

Characterization of Microbial Communities in Chinese Rice Wine Collected at Yichang City and Suzhou City in China

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Two typical microbial communities from Chinese rice wine fermentation collected in Yichang city and Suzhou city in China were investigated. Both communities could ferment glutinous rice to rice wine in 2 days. The sugar and ethanol contents were 198.67 and 14.47 mg/g, respectively, for rice wine from Yichang city, and 292.50 and 12.31 mg/g, respectively, for rice wine from Suzhou city. Acetic acid and lactic acid were the most abundant organic acids. Abundant fungi and bacteria were detected in both communities by high-throughput sequencing. *Saccharomycopsis fibuligera* and *Rhizopus oryzae* were the dominant fungi in rice wine from Suzhou city, compared with *R. oryzae*, *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae*, *Mucor indicus*, and *Rhizopus microsporus* in rice wine from Yichang city. Bacterial diversity was greater than fungal diversity in both communities. *Citrobacter* was the most abundant genus. Furthermore, *Exiguobacterium*, *Aeromonas*, *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Lactococcus* were highly abundant in both communities.

Keywords: Chinese rice wine, microorganism community diversity, *Rhizopus oryzae*, yeast, community construction, rice wine fermentation

Introduction

Chinese rice wine, a popular alcoholic beverage in China, is produced with glutinous materials, which are fermented with microorganisms [1]. The production of Chinese rice wine is associated with a unique aroma and abundant nutritional components such as glucose, polysaccharides, amino acids, vitamins, organic acids, ethanol, and biogenic amines [2, 3].

Fermentation microorganisms (called “Qu” in China) are important components of the production process of Chinese rice wine [4]. “Qu” is made by mixing rice and other components, such as laurel leaf flour, which are kneaded into a small mass, covered with rice straw, and fermented for 24–36 h at a suitable temperature. It is then air-dried and stored in sealed packaging. “Qu” is made in an open environment; therefore, it commonly includes abundant fungi and bacteria [1, 5]. In Shaoxing rice wine, 10 bacterial genera, including *Bacillus* and *Lactobacillus*, were found to

be predominant [6]. Similarly, five *Bacillus* species and three lactic acid bacteria were found to be the dominant bacteria in Hong Qu rice wine, a popular Chinese rice wine [7]. These microorganisms play important roles in fermentation and the production of special flavors [8].

Yeasts and molds are also commonly found in rice wine. Yeast, as the alcohol producer, is important for rice wine fermentation. *Saccharomyces cerevisiae*, *Pichia* sp., and *Trichosporon* sp. are the most frequently identified yeast species [5, 9, 10]. *Rhizopus*, *Mucor*, and *Absidia* are the typical fungi in “Qu”, and *Rhizopus* is the dominant fungal genus in most types of “Qu” [11]. These microorganisms sometimes function in the hydrolysis and saccharification of starch, contributing to the sweetness of the wine and providing a substrate for ethanol fermentation [12].

In most regions in China, including Zhejiang, Hubei, Yunnan, Jiangsu, and Sichuan provinces in the eastern, central, and southern parts of China, people have maintained the tradition of making Chinese rice wine [6, 13, 14]. Two

provinces in the eastern and central regions of China, Zhejiang and Hubei, are particularly well known for rice wine production. Typically, the microbial composition of “Qu” differs according to the location of production and the production process, conferring Chinese rice wines with a wide variety of flavors.

In this study, glutinous rice was fermented to produce Chinese rice wine, using two “Qu” samples, one from Suzhou (in Jiangsu province) and the other from Yichang (in Hubei province). The ingredients of the fermentation and structures of the microbial communities were investigated in order to elucidate the variety of microorganisms in the “Qu” and the effects of microbial diversity on the quality of Chinese rice wine.

Materials and Methods

Fermentation Experiment

Two “Qu” samples were bought in Yichang city and Suzhou city, which are in Zigui district and Xiangcheng district, respectively. The raw materials used for the “Qu” from Yichang city included rice flour, polygonum, licorice, poria, and cloves. The raw material used for the “Qu” from Suzhou city included rice and edible fungus.

A total of 1,000 g of glutinous rice was soaked in water for 12 h after rinsing with water several times. The rice was then cooked by steaming for 30 min and cooled down to room temperature (about 25°C). Next, 500 g of cooked glutinous rice was removed and mixed with 2 g of “Qu” from Yichang. Similarly, 500 g of cooked glutinous rice was mixed with 2 g of “Qu” from Suzhou. The rice samples from Yichang and Suzhou were then placed in closed boxes and cultured at 30°C for 2 days. Samples were then transferred to 10°C and incubated at this temperature until day 10. Cultures (30 g) were collected in 50-ml sterilized tubes on days 0, 2, 10 and stored at –80°C until measurement of the fermentation products and microbial content.

Physical and Chemical Component Determination

Two grams of each sample was removed, diluted 5-fold with sterilized ultrapure water, and vortexed thoroughly. The samples were then centrifuged at 9,100 ×g for 10 min, and the supernatants were collected for analysis of volatile fatty acids (VFAs), glucose, ethanol, and protein.

VFAs were analyzed by high-performance liquid chromatography (HPLC; LC5090; FULI, China) with a 300 × 7.8 mm Aminex HPX87H column (Bio-Rad, USA) and a UV detector (210 nm). The mobile phase was 5 mM H₂SO₄ (pH 2.2), and the program was as follows: a flow rate of 0.6 ml/min for 40 min. The samples were adjusted to pH 2.0 using H₂SO₄ to remove protein and filtered with a 0.45-µm membrane filter [15].

Ethanol was analyzed by gas chromatography with an HP-INNOWAX column (60 m, 250 µm, 0.5 µm; Agilent Technologies,

USA) and a flame ionization detector (FID). The temperatures of the injection, column, and FID were set at 220°C, 100°C, and 250°C, respectively. Nitrogen was the carrier gas, and the flow rate was set to 1 ml/min [16].

Sugar was measured by the 3,5-dinitrosalicylic acid (DNS) method. Briefly, 400 µl of sample, which was diluted from the original sample, was mixed with 600 µl of DNS reagent (1% NaOH, 20% Rochelle salt, 2% phenol, 0.005% sodium sulfite, and 1% 3,5-dinitrosalicylic acid) and incubated for 15 min at 100°C. Subsequently, the samples were placed on ice for 5 min and centrifuged, and the absorption was determined at 540 nm [17].

Total protein was analyzed by the Bradford method. Briefly, 100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol, and 100 ml of 85% (w/v) phosphoric acid was added. The samples were stored at 4°C until use. For analysis, some of the stock liquid was removed and diluted 5-fold, followed by filtration through a 0.45-µm filter membrane. Next, 200 µl of the sample was mixed with 1 ml of Coomassie Brilliant Blue G-250 and incubated at room temperature for 5 min. Finally, the absorbance was measured at 595 nm [18].

DNA Extraction and High-throughput Sequencing

Total DNA of bacteria was extracted using an E.Z.N.A Mag-Bind Soil DNA kit (OMEGA Bio-tek, China) according to the manufacturer’s directions. The primers 341F (barcode (CCT ACA CGA CGC TCT TCC GAT CTG) - CCT ACG GGN GGC WGC AG) and 805R (barcode (GAC TGG AGT TCC TTG GCA CCC GAG AAT TCC A) - GAC TAC HVG GGT ATC TAA TCC) were used to amplify the V3-V4 region of the 16S ribosomal genes. The primers ITS1F (barcode (CCC TAC ACG ACG CTC TTC CGA TCT N) - CTT GGT CAT TTA GAG GAA GTA A) and ITS2-R (barcode (GTG ACT GGA GTT CCT TGG CAC CCG AGA ATT CCA) - GCT GCG TTC TTC ATC GAT GC) were used to amplify the ITS1-ITS2 regions of fungal DNA. The first polymerase chain reaction (PCR) was carried out with 2× Taq Master Mix (15 µl), Bar-PCR primer F (10 µM; 1 µl), primer R (10 µM; 1 µl), genomic DNA (10–20 ng), and H₂O (volume to 30 µl) with the following protocol: 94°C for 3 min; five cycles of 94°C for 30 sec, 45°C for 20 sec, and 65°C for 30 sec; 20 cycles of 94°C for 20 sec, 55°C for 20 sec, and 72°C for 30 sec; and a 5-min hold at 72°C. The second PCR was carried out with 2× Taq Master Mix (15 µl), primer F (10 µM; 1 µl), primer R (10 µM; 1 µl), genomic DNA (20 ng), and H₂O (volume to 30 µl) with the following protocol: 95°C for 30 sec; five cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec; and 72°C for 5 min. The PCR products were purified and retrieved. The DNA was precisely quantified using a Qubit ssDNA Assay Kit (Thermo Fisher, USA). Ten nanograms of each DNA sample was mixed, and 20 pmol DNA was used for sequencing. Illumina sequencing was performed by Sangon Biotech Company (China).

The sequencing data were deposited in the NCBI Sequence Read Archive under BioProject Accession No. PRJNA369612 (BioSample Accession Nos.: SAMN06290039–SAMN06290050).

Analysis of Data

The samples were identified by barcoding, and quality control was carried out for every sample sequence. Serial contigs, redundant sequences, and chimeras were removed. The sequences were clustered by distance and structured operational taxonomic units (OTUs). The most abundant sequence was selected as a representative sequence. Graphing of the networks and principal component analysis were carried out using R with related packages.

Results

Changes in VFA, Ethanol, Sugar, and Protein Contents during Fermentation

The concentrations of ethanol, sugar, proteins, and VFAs during the fermentation of Chinese rice wine were measured (Figs. 1 and 2). Acetic acid and lactic acid were the primary VFAs in the fermented products from Suzhou and Yichang, respectively. Acetic acid levels were significantly increased from the start of culture, reaching peak values of 1.33 and 1.66 mg/ml for the Suzhou and Yichang samples, respectively, on day 2. Lactic acid was the second most abundant VFA,

reaching 0.48 mg/ml for the Suzhou samples and 0.79 mg/ml for the Yichang samples on day 2. Formic acid, propionic acid, and butyric acid were measured as well; however, the levels of these three components remained low during the entire fermentation process.

Ethanol is the main component of Chinese rice wine [19, 20]. The fermentation products from Suzhou and Yichang exhibited similar ethanol concentrations of 12.31 and 14.47 mg/ml, respectively, on day 2. During the fermenting process, many sugars were produced by both “Qu” types, and the yield from Suzhou samples was greater than that from Yichang samples; 292.50 mg/ml sugar was produced by Suzhou samples on day 2, which was 1.47 times that from Yichang samples. Similar trends were observed for proteins. However, in contrast, Yichang samples had higher levels than Suzhou samples, and the highest value of 410 µg/ml was observed on day 2. Protein levels can reflect the biomass of the microorganisms [21]; thus, the main microorganism communities showed different trends in growth.

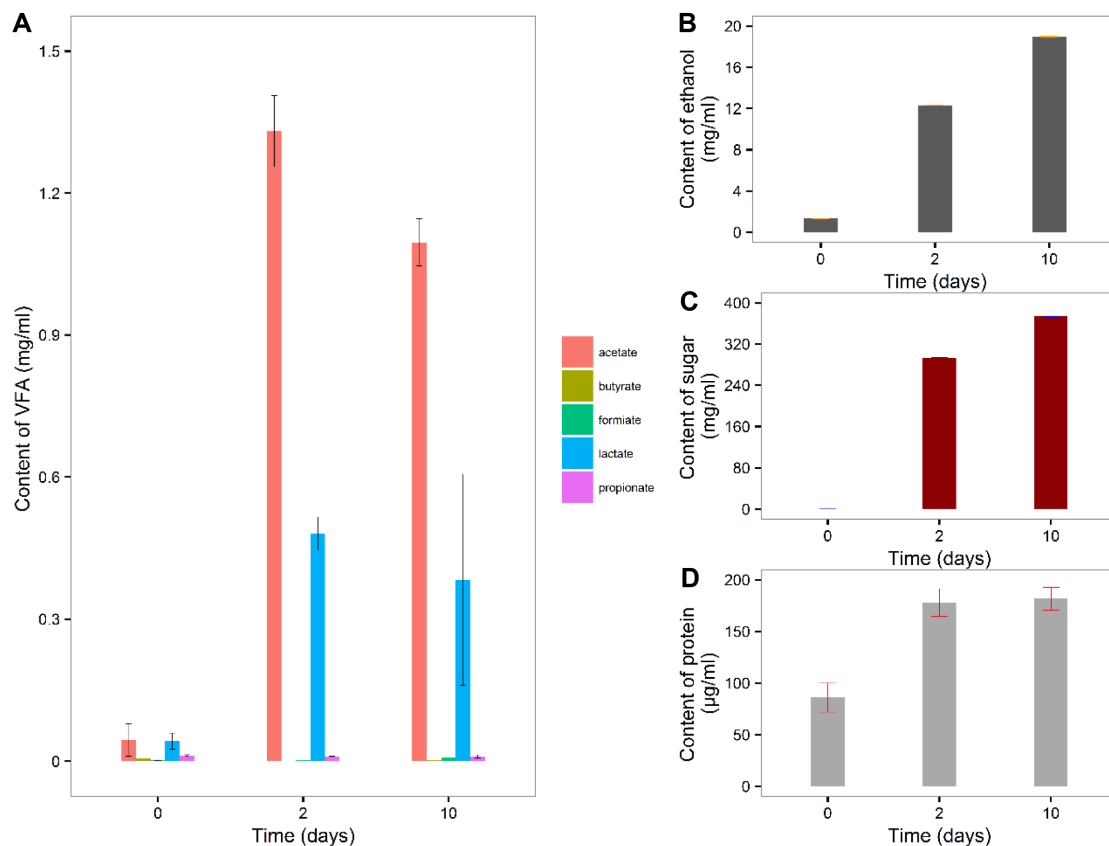


Fig. 1. Changes in volatile fatty acids (VFAs), ethanol, sugars, and proteins during fermentation by microorganism communities in rice wine samples from Suzhou city (S).

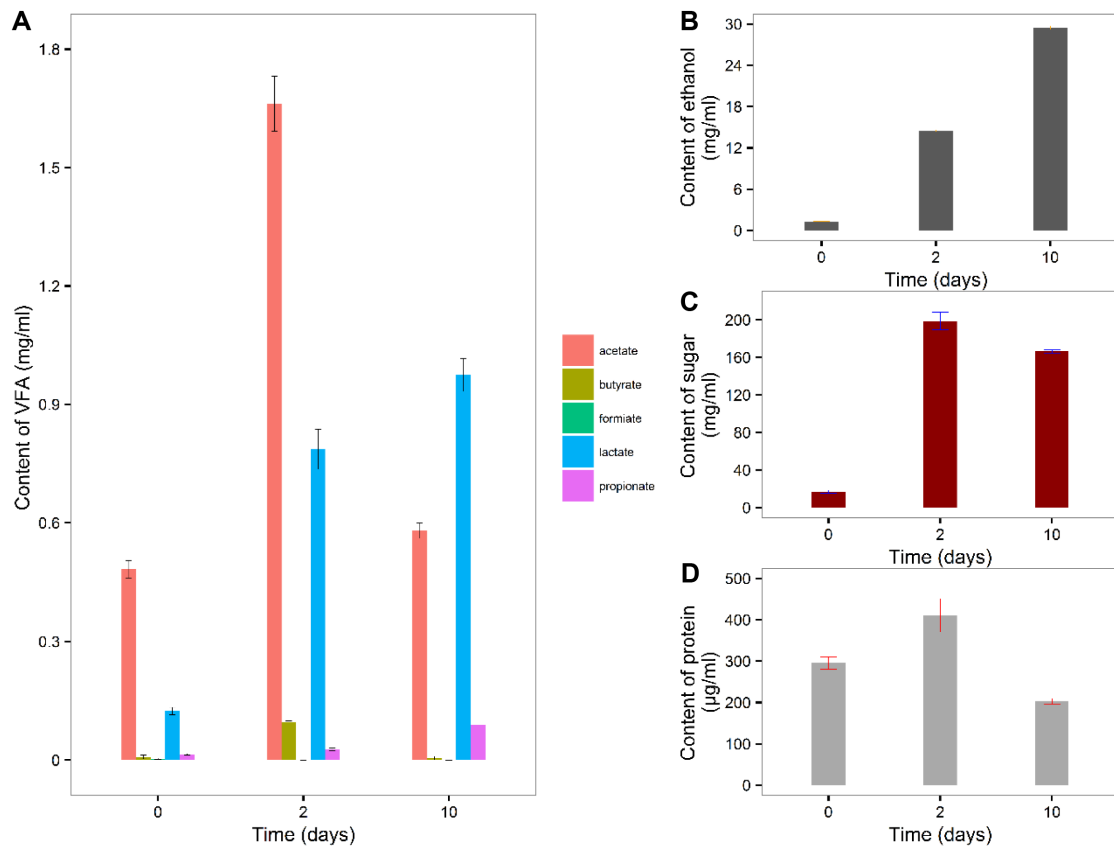


Fig. 2. Changes in volatile fatty acids (VFAs), ethanol, sugars, and proteins during fermentation by microorganism communities in rice wine samples from Yichang city (Y).

Analysis of Microbial Diversity

The V3-V4 regions of the 16S rRNA gene were sequenced using an Illumina Miseq platform, and 268,775 cleaned and filtered sequence reads were obtained. All of the sequences were clustered in 874 OTUs with 97% similarity. Of these, 242 OTUs were unique to Suzhou samples, and 173 OTUs were unique to Yichang samples; 459 OTUs were shared between the two locations.

The 875 OTUs represented 203 bacterial species. Twenty-two of the main bacterial genera are shown in Fig. 3. *Citrobacter* and *Exiguobacterium* were the most abundant bacteria in the Suzhou and Yichang samples. The abundances of the two genera were more than 55% and 65% in the Suzhou and Yichang samples, respectively. The two genera of bacteria remained dominant throughout the entire fermentation process in Chinese rice wine. In addition, *Acinetobacter*, *Aeromonas*, and *Bacillus* showed increased abundance in the Suzhou and Yichang samples. In particular, *Bacillus* sp. tended to increase during the fermentation process for both “Qu” types. In contrast, the abundance rates of *Cronobacter*, *Pantoea*, *Pediococcus*, and *Pseudomonas* were

dominant in the Suzhou samples and appeared to decrease during fermentation. *Pseudomonas*, *Leuconostoc*, and *Lactococcus* were the main bacteria in the Yichang samples; however, increased biomass was not observed during the fermentation process compared with that at the start of culture.

Similar to bacteria, fungi were analyzed based on sequencing of the ITS region. A total of 135,750 cleaned and filtered sequences of fungi were clustered in 110 OTUs. Thirty-six and 28 OTUs belonged to the Yichang and Suzhou samples, respectively, and 46 OTUs were shared. One hundred ten OTUs were associated with 14 species of fungi, and one was associated with “others” (Fig. 4).

As shown in Fig. 4, *Rhizopus oryzae* was the most abundant species in the Yichang samples. *Saccharomyces cerevisiae* was the second most abundant species and showed a significant increase during the final stage (10 days) of fermentation in the Yichang samples. In contrast, *Saccharomycopsis fibuligera* was the most abundant species in the Suzhou samples, and *R. oryzae* was also a dominant species. The Yichang samples showed more dominant strains than the Suzhou samples, including *Mucor indicus*, *Rhizopus microsporus*, and

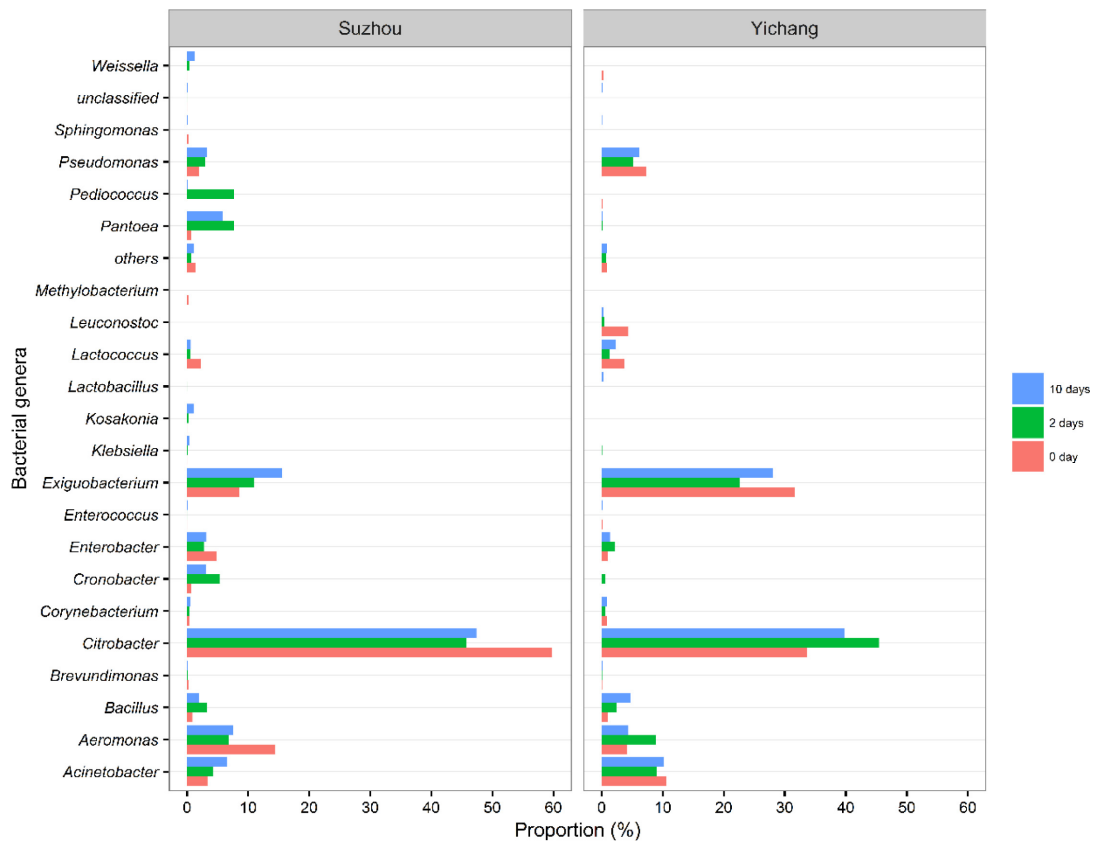


Fig. 3. Comparison of the abundance of bacterial genera in microbial communities from Suzhou and Yichang rice wine during the fermentation process.

Genera with greater than 0.1% sample sequence abundance are shown; genera with lower abundance were grouped as “others.”

Wickerhamomyces anomalus, which showed greater abundance rates in the Yichang samples than in the Suzhou samples. *R. oryzae* showed 94.8% abundance in the Yichang samples on day 2, whereas *S. fibuligera* showed 90.0% abundance in the Suzhou samples on day 2, suggesting that the microbial community in rice wine from Yichang was primarily a *R. oryzae*-based community and that the microbial community in rice wine from Suzhou was primarily a *S. fibuligera*-based community.

The network graph shown in Fig. 5 presents a more intuitive visualization of the distribution of bacteria. The types of microorganisms in the Suzhou and Yichang samples were similar. However, the abundances of the different types of bacteria were distinctly different. *Citrobacter*, *Exiguobacterium*, and *Aeromonas* were the three most predominant bacteria during the fermentation process in the Suzhou samples, with proportions of 59.73%, 8.6%, and 14.41%, respectively, at the start of culture. Similarly, *Citrobacter*, *Exiguobacterium*, and *Acinetobacter* were the three dominant bacteria in the Yichang samples, with proportions

of 33.58%, 31.61%, and 10.59% at the start of culture. These proportions were not altered dramatically during the fermentation process. Thus, despite the similarities in the bacterial communities of the Suzhou and Yichang samples, *Citrobacter* was the most common bacterium in the Suzhou samples, whereas *Citrobacter* and *Exiguobacterium* were the common bacteria in the Yichang samples.

The community structures of fungi were less complex than those of bacteria (Fig. 5B). In the microbial community in rice wine from Suzhou, *S. fibuligera* was obviously the dominant strain, with an abundance of 60.87% at the start of culture (day 0), increasing up to 89.99% on day 2. *R. oryzae* decreased from 37.61% on day 0 to 9.50% on day 2. Other fungi were present in very low numbers (*i.e.*, under 1%). In the microbial community in rice wine from Yichang, *R. oryzae* was the most dominant species, with an abundance of 87.33% on day 0, which increased to 94.81% on day 2. Other species, such as *Mucor indicus* and *R. microsporus*, were present at levels of only slightly more than 1%. However, the ethanol fermentation strain *W. anomalus* was only

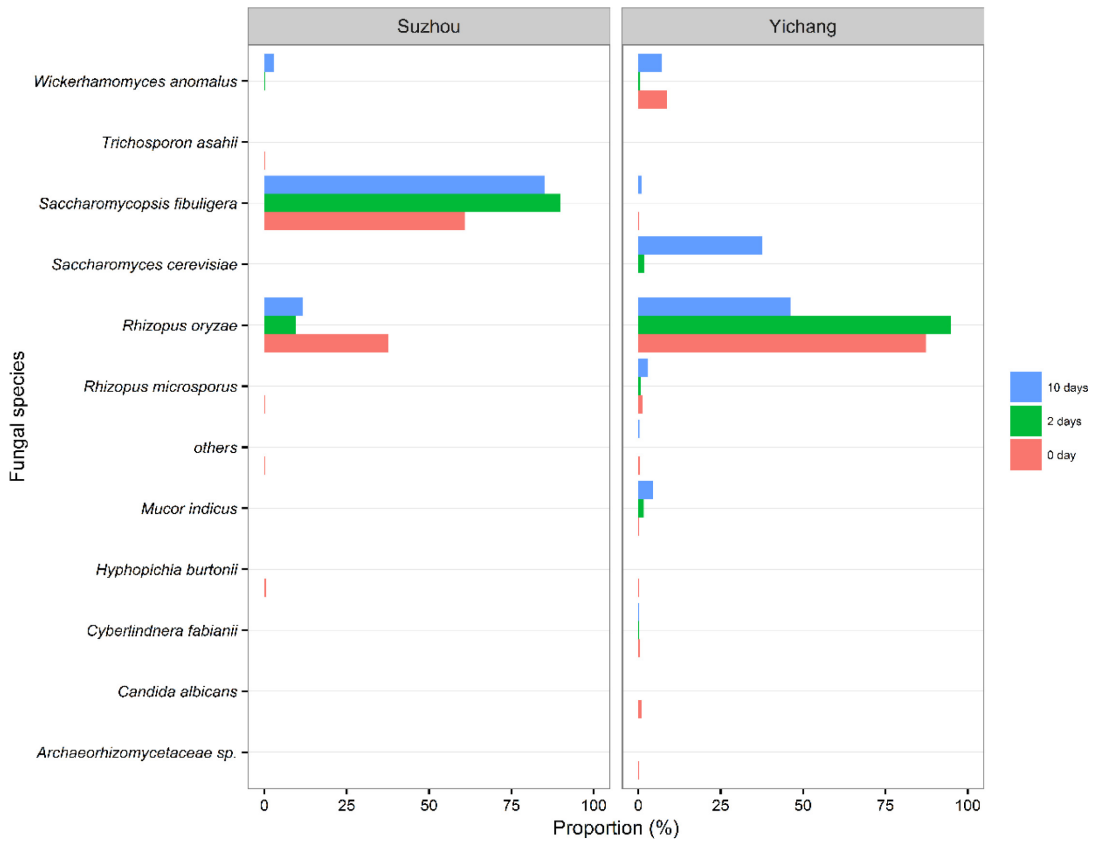


Fig. 4. Comparison of the abundances of fungal communities in the rice wines during the fermentation process. Genera with greater than 0.1% sample sequence abundance are shown; genera with lower abundance were grouped as “others.”

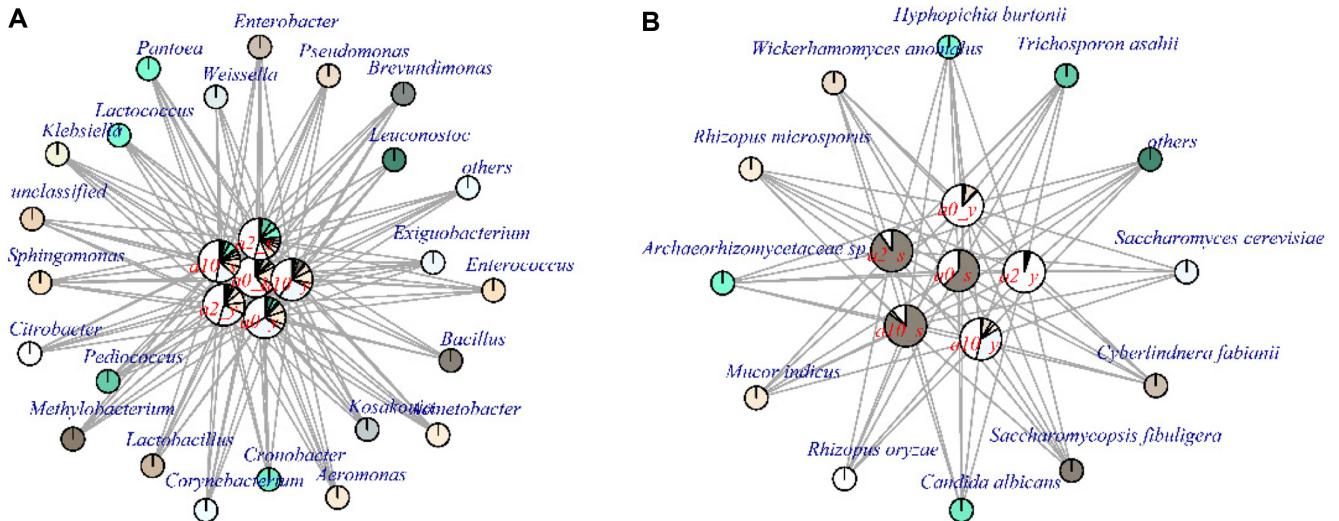


Fig. 5. Analysis of the bacterial and fungal community structures during rice wine fermentation, using a network map. (A): Bacterial community; (B): fungal community.

present at 8.66% in the starting culture, different from that in the Suzhou samples.

Correlations of the primary bacterial and fungal components of the Suzhou and Yichang samples at different

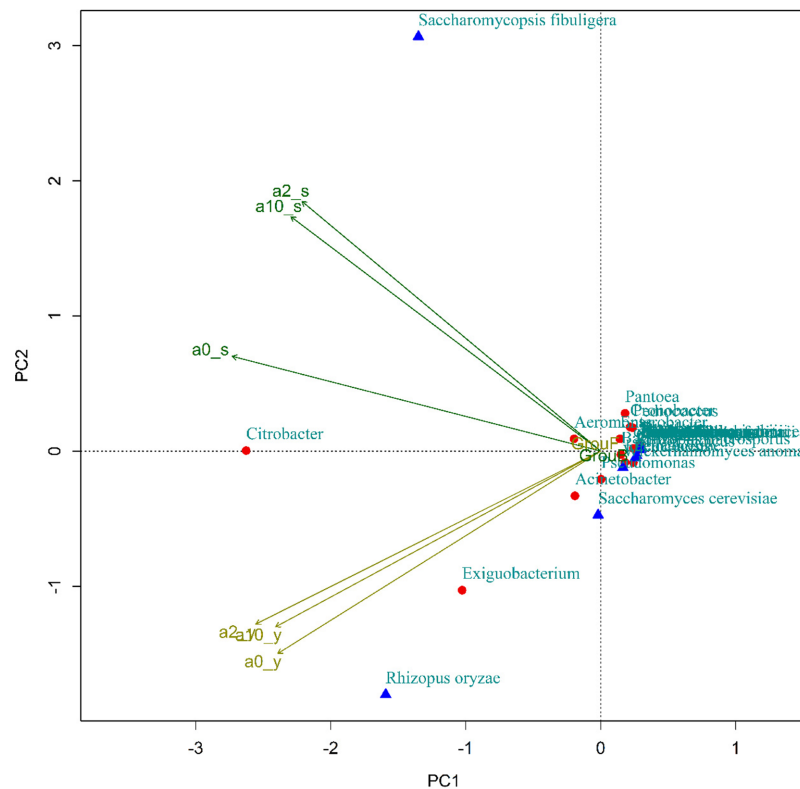


Fig. 6. Variations in the structures of microorganism communities in rice wines from Suzhou and Yichang during the fermentation process.

Circles indicate bacteria, triangles indicate fungi, and arrows indicate Suzhou (s) and Yichang (y) microorganism communities at different fermentation times (0, 2, 10 days).

fermentation times were analyzed by principal component analysis (Fig. 6). Consistent with the above analysis, *R. oryzae*, *S. fibuligera*, *Citrobacter*, and *Exiguobacterium* accounted for the majority of the diversity between the Suzhou and Yichang samples. Additionally, these organisms may be associated with the quality of fermentation and the unique flavor of Chinese rice wine. From the data shown in Fig. 6, *R. oryzae* and *Exiguobacterium* grouped near the Yichang group, whereas *S. fibuligera* grouped near the Suzhou group, suggesting that *R. oryzae* and *Exiguobacterium* directed the fermentation process in the Yichang samples, whereas *S. fibuligera* directed the fermentation process in the Suzhou samples. Furthermore, *Citrobacter* was a unique bacterium in the Suzhou and Yichang communities and was highly abundant in both (Fig. 5A).

In addition, the variations in the community structure between days 2 and 10 were minor; however, the community structures on both days were significantly different from that at the beginning of culture (day 0). This phenomenon was clearly observed in the Suzhou samples, indicating that when the fermentation was started, the microorganism

community would show changes in biomass to reflect a richer balance, with the changes becoming more stable after 2 days. The communities in the samples from Yichang were stable during the fermentation of Chinese rice wine.

Discussion

In this study, we evaluated the differences in the microbial communities between “Qu” samples obtained from two locations in China (Suzhou and Yichang). Despite the similar functional fermentation of glutinous rice to Chinese rice wine by both communities, the microorganism community structures were significantly different. Our data showed that there was major variance in the microbial compositions of the samples from Suzhou and Yichang, which are located in central and eastern China, respectively.

The composition of “Qu” can differ significantly as a result of differences in the producing areas, raw materials, the microbial communities. Liu’s investigation showed that *Bacillus*, *Leuconostoc*, *Lactococcus*, *Weissella*, *Thermoactinomyces*, *Pseudomonas*, *Saccharopolyspora*, *Staphylococcus*, *Enterobacter*,

and *Lactobacillus* were the dominant genera during the Shaoxing rice wine fermentation process [6]. Yu *et al.* [7] found that *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Aspergillus oryzae*, and *S. cerevisiae* were the major components of the fungal flora in wheat Qu. In this study, *S. fibuligera* and *R. oryzae* were the dominant fungi in rice wine from Suzhou city. *R. oryzae*, *W. anomalus*, *S. cerevisiae*, *Mucor indicus*, and *R. microsporus* were the dominant fungi in rice wine from Yichang city. “Qu” is always produced in an open environment [2, 22] and is therefore easily affected by the local environment and climate, resulting in variations in the microorganism community structure [8]. Suzhou is at a lower elevation and is near Taihu Lake, so its environment is more humid. However, Yichang city is located in the high mountain region in the middle of China and has a more distinct diurnal amplitude. These environmental differences may be one factor that caused the differences in the microbial community compositions in the “Qu” from Suzhou and Yichang.

Almost all “Qu” include three types of filamentous microorganisms, including molds, yeasts, and bacteria [23], for saccharifying amyllum, resulting in the production of ethanol and flavors [24, 25]. Accordingly, the diversity of microorganisms within “Qu” is not expected to be great. However, our results showed that although the types of microorganisms were similar, their abundances varied. Despite the variance in microorganism community structures between “Qu” from Yichang and “Qu” from Suzhou, the three functions of saccharifying starch, fermenting ethanol, and providing flavor were all sufficient in both types of “Qu”; the unique microorganism community structures led to the unique flavors of each of the produced Chinese rice wines.

Fungi sometime play important roles in the production of ethanol and flavor substances during the fermentation process [26]. Yeasts are also commonly found as dominant functional strains in wine fermentation [27–29]. In this study, *S. fibuligera* and *Rhizopus oryzae* were the most abundant fungi in the Suzhou samples, at abundances of 60.87% and 37.61%, respectively, at the start of culture. Furthermore, *S. fibuligera* showed a clear dominance and reached an abundance of 89.99% on day 2. Notably, in the Yichang samples, *R. oryzae* was the only favored fungus during the fermenting process, reaching an abundance of over 87% at 2 days after the start of culture. *R. oryzae* is a common strain widely found in “Qu” used for Chinese rice wine [11, 24] and can produce higher glucoamylase and α -amylase activities, thereby improving the saccharification of glutinous rice. In addition, *R. oryzae* can produce L-lactic

acid, with a maximum productivity of 5.45 g/(l·h) [30], as well as volatile compounds such as ethanol, ethyl acetate, and acetaldehyde [31]. Therefore, similar to yeast, when *R. oryzae* is the abundant strain in the fermentation microorganism communities, it can result in the production of high-quality rice wine.

The fermentation products of Suzhou and Yichang “Qu” were also compared. Both communities could produce high levels of sugars and organic acids by day 2 (sometimes, when rice wine is fermented for 2 days, it can be used for food [32]). However, Yichang “Qu” produced more organic acids. The contents of lactic acid and acetic acid were 63.6% and 24.88% greater than those in the Suzhou samples, respectively. However, Suzhou “Qu” produced more sugars than Yichang “Qu”; indeed, 47.23% more sugar was produced by Suzhou “Qu” than by Yichang “Qu” on day 2. Moreover, the ethanol contents of both samples were similar on day 2. Organic acids, sugars, and ethanol are important factors affecting the flavor of rice wine [25, 33]. Additionally, of the organic acids produced in rice wine, acetic acid and lactic acid are always the most abundant acids, ranging from 0.47 to 6.90 mg/g and 2.39 to 5.91 mg/ml, respectively [34, 35]. Ethanol and total sugar contents ranged from 0 to 123.08 mg/ml and 5.2 to 125 mg/ml, respectively [19]. Thus, compared with other rice wines, Chinese rice wines produced from Suzhou and Yichang “Qu” were the same type of wine with low acetic acid and ethanol contents and high sugar contents. These results can be explained by the features of the fermentation communities, which included more fungi [2].

In summary, two Chinese rice wine-fermenting microorganism communities (“Qu”) from Suzhou and Yichang were compared. The Suzhou and Yichang samples showed similar bacterial community structures, and *Citrobacter* was a major component in both communities. The fungal community structures of the Suzhou and Yichang samples varied significantly, particularly with regard to the abundances of the fungi and the dominant strains. Compared with that in the Suzhou samples, for which *S. fibuligera* and *R. oryzae* were the most abundant fungi in the fermentation, only *R. oryzae* was highly abundant in the Yichang samples. Although the Suzhou and Yichang microorganism communities were both high-sugar-producing communities for rice wine fermentation, Suzhou “Qu” was more likely to produce more sugars, whereas Yichang “Qu” was more likely to produce more organic acids.

This study can also provide insights into the construction of appropriate microorganism communities containing bacteria and fungi by laboratories or factories to produce

high-quality or uniquely flavored rice wine by adjusting the structure of the microorganism community. Furthermore, using this method, we can control the types of microorganisms and prevent the inclusion of harmful bacteria in “Qu.”

Acknowledgments

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