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Study on fungal community diversity of shilixiang baijiu *Daqu* based on high-throughput sequencing

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Abstract
Purpose
The diversity and relative abundance of fungi in shilixiang baijiu Daqu were analyzed. An understanding of the composition and structure of fungi communities of the shilixiang baijiu Daqu can provide a theoretical basis for the stability and quality evaluation of shilixiang baijiu Daqu-making technology.
Result
The results showed that the fungi community structure was different among Daqu samples from different fermentation batches, and at different fermentation days within the same fermentation batch. In general, the abundance and diversity of fungi found in Daqu samples from the F, G and K batches increased after 30 days of fermentation. But the abundance and diversity of fungi found in Daqu samples from the H and I batches decreased after 30 days of fermentation.The analysis of community structure showed that after 30 days of fermentation, Ascomycota was the predominant phylum in the five batches of the shilixiang baijiu Daqu samples. After 30 days of fermentation,the proportion of Thermoascus, Candida and Rhizomucor increased, while the proportion of Aureobasidium and Gibberella decreased; Thermoascus and Candida was the dominant microorganism in batches F, G, I and K of shilixiang baijiu Daqu samples, while Thermoascus and Rhizomucor was the dominant microorganism in batches H of shilixiang baijiu Daqu samples. In addition, there are unique genera in each batch of Daqu samples.
Conclusion
By analyzing the fungal community structure of shilixiang baijiu Daqu, we obtained the dominant fungi and their possible functions in the fermentation process of Daqu, and then understand the influence of functional fungi on the quality of Daqu.
Key words: *Shilixiang baijiu Daqu; Fungal diversity; Illumina MiSeq sequencing; ITS region; Alpha diversity; Beta diversity*

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I. Introduction

Daqu is one of the most indispensable raw materials in the process of liquor brewing, as a fermentation agent and saccharifying agent, which is of great importance to the quality of Chinese liquor. During the production process, *Daqu* relies on various microorganisms brought in by nature to enrich, grow and propagate in the starchy raw materials, among which the main microorganisms include bacteria, mold and yeast (Wang et al., 2011). It is well known that the crude enzyme preparation and various microorganisms in *Daqu* are necessary for hydrolysis of macromolecules and metabolism. In addition, it also provides a large number of flavor compounds and precursors, as well as beneficial metabolites for liquor brewing(Li et al., 2020). *Daqu* itself is made of raw materials, natural inoculum, and open culture, leading to a very complex microbial community. The manufacturing process is unpredictable and uncontrollable, which leads to unstable quality of wine. Therefore, it is an important content of brewing microorganism research.

Based on the current research status of Daqu microorganisms, it can be seen that the research methods are gradually transitioning from the traditional culturable microorganism research methods to the culture independent techniques (Gu et al., 2020). High-throughput sequencing technology can detect low-abundance microorganisms without the need for isolation and culture of microorganisms, and at the same time can more fully and accurately reflect the microbial community composition and diversity of the sample. Although the HTS thechnology is widely used in microbial studies, it was scarcely employed on fungal community analysis

of *Daqu*. We can see some less examples as follows: Li et al analyzed of the fungal community structure of luzhou-flavor and sesame-flavor liquor *Daqu* based on HTS technology (Li et al., 2019). Yang et al analyzed the bacterial and fungal community structure of *Daqu* at high temperature in liquor by HTS (Yang et al., 2020).

In the process of liquor saccharification and fermentation, fungi are the main functional microorganism in alcohol and aroma production, as well as the main enzyme power and fermentation power in the brewing process, and play an important role in regulating the production quality of liquor (Liu et al., 2012; Liu et al., 2017). Among them, mold can secrete a variety of hydrolases, decompose starch, protein and other macromolecules substances in brewing raw materials, but also provide nutrients and basic substances for the growth and metabolism of other microorganisms, making an important contribution to the formation of liquor body flavor (Wang., 2016; Guo et al., 2017). Yeast is an important aroma-producing microorganism group in the process of liquor brewing, which is directly related to the rate of liquor production and the formation of trace aroma components. Various flavor substances produced in the metabolic process play a vital role in the formation of liquor aroma (Q. Wu, 2012). Molds and yeasts are the important microflora that affect the liquor brewing process, which can ensure the normal fermentation process, and the microorganisms that constitute the community (whose presence and abundance contribute to the community structure shape) may promote or inhibit the growth of other microorganisms (by a wide array of secreted molecules), and their different microflora composition will affect the quality of liquor.

In this study, the structure of fungal community in shilixiang baijiu *Daqu* was taken as the research object. HTS combined with mathematical statistics software was used to comprehensively analyze the composition and diversity of fungal community in different fermentation batches of shilixiang baijiu *Daqu*, and to determine the influence of fermentation process on fungal. This study provided theoretical basis and reference for scientific understanding of the changing trend of microbial community and the fermentation mechanism during the fermentation of shilixiang baijiu *Daqu*.

II. Materials and methods

2.1 Sample collection

The wheat was crushed and added with 34% to 36% water, and then cultured in an embryo-making factory at 40-45℃ for 48 hours. 24 hours after opening the mold, *Daqu* was turned over 2-3 layers and it was cultured for 5-6 days. When the temperature of the three layers was below 60℃, it was changed from three to five layers and was incubated for 8-12 days during a high temperature culture period between 58℃and 62℃. On 18th day, when the temperature of the fermenting grains began to drop, it goes from five layers to six. When the temperature of the fermenting grains dropped below 40℃, it was changed from six to eight layers. When the temperature of the fermenting grains reduced to room temperature and the production was dry, it was mature and completed. The total number of days was about 30 to 35 days.

The samples from batches F, G, H, I and K of shilixiang baijiu *Daqu* (batches F, G, H, I and K were sampled in May, June, July, August and September respectively) were collected separately at the Day0, 2, 9, 18 and 30 based on the temperature control during the fermentation process under similar conditions, and stored on ice for transport, then stored in the laboratory at -80℃ before DNA extraction. The samples of each batch were tested in three replicates.

2.2 DNA extraction

The extraction of total microbial DNA in *Daqu* was carried out according to the methods and steps in the instructions of E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, USA).

2.3 Polymerase chain reaction and sequencing

With total DNA as template, ITS1F (5 '- CTTGGTCATTTAGAGGAAGTAA -3') and ITS2R 5'-GCTGCGTTCTTCATCGATGC - 3'), the general primers of Miseq sequencing platform, combined with adapter sequence and barcode sequence, were used to amplify the ITS sequence of fungi. PCR amplification system: 10×PCR Buffer 5 μL, dNTPs 0.1mmol /L each, DNA 10 ng, ITS1 0.5 μmol/L, ITS2-Rev 0.5 μmol/L, Plantium Taq 0.05 U, the PCR mixture is in a final volume of 50 μL(Li et al., 2019) . PCR amplification conditions: pre-denaturation at 94℃ for 3 min; There were 5 cycles of denaturation at 94℃ for 30 s, annealing at 45℃ for 20 s, and extension at 65℃ for 30 s. Then, there are 20 cycles of denaturation at 94℃ for 20 s, annealing at 45℃ for 20 s, and extension at 65 ℃for 30 s. Final extention at 72 ℃for 5 min(Li et al., 2019).

The PCR amplicon products were tested on 2% agarose gel electrophoresis and purified through Gel Recycling Kit from Axygen, and then quantified with Quant-iT PicoGreen dsDNA Assay Kit (Shanghai Maokang Biotechnology) according to the preliminary quantitative results of electrophoresis. According to the fluorescence quantitative results, each sample was mixed according to the amount of sequencing required for each sample and the qualified samples were sent to the company of Personalbio for sequencing.

2.4 Statistical analysis

QIIME2 (2019. 4) (Bolyen et al. 2018) software was used to process the sequencing data, and UCLUST (Edgar, 2010) was used to classify OTUs at 97% similarity level, and the highest abundance sequence of each OTUs was selected as the representative sequence of OTUs (Blaxter et al., 2005). Then, based on the number of each OTU sequence in each sample, a matrix file of OTU abundance in each sample is constructed. For each OTU representative sequence, the default parameters were used in the QIIME software and then the OTU representative sequence was compared with the template sequence of the Greengenes database (DeSantis et al., 2006) to obtain the OUT taxonomic assignation. In order to ensure the reliability and accuracy of the analysis results, remove the abundance of OTU below 0.001% of the total sample sequencing (Bokulich et al., 2013), remove the abundance matrix of rare OTU, and further generate OUT table to record the abundance of each OTU in each sample and the classification of these OTUs.

The QIIME software was used to randomly sample the total sequence number of each sample in the OTU abundance matrix at different depths, and the sequence number extracted at each depth and the corresponding OTU number were used to draw the sparse curve. The α-diversity index at OTU level included Chao1 (Chao, 1984), ACE (Chao and Yang, 1993), Simpson (Simpson, 1949), and Shannon (Shannon, 1948a, b). Chao1 and ACE index focused on community richness, while Simpson and Shannon index focused on community diversity. QIIME software was used to obtain the composition and abundance distribution table of each sample at three taxonomic levels of phylum, family and genus, and the analysis results were presented in histogram form. NMDS analysis based on Weighted UniFrac distance matrix (C. and Knight, 2005) was performed with R software, and the structure distribution of community samples was described by two-dimensional ordering diagram.

III. Results

3.1 Alpha diversity analysis

After quality filtering, 2607 high-quality reads were obtained from the initial ITS sequences. All the rarefaction curves based on the observed species were saturated, indicating that the obtained sequencing reads were sufficient to represent community diversity in these samples (Figure 1). To describe the α-diversity of fungal community in different samples, the measures used here included Chao1 index and ACE index, which focused on community richness, and Shannon index and Simpson index, which considered community diversity and evenness (Table 1). The abundance and diversity of microorganisms in batches F, G, I and K decreased first and then increased, while the abundance and diversity of microorganisms in batches H decreased sharply during the fermentation process. The sampling time of batch H was in July, so it may be caused by excessive temperature. According to the data analysis in Table 1, the Simpson index values (0.54) and Chao1 index values (27.75) of H30 were the smallest among all samples, indicating that the fungal community richness and diversity in batch H of shlixiang baijiu *Daqu* were the lowest after 30 days of fermentation. The Chao1 index values (110.27) and Simpson index values (0.83) of sample K30 were the highest, indicating that the fungal community richness and diversity in batch K of shilixiang baijiu *Daqu* were the highest after 30 days of fermentation.

3.2 Analysis of fungal community structure

Based on the ITS gene, the fungal diversity in different batches of shilixiang baijiu *Daqu* samples were analyzed at the phylum and genus levels by using QIIME software.

According to the taxonomic analysis, three phyla, Ascomycota, Zygomycota and Basidiomycota, were detected in the Shilixiang baijiu *Daqu* samples (Figure2). In different fermentation batches and different fermentation days in the same batch, Ascomycota was dominant. So notable changes were not observed in the phyla composition. We also found that the relative abundance of Zygomycota was high at the middle stage of batch H fermentation. Studies have shown that Zygomycota mainly grow in eutrophic environments, and the rising temperature accelerates the decomposition of organic matter and provides sufficient carbon sources for the growth and reproduction of fungi. However, batch H was the sample collected in July and the temperature was high, so the relative abundance of Zygomycota in this batch was relatively high.

According to the taxonomic analysis, among the 20 listed genera (relative abundance $\geq$ 0.1%), 11 genera were shared by five batches, including *Thermoascus*, *Candida*, *Pichia*, *Rhizomucor*, *Rhizopus*, *Aspergillus*, *Aureobasidium*, *Thermomyces*, *Trichosporon*, *Gibberella*, *Lichtheimia*. *Thermoascus* and *Candida* were extremely abundant in all samples (Figure3). Studies have shown that *Thermoascu*s can produce xylanase, superoxide dismutase, keratinase and other heat-stable hydrolases, which play an important role in accumulation of products for the saccharification and fermentation and in the formation of flavor (Sun et al., 2019; Kumar et al., 2015). Many members of the *Candida* family are capable of alcoholic fermentation (Li et al., 2019). Secondly, there are a lot of dominant filametous fungi in *Daqu*. The growth and metabolism of mold can produce a variety of enzymes. The liquefied and saccharified amylase produced by their metabolism are mainly

used in *Daqu* wine brewing as saccharifying agent in production and fermentation. The filamentous fungi that can metabolize to produce amylase are: *Rhizomucor*, *Rhizopus*, *Aspergillus* and so on. The acid hydrolase secreted by *Aspergillus* can decompose cellulose and starch macromolecules in raw materials and catalyze the production of aromatic lipids, which can enhance and improve liquor body flavor (Huang, 2014; Guo, 2018).

After 30 days of fermentation, *Thermoascus* was the dominant microorganisms in the shilixiang baijiu *Daqu* samples. *Guehomyces*, *Wickerhamomyces* and *Mucor* are endemic to batch F. Although *Wickerhamomyces* belongs to non-Saccharomyces cerevisiae and has a low fermentation capacity, it can synthesize a variety of enzymes to convert precursor substances in raw materials into flavor substances such as esters, acids, higher alcohols and aldehydes. It plays an important role in the formation of liquor flavor. *Fusarium*, which is endemic to batch G and H, is one of the most important large fungal genera in the world. It mainly infects important agricultural grains and produces Fusarium mycotoxins with acute and chronic toxic effects. Therefore, attention should be paid to the prevention and control in the production of *Daqu*. We also found that the abundance of *Gibberella* and *Pichia* was high at the early stage of fermentation, and the proportion of all batches decreased after 30 days of fermentation. This was because the environment and *Daqu* temperature were low at the early stage of fermentation, which was suitable for the growth and reproduction of mold and yeast (Liu, 2020). After 30 days of fermentation, the proportion of Mucor in batches F, G, H and K of *Daqu* samples increased. It's possible that the fungus can tolerate high temperatures. In each batch of shilixiang baijiu *Daqu* samples, the abundance of *Candida* was high at about the second day of fermentation, and then decreased gradually, so *Candida* played a major role at about the second day of fermentation.

In general, the analysis results from phyla and genus levels showed that the dominant fungal microflora in the each batch of shilixiang baijiu *Daqu* was similar but the abundance was significantly different, and there were unique microflora in each batch. The main reasons may be the composition of raw materials, brewing environment and temperature differences. In addition, fermentation days had a certain effect on the microbial community structure of shilixiang baijiu *Daqu* samples.

3.3 Beta diversity analysis

The similarity of microbial communities was analyzed using a non-metric multidimensional scale (NMDS) based on uniform distance (Figure 4). As can be seen from the NMDS of uniform distance matrix, the difference between different fermentation days in batches F, I and K is relatively small, and the distance between the three batches is relatively close, indicating that the microbial community structure between different samples has a high similarity, while the difference is small. However, the difference between batches G and H is relatively large, which may be caused by external environmental conditions. The sampling time of batch G is from mid-June to mid-July, while that of batch H is from early July to early August. The sampling temperature of these two batches is higher than that of other batches. Therefore, the number and abundance of bacteria that cannot withstand high temperature in batches G and H may be relatively less than that of other batches.

IV. Discussion

Daqu is made from different ingredients and is produced in different parts of China, and different fermentation conditions are applied in each place. The easiest parameter to change in environmental conditions is temperature. The change of temperature will have a significant impact on the diversity of *Daqu* fungal community, and then the quality of liquor production. In this study, the sampling time of shilixiang baijiu *Daqu* was distributed in May, June, July, August and September, and the temperature and environmental conditions in the five periods were different. Therefore, different fermentation batches of shilixiang baijiu *Daqu* had different fungal community diversity and abundance.

In this study, HTS technology was used to analyze the fungal community diversity in the production process of shilixiang baijiu *Daqu*. The fungal community in the sample of shilixiang baijiu *Daqu* was mainly composed of *Thermoascus*, *Candida*, *Rhizomucor*, and other unknown genera. This result is consistent with the research of Zhang on the change of flavor substances and taxonomic composition of fermented grains in Shilixiang (Zhang et al., 2020). Using nested PCR-DGGE, Zhang studied the microbial community structure of three typical *Daqu* samples, and found that Aspergillus was the dominant mold in the fungal community (Zhang et al., 2014) . There were some differences in microbial community structure among different *Daqu* liquor enterprises, which might be attributed to the different peak production temperature, raw material composition and micro-habitat around liquor enterprises. Liu were the first to detect *Thermoascus* in the starter culture used in the production of Maotai (Liu et al., 2012). Sun used MiSeq HTS technology to analyze the microbial flora structure of *Daqu* of different Maotai-flavor distilleries, and the results showed that the dominant fungal flora were *Thermoascus*, *filametous fungi* and *Thermomyces* (Sun et al., 2020). The above research results are almost consistent with the results obtained in this study.

It is well known that the saccharization and alcoholic fermentation of Chinese baijiu are the result of the combined action of several yeasts and moulds, which grow more or less continuously throughout the

brewing process. A variety of moulds, including *Rhizomucor*, *Rhizopus*, *Aspergillus*, *Gibberella* and *Tripterygium*, were identified in the samples of shilixiang baijiu *Daqu*. Candida and Pichia pastoris were also identified. So *Daqu* is rich in mold and yeast. Existing studies have shown that in the early stage of alcohol fermentation, many moulds can produce a large amount of glucoamylase and α-amylase, which contribute to the accumulation of glucose, and yeast can use glucose fermentation to produce alcohol. The study on the characteristics and functions of fungi in *Daqu* is of great importance to guide and optimize the liquor production process (Ma et al., 2011).

Using QIIME software, the analysis of fungal community structure showed that Ascomycota were the most dominant phyla. At the genera level, *Thermoascus*, *Candida* and *filametous fungi* were the dominant genera. At the early stage of fermentation, the relative abundance of *Candida* is relatively high and it is well known that members of this genus the ability of alcohol fermentation. So *Candida* is the main producer of alcohol at this stage. At the last stage of fermentation, *Thermoascus* become the dominant group, probably due to their tolerance to low water activity and high temperatures. *Thermoascus*, *Candida* and *Rhizomucor* were the main strains that existed from the beginning of fermentation to the end of fermentation.

By analyzing the diversity of microorganisms in different batches and screening the key species, it is helpful to develop effective strategies to regulate microorganisms in the brewing process and further improve the flavor of Chinese liquor. In the study of He, after inoculation with Bacillus, the microbial community structure changed through the synergistic or repulsive interactions among microorganisms (He et al., 2019). The contents of aromatic compounds and major esters in fermented liquor were high, which were mainly related to *Bacillus*, *Aspergillus*, *Lactobacillus* and *Candida*. This study reveals correlations between key microorganism and major flavor compounds.

This study revealed the diversity and abundance distribution of the fungal community in shilixiang baijiu *Daqu*. Through the analysis of all samples, the fungal community richness and diversity in shilixiang baijiu *Daqu* were related to fermentation days. There were some differences in the richness and diversity of microorganisms in the same batch of shilixiang baijiu *Daqu* samples at different fermentation days. This dynamic change may be caused by the bending operation and temperature variation during *Daqu* production. With the increase of fermentation days, the variation trends of the abundance and diversity of microorganisms in the five batches were also different. So in the process of Daqu-making, we should pay attention to the control of fermentation time and conditions, especially the control of the temperature. However, for the dominant microorganisms pointed out in this study and the unique microorganisms in each batch, it is necessary to further follow up the study on the flavor substances formed after each batch of *Daqu* is used in the brewing of shilixiang baijiu. To obtain more detailed information on the specific function of each microbe and its contribution to the eventual formation of the distinctive aroma of Chinese baijiu.

V. Conclusion

In this study, HTS technology was applied to analyze and study the structural diversity and characteristics of fungal flora in the shilixiang baijiu *Daqu* samples. The results showed that 3 phyla and 73 fungal genera were detected from *Daqu* samples. According to the relative abundance of each phyla and genus, Ascomycota is the dominant phyla of shilixiang baijiu *Daqu* samples. The dominant genera mainly include *Thermoascus*, *Candida*, *filametous fungi* among which *Thermoascus* and *Candida* are the absolute dominant genera. Through the distribution difference analysis on the level of fungi, it was found that there were significant differences in the diversity and abundance of fungal flora in different batches of *Daqu* samples, and each batch had its own unique fungi genus. The difference between *Daqu* samples may be related to the changes of environmental conditions such as pH, temperature and moisture content, among which temperature is the main influencing factor.

This study revealed the diversity and characteristics of fungi in shilixiang baijiu *Daqu*, which laid a foundation for further research on optimizing the *Duqu* making process of shilixiang baijiu and improving the quality of *Daqu*.

ETHICAL STATEMENT

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Competing interests
The authors declare they have no conflict of interests.
Funfing
Not applicable.
Authors' contribution
Yijing Wang, implementation of the experiment and process of the data. Hui Tang, instruction and design of the experiment.
All authors read and agree on the final text.

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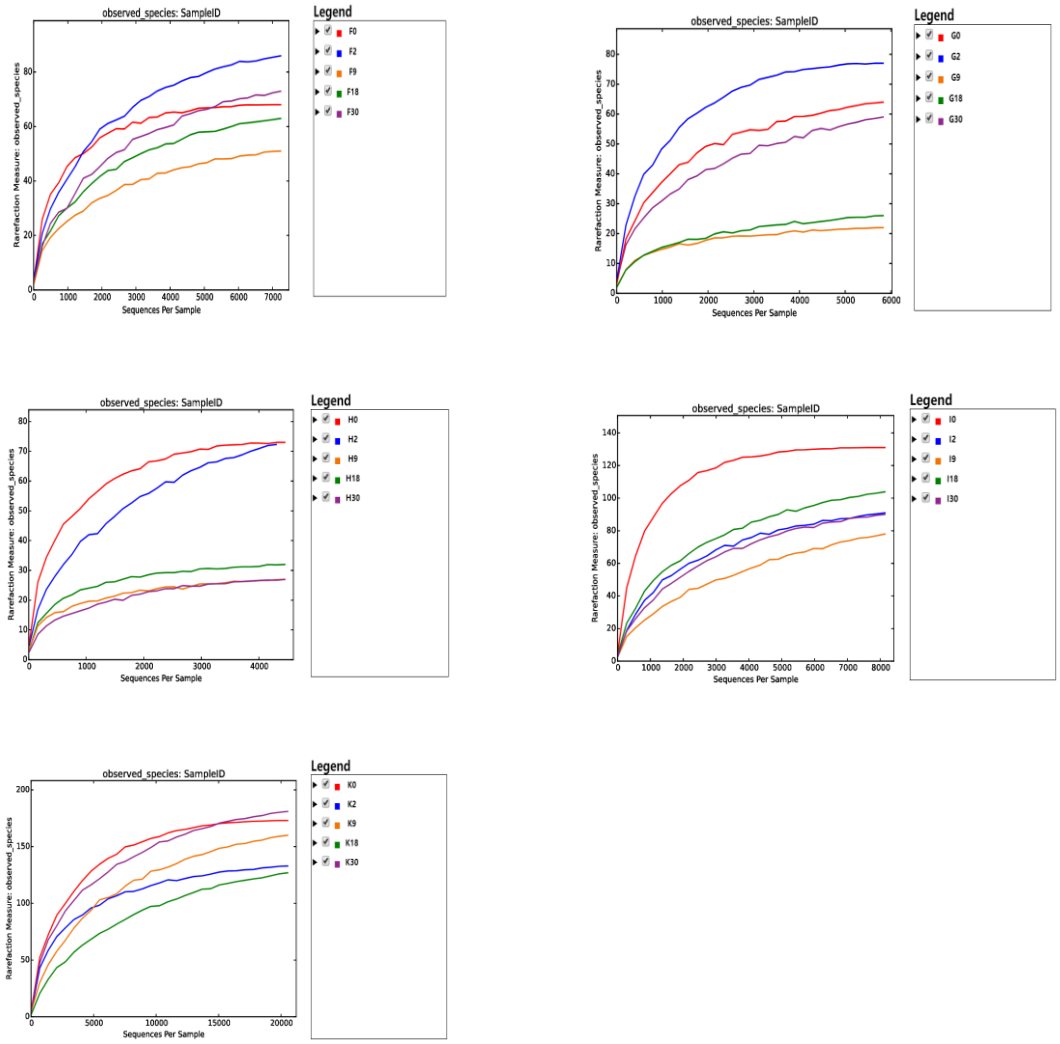

FIGURE 1 Rarefaction curves of shilixiang baijiu *Daqu* samples

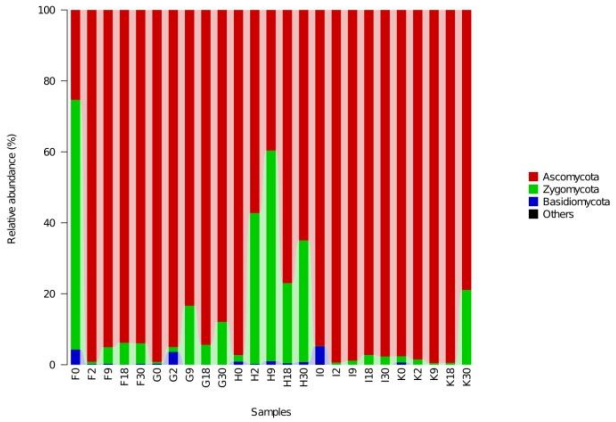

FIGURE 2 Fungal phyla distribution in shilixiang baijiu *Daqu* samples

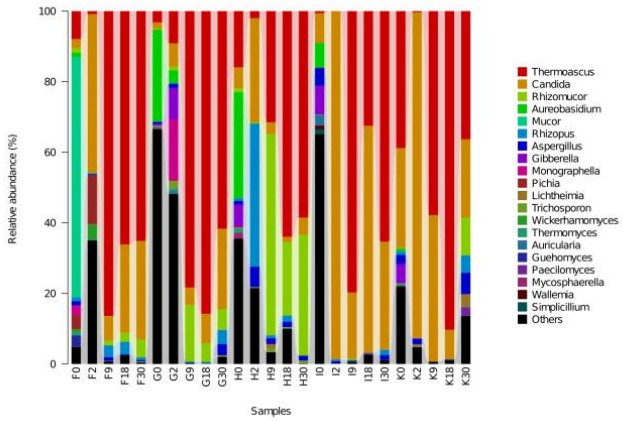

FIGURE 3 Fungal genus distribution in shilixiang baijiu *Daqu* samples

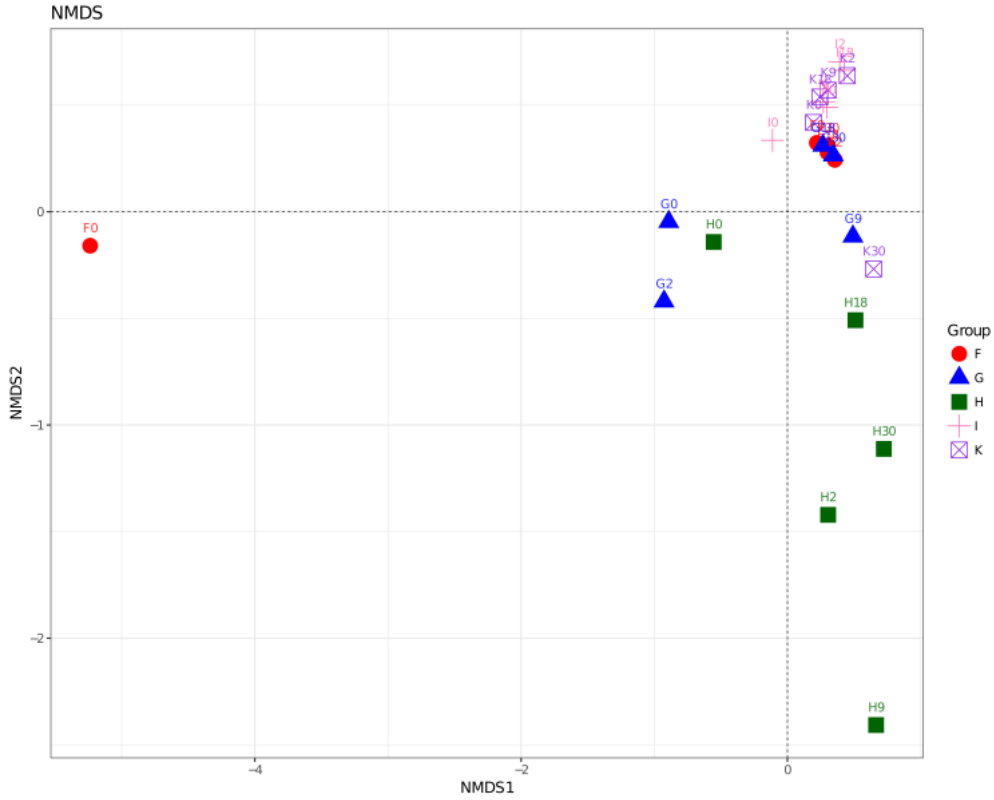

FIGURE 4 NMDS analysis diagram of shilixiang baijiu *Daqu* samples

TABLE 1 Abundance and diversity indexes of microbial population

Batch	Sample	Simpson	Chao1	ACE	Shannon
F	F0	0.531790	61.14	61.27	2.28
	F2	0.758740	74.06	79.11	2.66
	F9	0.266101	38.62	40.55	1.13
	F18	0.558624	48.55	50.54	2.06
	F30	0.561854	62.55	66.81	2.03
G	G0	0.589286	60.25	61.51	2.01
	G2	0.746181	71.40	75.66	2.93
	G9	0.365867	21.60	23.05	1.13
	G18	0.262731	24.50	27.02	0.97
	G30	0.611009	46.10	47.91	2.33
H	H0	0.835952	60.00	60.00	3.49
	H2	0.775308	82.00	87.58	2.92
	H9	0.580710	28.20	29.53	1.81

	H18	0.557770	32.75	33.24	1.81
	H30	0.543913	27.75	29.78	1.52
I	I0	0.800255	89.00	88.33	3.59
	I2	0.633933	72.07	74.84	1.99
	I9	0.367429	44.93	52.69	1.35
	I18	0.749261	84.56	87.42	2.66
	I30	0.556296	73.75	73.79	1.97
K	K0	0.796738	99.55	109.81	3.21
	K2	0.622938	79.88	83.74	2.43
	K9	0.631469	77.00	73.42	2.15
	K18	0.200880	51.17	55.22	0.82
	K30	0.832249	110.27	106.48	3.49

Hui Tang, et. al. Study on fungal community diversity of shilixiang baijiu Daqu based on high-throughput sequencing." *IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)*, 16(03), (2022): pp 34-42.