus*) and eukaryotes (*Aspergillus*, *Candida*, and

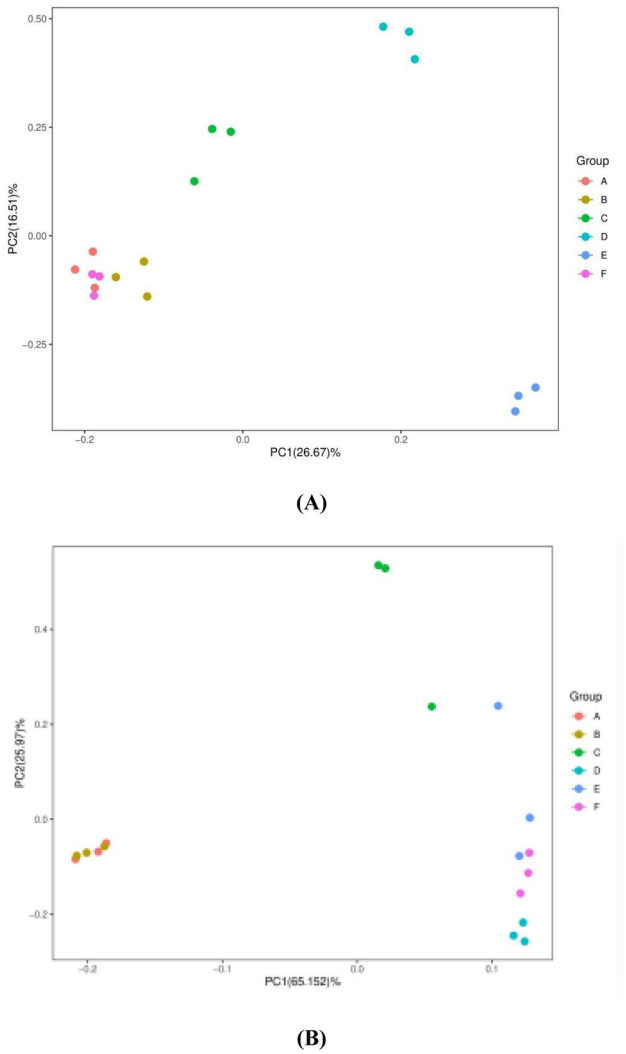


Fig. 4 Principal coordinate analysis (PCoA) of Daqu flora among the six groups (three biological replicates in each group). **a** Bacteria, **b** Fungi. The *x* and *y* axes are the first and second coordinates, and the values in parentheses show the percentage of the community variation explained. Sample A was collected at Daqu entry; Sample B was collected at the first Daqu flipping; Sample C was collected at the second Daqu flipping; Sample D was collected at the Daqu dry-fire period; Sample E was collected at the Daqu post-fire period and Sample F was collected at Daqu exit

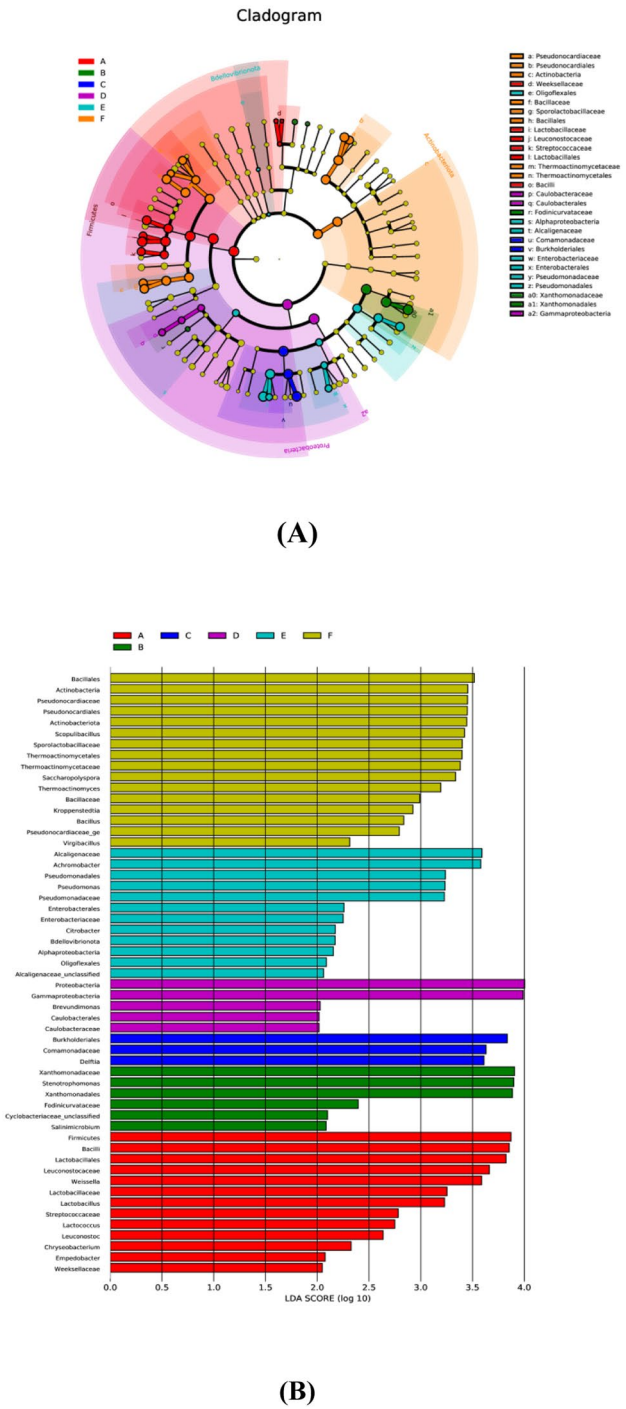


Fig. 5 The key bacterial phyla in the six Daqu samples during fermentation, as determined via LEfSe. **a** The bacterial indicator groups with LDA values higher than 2.0 in the six Daqu samples. **b** A cladogram indicating the phylogenetic distribution of the microbial species associated with the six types of Daqu

Thermoascus) displayed relatively high abundance. In addition, most of the esters were mainly positively correlated with the *Lactobacillus* and *Candida* at the bottom of 50% fortified Daqu fermentation. When 50% fortified Daqu was used, the aromatic compounds in the middle Daqu layer were positively correlated with *Bacillus* and *Aspergillus*. After adding 100% fortified Daqu, hexyl hexanoate was positively correlated with the higher abundance of *Ruminococcus* (He et al. 2019). During the fermentation of Shaoxing rice wine, ten genera, including *Bacillus*, *Leuconostoc*, *Lactococcus*, *Weissella*, *Thermoactinomyces*, *Pseudomonas*, *Saccharopolyspora*, *Staphylococcus*, *Enterobacter,* and *Lactobacillus* were identified, with *Bacillus* and *Lactobacillus* in the highest abundance (Liu et al. 2015). Zhang et al. proposed a three-phase model in 2017 to describe the

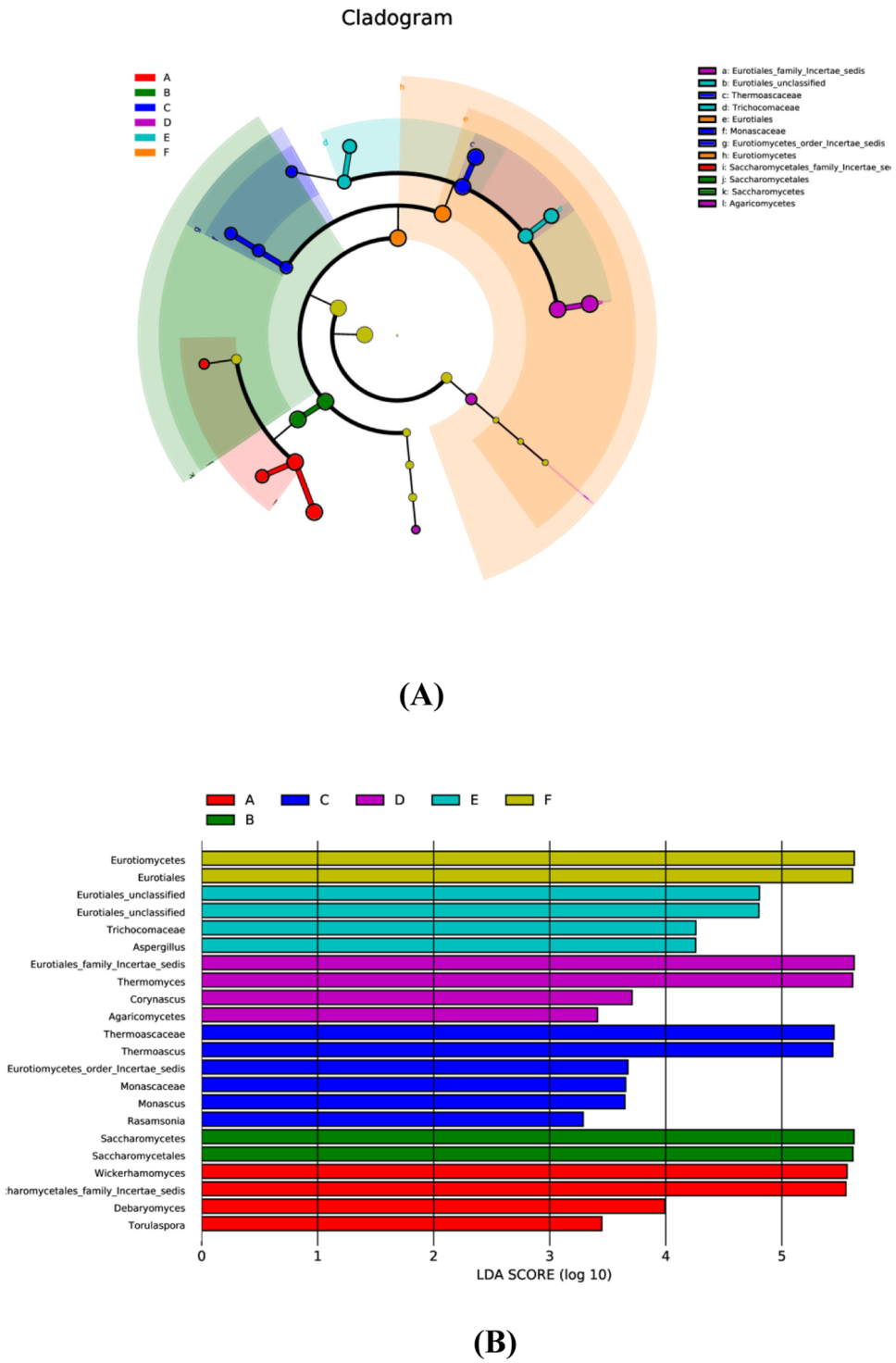


Fig. 6 The key fungal phyla in the six Daqu samples during fermentation, as determined via LEfSe. **a** The bacterial indicator groups with LDA values higher than 2.0 in the six Daqu samples. **b** A cladogram indicating the phylogenetic distribution of the microbial species associated with the six types of Daqu

fermentation process of CSFL. During the initial fermentation stage, glucose was produced from macromolecular carbohydrates, and the prokaryotic biodiversity was significantly reduced. The Lactobacillaceae gradually started to dominate the prokaryotic community, while the diversity of eukaryotes increased significantly. *Thermoascus*, *Aspergillus*, *Rhizopus,* and unidentified Saccharomycetales dominated the eukaryotic cells. During the mid-fermentation stage, the prokaryotic community was dominated by *Lactobacillus*, while the eukaryotic community was mainly composed of *Thermoascus*, *Emericella,* and *Aspergillus*. Furthermore, the ethyl caproate concentration increased significantly during the late fermentation period. It was also proposed that CSFL fermentation can be divided into three

stages: saccharification, glycolysis, and esterification. *Saccharomyces*, *Monascus,* and *Rhizopus* were positively correlated with the glucose concentration, highlighting their vital role in starch saccharification. Lactobacillaceae, Bacilli, Botryotinia, Aspergillus, unidentified Pleosporales, and Capnodiales contributed to glycolysis and esterification since they were positively correlated with most organic acids and ethyl esters. Four genera, namely *Emericella*, *Suillus*, *Mortierella,* and *Botryotinia*, which possibly played a vital role during the fermentation process, were observed for the first time (Zhang et al. 2017). Our previous research used high-throughput sequencing technology to analyze the microbial community diversity in three Daqu samples with different characteristics in the same Daqu fermentation tank containing sesame-flavored Baijiu. The results showed that the microbial diversity and abundance of the Black sample were significantly higher than the White and Yellow samples. The dominant bacteria in the samples were *Saccharopolyspora*, *Bacillus*, *Lentibacillus*, *Staphylococcus*, *Kroppenstedtia,* and *Thermoactinomyces*, while the main fungal groups were *Thermomyces*, *Thermoascus,* and *Aspergillus*. The dominant flora in the Black sample was *Thermoactinomyces*, *Saccharopolyspora*, and *Pseudomonas*. *Thermoactinomycetes* were the most abundant in the White sample, followed by *Saccharopolyspora* and *Lentibacillus.* The Yellow sample was dominated by *Staphylococcus*, followed by *Bacillus* and *Kroppenstedtia*. Regarding the fungi in the three samples, *Thermomyces* accounted for 93.70% in the Black sample, and *Aspergillus* dominated in the White sample, while the *Thermoascus* and *Aspergillus* content were similar in the Yellow sample (Wu et al. 2020).

The Daqu microbial community structure in Northern Jiang-flavored Baijiu is significantly different from that in other flavored liquors, affecting its taste. The CSFL Daqu, fermented at a medium temperature of 50–60 ℃ (Jin et al. 2017), displayed dominant bacteria that included *Bacillus*, *Weissella*, *Lactobacillus*, *Staphylococcus*, *Kroppenstedtia*, *Thermoactinomycetes*, *Lactococcus*, *Enterobacter*, *Pseudomonas,* and *Klebsiella*. The dominant fungi were *Pichia*, *Saccharomyces*, *Aspergillus*, *Rhizopus,* and *Geotrichum* (Wang et al. 2011; Gou et al. 2015; Zou et al. 2018b). The same strains were also found in Northern Jiang-flavored Baijiu Daqu. The bacteria included *Bacillus*, *Weissella*, *Lactobacillus*, *Kroppenstedtia*, *Saccharopolyspora*, and *Thermoactinomyces*, while *Saccharomyces* represented the fungus. Light-flavored Daqu was fermented at a lower temperature of 40–50 ℃ (Jin et al. 2017). The main bacteria were *Pantoea*, *Klebsiella*, *Lactobacillus*, *Bacillus*, *Kroppenstedtia*, *Staphylococcus*, *Weissella*, *Acinetobacter*, and *Lentibacillus*, while the fungi were represented by *Saccharomycopsis*, *Pichia*, and *Aspergillus* (Fan et al. 2018; Wang et al. 2019a, b; Huang et al. 2020). The same bacteria were present in both the Northern Jiang-flavored Baijiu Daqu and the light-flavored Daqu and included *Bacillus*, *Lactobacillus*, *Kroppenstedtia,* and *Weissella*, while *Saccharomycopsis* represented the fungus.

According to previous studies, the primary bacteria in Maotai-flavored Baijiu Daqu included *Thermoactinomyces*, *Saccharopolyspora*, *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, and *Weissella*, and the fungi were *Aspergillus*, *Monascus*, *Thermoascus*, and *Thermomyces*, *Saccharomyces,* and *Pichia* (Wang et al. 2016; Jin et al. 2017). In this experiment, it was found that the same bacteria in Northern Jiang-flavored Daqu and Maotai-flavored Baijiu Daqu were *Thermoactinomyces*, *Saccharopolyspora*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, and *Weissella*, and the same main fungus was *Saccharomycopsis*.

High-throughput sequencing technology indicated that the bacteria in the high-temperature Daqu of Northern Jiang-flavored Baijiu mainly involved Firmicutes, Actinobacteriota, and Proteobacteria. Of these, Proteobacteria were the most dominant during the six stages of Daqu production. The results showed that *Lactobacillus*, *Weissella*, *Bacillus*, *Delftia*, *Achromobacter*, *Saccharopolyspora*, *Thermoactinomyces*, *Scopulibacillus*, *Kroppenstedtia*, *Stenotrophomonas,* and *Pseudomonas* were the main bacterial groups in Northern Jiang-flavored Daqu, while the fungi mainly involved Ascomycota, Zygomycota, and Basidiomycota. The main fungal genera in the Daqu were *Wickerhamomyces*, *Saccharomycopsis*, *Thermoascus*, and *Thermomyces*.

This study exhibited significant differences in the microbial diversity of the six Daqu samples, affecting the flavor of the subsequent Baijiu. *Lactobacillus*, *Weissella,* and *Bacillus* are typical bacterial groups frequently detected during the fermentation of different Baijiu types in China, playing an essential role in producing aroma components (He et al. 2019). *Saccharomycopsis* is crucial in most liquors while significantly impacting the taste (Huang et al. 2020). However, the dominant bacteria in Northern Jiang-flavored Daqu, namely *Achromobacter*, *Stenotrophomonas*, *Delftia*, and *Scopulibacillus,* were unique to other flavored liquors, reflecting the significant difference in microbes between these Baijiu products. This may have a crucial micro-ecological effect on the unique flavor of the Northern Jiang-flavored Baijiu. Previous results indicated that *Lactobacillus* was the primary functional contributor to alcohol and acid production (ethanol, lactic acid, and acetic acid) (Song et al. 2017). *Lactobacillus* can produce lactic acid from glucose, which is further converted into ethyl lactate via enzymatic reactions, enhancing the mellow flavor of Baijiu (Liu and Miao 2020).

Bacillus can utilize various hydrolytic enzymes (including amylase, protease, and lipase) to hydrolyze macromolecules and produce flavor compounds during the brewing process (Li et al. 2014; Zhao et al. 2019). The solid-state fermentation of *Bacillus licheniformis* can produce

aromatic compounds, C4 compounds, pyrazine compounds, and volatile acids, providing Jiang-flavored Baijiu with its unique flavor characteristics. The results showed that these aromatic compounds are positively related to *Bacillus* (He et al. 2019). Some of the *Bacillus* produced from Daqu have been applied for biological enhancement during Chinese Baijiu production to increase the acid in the liquid culture (Yan et al. 2013). Changes in microbial communities, such as *Kroppenstedtia*, increased the acetic acid, lactic acid, malic acid, and ethyl acetate levels during Baijiu fermentation while decreasing the ethyl lactate content (Wang et al. 2017a). *Thermoactinomycetes* can secrete esterase, amylase, and phosphatase, denoting enzymes that are considered crucial for brewing Baijiu (Xiao et al. 2017).

Saccharomycopsis can adapt to highly acidic conditions and has been proven to play an important role during Chinese Jiang-flavored Baijiu production. *Saccharomycopsis* is the core fungal microorganism in the fermentation process, and its origin can be traced back to the production stage of Daqu. *Wickerhamomyces* is the dominant fungus during the fermentation process and displays an excellent ester-producing ability (You et al. 2016). It can also produce intracellular and extracellular glycoside hydrolases, arabinosidases, and xylosidases. These enzymes may be crucial for the aroma of Northern Jiang-flavored Baijiu (Zou et al. 2018b). *Thermoascus* can produce xylanase, heat-resistant alkaline catalase, superoxide dismutase, cutinase, and other enzymes, playing a crucial role in the subsequent accumulation during saccharification and fermentation and the development of the sauce flavor. *Thermomyces* and *Thermoascus* colonize at high temperatures, producing various thermos-tolerant enzymes that help to degrade plant materials (McClendon et al. 2012; Winger et al. 2014; Liu and Miao 2020).

Although *Delftia*, *Achromobacter*, *and Stenotrophomonas* denote the dominant strains during the mid-stage of Northern Jiang-flavored Daqu production, not many studies are available regarding their microbial diversity and biological functions during the fermentation process of Baijiu. Current research indicates that *Delftia* can promote rapeseed root development and increase crop yield (Perry et al. 2017). *Achromobacter* displays biodegradation and chitinase production functionality (Bholay et al. 2015), while *Stenotrophomonas* promotes plant growth and exhibits antibacterial properties (Sánchez-Castro et al. 2017). *Scopulibacillus* was highly abundant during the final period of the production of Northern Jiang-flavored Daqu, but minimal research is available involving its microbial diversity and biological functionality. *Saccharopolyspora* has been detected in a variety of Daqu and can metabolize to produce important biologically active substances, such as antibiotics, microorganisms, enzymes, and cellulose degradation-promoting factors, all of which can render the Daqu flavor more complex.

The Northern Jiang-flavored Daqu also exhibited microbial succession during the production process. In the early stage of Daqu production, the dominant bacteria were *Lactobacillus* and *Weissella*, which might be derived from raw materials. Due to an increase in the fermentation temperature of Daqu, many bacteria were eliminated during the first and second flipping period, as well as the dry-fire period. The optimum growth temperature for *Lactobacillus* and *Weissella* was 30–40 ℃. The temperature rose after the first Daqu flipping period, eliminating these bacteria. Furthermore, the contact surface increased after the first Daqu was flipped, exposing the bacteria to more oxygen. *Stenotrophomonas* and *Delftia* are aerobic bacteria and consequently grew to become dominant. The bacterial diversity declined, and *Achromobacter*, *Stenotrophomonas*, and *Delftia* became dominant. *Wickerhamomyces* represented the most dominant fungus during the early stage of production. As the temperature rose, *Wickerhamomyces* was eliminated, while *Saccharomycopsis* and *Thermoascus* increased. *Thermomyces* then gradually accumulated in the D, E, and F samples, becoming dominant during the Daqu exit period. The superiority and metabolic activity of the bacteria mentioned above allowed the formation and accumulation of aromatic Daqu substances. It is worth noting the difference between the microbial composition in the Northern Jiang-flavored Baijiu and other flavored liquors. *Delftia*, *Achromobacter*, *Stenotrophomonas*, and *Scopulibacillus* may play a vital role in the unique flavor of the Northern Jiang-flavored Baijiu.

This study used high-throughput sequencing technology for the first time to characterize the microorganisms in Northern Jiang-flavored Baijiu Daqu. It also revealed various low-abundance microbial populations in high-temperature Daqu, enhancing the understanding of the microbial diversity and dynamic succession during the brewing process of Northern Jiang-flavored Baijiu Daqu. Here, the main bacterial groups in the six samples were *Kroppenstedtia*, *Lactobacillus*, *Weissella*, *Bacillus*, *Achromobacter*, *Delftia*, *Stenotrophomonas*, *Saccharopolyspora*, *Thermoactinomyces, and Scopulibacillus*. The structure and diversity of these microbial communities presented dynamic succession during the Daqu production process. The abundance of *Lactobacillus* and *Weissella* was higher during the early stage, while *Achromobacter*, *Delftia*, and *Stenotrophomonas* increased significantly after the Daqu flipping period. Finally, *Saccharopolyspora*, *Thermoactinomyces*, *Scopulibacillus*, *Kroppenstedtia*, and *Bacillus* represented the dominant groups. The main fungi were *Wickerhamomyces*, *Saccharomycopsis*, *Thermoascus,* and *Thermomyces*. *Wickerhamomyces* displayed the highest abundance during the Daqu entry period. *Saccharomycopsis* and *Thermoascus* increased during the first and second Daqu flipping periods. Finally, *Thermomyces* became the dominant fungus during the dry-fire period, the post-fire period, and the Daqu exit period.

Daqu is central to Baijiu brewing, providing raw materials, enzymes, and microorganisms. In this study, the microbial community structure of the different flavor substances in Daqu was examined, providing a foundation for investigating the relationship between the flavor substances and microorganisms in Baijiu. Consequently, the main bacteria affecting the flavor of Daqu can be selected for functional mechanism evaluation and safety assessment, allowing microbial control to be exerted according to the functionality of the specific microorganisms. For example, adding microorganisms during the different stages of Daqu fermentation or improving the process to enhance the flavor characteristics of Northern Jiang-flavored Baijiu to refine future production technology.

Conclusion

This study examines the microbial diversity and dynamic succession in six Northern Jiang-flavored Baijiu Daqu samples, revealing that Firmicutes denotes the most dominant phylum. *Lactobacillus*, *Weissell*a, *Bacillus*, *Delftia*, *Achromobacter*, *Saccharopolyspora*, *Thermoactinomyces*, *Scopulibacillus*, *Kroppenstedtia*, *Stenotrophomonas,* and *Pseudomonas* represent the central bacterial communities in Northern Jiang-flavored Baijiu Daqu. The most dominant fungal phylum is Ascomycota, while the primary fungal genera include *Wickerhamomyces*, *Saccharomycopsis*, *Thermoascus,* and *Thermomyces*. Furthermore, the study reveals that the microbial changes in the Daqu samples show dynamic succession.

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Authors' contributions QEJ and XYW designed and participated in all experimental procedures, performed data analysis, and drafted the manuscript. YQX and ZSW participated in data analysis and draft writing. YLZ, LYS and WJY participated in the Daqu samples preparation and collection. JYS and YL supervised the study and critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials All data generated or analyzed during this study are included in this published article and its additional information files.

Declarations

Consent for publication Not applicable.

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.

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