



Dynamic analysis of volatile metabolites and microbial community and their correlations during the fermentation process of traditional *Huangjiu* (Chinese rice wine) produced around Winter Solstice

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ABSTRACT

Traditional *Huangjiu* produced around Winter Solstice has higher quality and a more harmonious aroma. To investigate the variations of volatile metabolites and microbial communities during fermentation, gas chromatography–ion migration chromatography (GC–IMS), gas chromatography–mass spectroscopy (GC–MS) and high-throughput sequencing were employed. Aroma compounds results showed that alcohols and phenols increased before 45 days of fermentation and then decreased after 45 days, while esters gradually increased. Fungal genera *Saccharomyces*, *Aspergillus*, and *Rhizomucor* were dominant, whereas *Staphylococcus*, *Pediococcus* and *Weissella* were the dominant bacterial genera in the late stage. In addition, 11 genera such as *Lactobacillus*, *Saccharopolyspora* and *Aspergillus* ($|r| > 0.6$, $p < 0.05$) may contributed to traditional *Huangjiu* ecosystem stability. Moreover, correlation analysis indicated the dominant microorganisms (*Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, *Saccharomyces* and *Aspergillus*) were positively correlated with key compounds. These results provided theoretical guidance for further study on the flavor regulation of traditional *Huangjiu* via microbial community level and microbial augmentation.

1. Introduction

Traditional *Huangjiu* (a kind of Chinese rice wine) is extremely popular among consumers in East Asia due to its flavor characteristics of mellow, soft, sweet, fresh and clean (Mao, 2018). The old saying that “Traditional *Huangjiu* starts from the winter begins, and ends in the spring begins” (Fu, 2018) intuitively reveals that it is brewed under a low ambient temperature and its brewing process lasts for almost 90 days, namely winter brewing. Traditional *Huangjiu* is fermented in an open and non-sterile environment and therefore low ambient temperature conditions during winter brewing could be conducive to controlling the fermentation process and inhibiting the proliferation of infective bacteria which can cause rancidity and decrease the production of flavor compounds (Mao, 2018). Moreover, winter brewing is also beneficial to the long-term action of yeasts, bacteria and other microorganisms at low temperature to accumulate unique flavor substances (Mao, & Xuan, 2006). Traditional *Huangjiu* put into production near Winter Solstice, the coldest time of year, had better quality and more harmonious flavor according to the production experience. In our previous study (Yu et al.,

2021), aroma characteristics of finished traditional *Huangjiu* (after fermentation) produced under different ambient temperature conditions were revealed and proved that the traditional *Huangjiu* produced around Winter Solstice had better aroma quality. However, there are no scientific explanations for this phenomenon and the potential relationships between microbial community and flavor compounds during fermentation are still unknown.

Aroma characteristics is an important factor affecting the perceived flavor quality of *Huangjiu* and consumers' preferences. The aroma characteristics are the result of the joint action of various volatile compounds (Yang et al., 2020). Esters, alcohols, acids and aldehydes are the skeleton components of aroma in *Huangjiu* (Chen, Xu, & Qian, 2013), which are mostly generated during fermentation. During mechanized *Huangjiu* fermentation, most alcohols increased at first and then decreased, most esters increased continuously and then decreased slightly, while acids increased firstly and then decreased rapidly (Wang et al., 2014; Chen et al., 2018). The changes of aroma compounds in the whole fermentation process of millet Chinese rice wine were also described (Ye, Wang, Zhan, Tian, & Liu, 2022), in which the contents of

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alcohols and esters gradually increased, and acids increased firstly and then decreased with increasing fermentation time, while the aldehydes were inversely proportional to the fermentation time. Moreover, gas chromatography–ion mobility spectrometry (GC–IMS) recently has gradually been applied to characterize the aroma compounds variations and distinguish subtle differences by means of volatile fingerprint in Chinese rice wine (Yu et al., 2021; Zhang et al., 2022). Nevertheless, there are few studies on the analysis of aroma profiles in the fermentation process of traditional *Huangjiu*.

Complex microbial metabolism played a decisive role in the production of volatile compounds (Liu et al., 2019). *Saccharomyces* was responsible for the production of alcohol and contributed to aroma compounds in Chinese rice wine (Tian, Zeng, Fang, Zhou, & Du, 2022). Bioaugmentation inoculation of *Saccharomyces cerevisiae* could increase the content of alcohol and ester (Su, Zhang, Cao, & Yang, 2020). Some genera of moulds including *Aspergillus* and *Rhizopus* contributed to the saccharification process through their extracellular enzymes, including amylase and saccharifying enzymes. (Cai et al., 2018; Zhang, Wu, & Yan, 2020). Additionally, bacteria also acted on the flavor formation of *Huangjiu*. The primary role of the bacteria was to produce various flavoring compounds or precursor of volatile compounds (Yang et al., 2020; Zou, Zhao, & Luo, 2018). In particular, *Bacillus* played an important role in the formation of esters and pyrazines (Huang et al., 2018). The genera *Lactobacillus*, *Candida* and *Enterobacter* were the major contributors of organic acids (Wang et al., 2014). And *Saccharopolyspora* was found to be the dominant bacterium in the fermentation process of mechanized *Huangjiu* (Liu et al., 2019). The microbial succession in *Huangjiu* revealed that *Leuconostoc*, *Pediococcus*, *Bacillus*, and *Lactobacillus* dominated in the late stage, while *Rhizopus* and *Saccharomyces* were the predominant fungal genera throughout fermentation (Zhao et al., 2020). To our knowledge, the dynamics of microbial community, and relationships between microbial community and flavor compounds during the long-term fermentation process at low temperature are still not completely clear. Therefore, it is necessary to reveal the succession rule of microbial community during fermentation and to further study on their potential correlations.

The objectives of this study were (1) to analyze the dynamics of aroma compounds in traditional *Huangjiu* put into production around Winter Solstice by using a combination of GC–IMS and GC–MS analysis, (2) to compare the microbial community diversity and dynamic by using high-throughput sequence, (3) to further reveal the correlation between the dominant community and key aroma compounds during fermentation. This study provided detailed information on the dynamic of flavor compounds and microbial community during traditional *Huangjiu* fermentation, and might help better clarify the complex relationship between microbial community and aroma compounds, expecting to improve the quality of traditional *Huangjiu* by means of bioaugmentation with the identified beneficial strains in the future.

2. Materials and methods

2.1. Samples and chemicals

All the samples were provided by Zhejiang Pagoda Brand *Huangjiu* Co., Ltd. (a representative enterprise focusing on traditional *Huangjiu*), Shaoxing city, Zhejiang Province, China. According to the actual brewing process of the brewing factory and the requirements of the national standard of China (GB/T 17946–2008), the traditional *Huangjiu* samples in this research were brewed using glutinous rice, Jianhu water, wheat *Qu* (a saccharification starter) and *Jiuyao* (a fermentation starter) as the materials, and put into production with unique brewing technology of simultaneous fermentation and saccharification around Winter Solstice. And the traditional *Huangjiu* manufacturing involved soaking rice (15–20 days for water absorption), steaming rice, cooling to indoor temperature, mixing with saccharification and fermentation starters (wheat *Qu* and *Jiuyao*), and fermenting in pottery jars (primary

fermentation at 28 °C for 3–5 days, then secondary fermentation in an open environment at medium–low temperatures (5.76 ± 0.68 °C– 18.06 ± 0.66 °C) for about 90 days until the fermentation is complete). The fermenting mash samples were collected during the above long time period of secondary fermentation at different fermentation days (0d, 22d, 45d, 67d, and 90d, respectively), corresponding to sample C1, C2, C3, C4 and C5 in sequences. After mixing uniformly the pottery jars, three different fermentation pottery jars were sampled and mixed evenly as one sample (approximately 1,000 g), and triplicate above samples were collected at each sampling point. The collected samples were divided into two parts: one was immediately stored at –20 °C for flavor analysis, and the other was kept at –80 °C for the analysis of microbial communities. Each example was sealed in the sterile sampling bag with a mark.

The reagents including ethanol ($\geq 99.7\%$), 2-octanol ($\geq 99.0\%$, internal standard), dichloromethane ($\geq 99.5\%$) and *n*-alkane mixture (C₅–C₃₀) were of chromatographic grade and obtained from Sigma–Aldrich (Shanghai, China). TIANamp Stool DNA Kit was purchased from Sangon Biotech Co., Ltd (Shanghai, China).

2.2. GC–IMS analysis

The GC–IMS (Flavorspec®, Gesellschaft für Analytische Sensortechnik mbH, Dortmund, Germany) equipped with an MXT–WAX column (30 m × 0.53 mm × 0.1 μm, Restek, Beijing, China) was applied to analyze the volatile fingerprints of the samples. The parameters and procedures were described in our previous study (Yu et al., 2021). Traditional *Huangjiu* sample (1 mL) was introduced into a 20 mL headspace sample vial and incubated at 50 °C for 10 min, and then the headspace gas was injected into the inlet by heated syringes (85 °C). Nitrogen (99.99% purity) was used as the carrier gas and the flow rate was 1.0 mL/min. The programmed temperature procedure was set as follows: the column was held at 60 °C, then 2 mL/min for 2 min, then an increase to 100 mL/min in the next 18 min, finally maintained at 100 mL/min until 40 min. All analysis was conducted in triplicate. The GC × IMS Library Search software equipped with NIST and IMS database was applied to qualitatively analyze the volatile compounds by matching the retention indexes and the drift time to standards in the GC–IMS library.

2.3. GC–MS analysis

Volatile compounds of the samples were extracted by headspace solid phase microextraction (HS–SPME) and solvent assisted flavor evaporation (SAFE), and then they were analyzed by GC–MS. The HS–SPME analysis was carried out according to our previous study (Yu, Xie, Xie, Ai, & Tian, 2019). The samples (5 g) and 20 μL internal standard (2-octanol, 315 μg/mL) were put into a 20 mL vial. After equilibrating at 50 °C for 5 min, the SPME fiber (100 μm, Supelco, Inc., Pennsylvania, USA) coated with DVB/CAR/PDMS was exposed to the headspace of the vial for 50 min at continuous stirring of 250 r/min. Specific SAFE conditions and parameters referred to our previous study (Yu, Guo, Ai, Chen, & Tian, 2022). The samples (60 mL), 200 μL internal standard (2-octanol, 315 μg/mL) and dichloromethane (60 mL) were placed in a 250 mL conical flask. After extraction, separation and centrifugation, the collected dichloromethane extract (about 180 mL) was dried and separated. Finally, the SAFE fraction was concentrated to 1 mL by nitrogen and stored at –20 °C.

The procedure parameters for GC–MS were described in our previous research (Yu et al., 2021). A 7890 GC equipped with an MS (model 5973C, Agilent Technologies, Santa Clara, CA) was used. An HP–Innowax column (60 m × 0.25 mm × 0.25 μm) from Agilent Technologies was applied. The GC temperature procedure was set as follows: the oven temperature was held at 40 °C for 3 min at first, then increased at 3 °C/min to 120 °C and held for 5 min, and finally raised at 3 °C/min to 200 °C and held for 4 min. The temperature of the detector was 250 °C, the carrier gas was helium (99.999%) at a flow rate of 1 mL/

min. The electron ionization energy was set at 70 eV, and the mass range was from 30 to 450 amu. The analyses were conducted in triplicate.

The MS spectra and retention indexes (RI) were compared with those in the NIST11 libraries and literatures to identify aroma compounds, where RIs were calculated according to the C₅–C₃₀ alkane standards. The aroma compounds were quantitatively analyzed by external standard method.

2.4. Total DNA extraction, PCR amplification and sequencing

Total DNA of the samples were extracted using TIANamp Stool DNA Kit (Sangon Biotech, Shanghai, China) according to manufacturer's protocol. Primers 338F/806R and ITS1F/ITS2R were used to amplify the V3-V4 hypervariable region of the bacterial 16S rRNA genes and fungal ITS1 regions, respectively. The PCR products were extracted and

purified, and then the purified amplicons were used to construct a PCR amplicon library and sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The experiments were conducted in triplicate.

2.5. Bioinformatics and statistical analysis

Raw reads were processed and analyzed via QIIME software package (V1.9.1) (Yang et al., 2022). The valid sequences with similarities > 97% were clustered into an OTU (operational taxonomic units) for microbial classification using Uparse software (V7.0.1090). Representative sequences for each OTU were screened for further annotation. For the representative bacterial OTU sequences, they were annotated and classified using the SILVA's SSUrRNA database. And UNITE fungal ITS database was used to annotate and compare the representative fungal

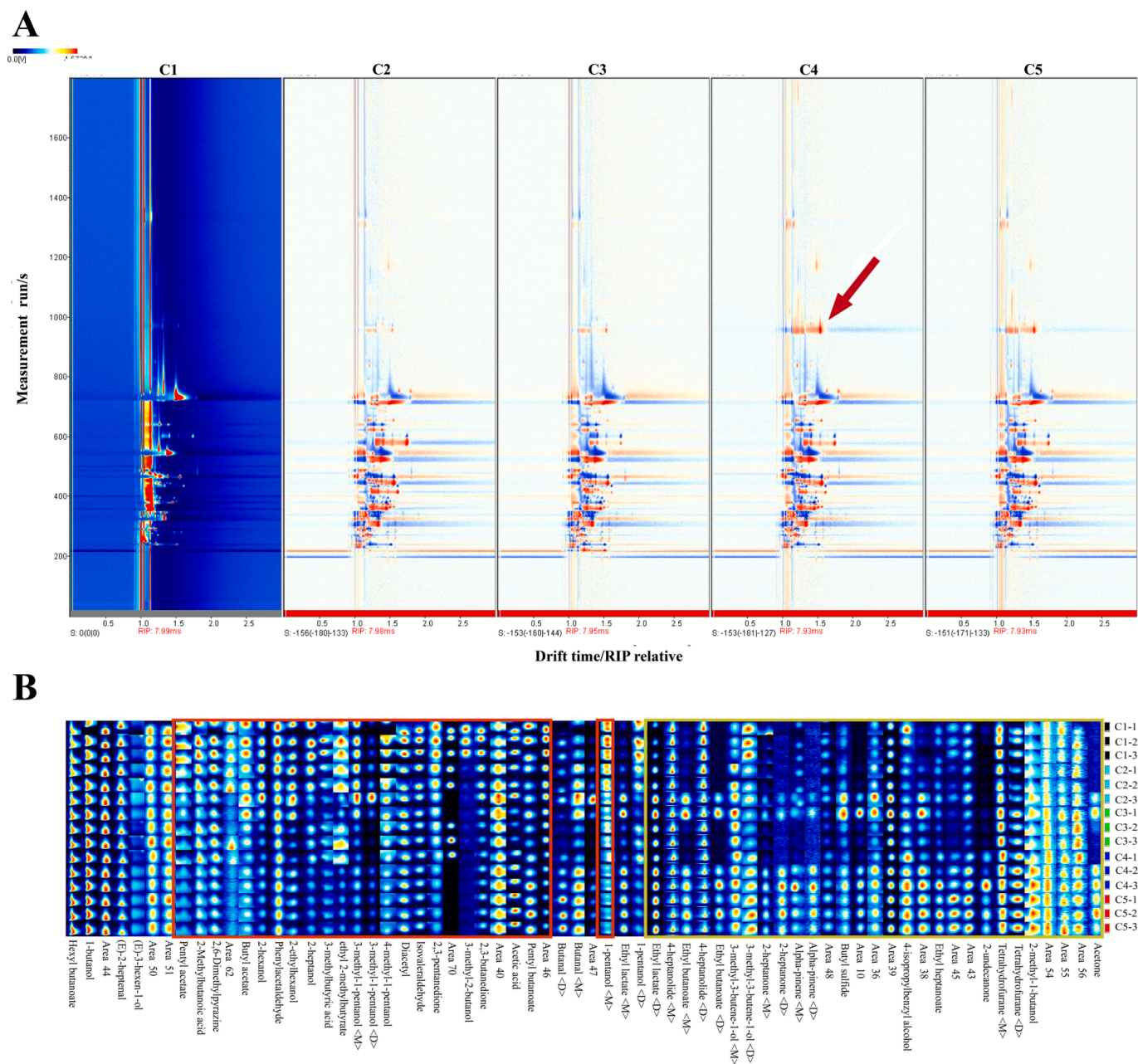


Fig. 1. 2D-topographic plots of volatile organic compounds in traditional *Huangjiu* during fermentation (A); The fingerprint of volatile profiles in traditional *Huangjiu* during fermentation (B) (Each row represents all the signal peaks selected in a traditional *Huangjiu* sample, and each column represents the signal peaks of the same volatile compounds in different samples. C1, C2, C3, C4 and C5 correspond to the *Huangjiu* samples fermented for 0d, 22d, 45d, 67d, and 90d, respectively).

OTU sequences. The amplicon databases were submitted to NCBI Sequence Read Archive (SRA) and were available under accession numbers PRJNA880416.

The alpha-diversity indices including Chao1, Shannon, Simpson and ACE in each sample were analyzed for comparing the species diversity and richness via Mothur software (V.1.30.1). And R software (V2.15.3) was used to visualize those data of alpha diversity. For β -diversity analysis, QIIME software (V1.7.0) was applied to calculate UniFrac distances based on weighted and unweighted. Cluster analysis was performed using ggplot2 in R software. Principal coordinate analysis (PCoA) was performed using SIMCA (V14.1) to capture principal coordinates and visualize complex multidimensional data.

2.6. Statistical data analysis

The data were analyzed by Duncan's multiple range tests using SPSS Statistics 21 (SPSS Inc., Chicago, USA) and differences at a significant level of 0.05 were considered significant. The heatmap was generated and Pearson correlation coefficient between dominant bacteria and major aroma compounds were generated using R (V2.15.3), and then visualized with Cytoscape 3.4.0 (NIGMS, Seattle, USA).

3. Results and discussion

3.1. Variation tendency of the volatile fingerprints during traditional *Huangjiu* fermentation

To reveal the differences and dynamics of volatile compounds during fermentation, GC-IMS was applied to analyze the volatile fingerprints of the traditional *Huangjiu* samples. Fig. 1A is a comparison plot of the differences in volatile compounds in the samples, in which the vertical coordinate represents the retention time of the gas chromatographic peaks, and the horizontal coordinate represents the ion migration time (normalized). The topographic plot of the sample (C1) was selected as a reference, it can be seen that the types of volatile compounds in the samples were basically the same, whereas the contents were different. To visually reveal the dynamic changes in each substance, all peaks were extracted to form a characteristic fingerprint for comparison (Fig. 1B). It can be seen from Fig. 1B that the trends of compounds at different stages of fermentation were different. Most of the detected alcohols (2-hexanol, 2-ethylhexanol, 3-methyl-1-pentanol, 1-heptanol, 1-pentanol, and so on) in the red frame gradually decreased, while the contents of esters (ethyl lactate, ethyl butanoate, ethyl heptanoate and pentyl acetate) and ketones in the yellow frame increased in general

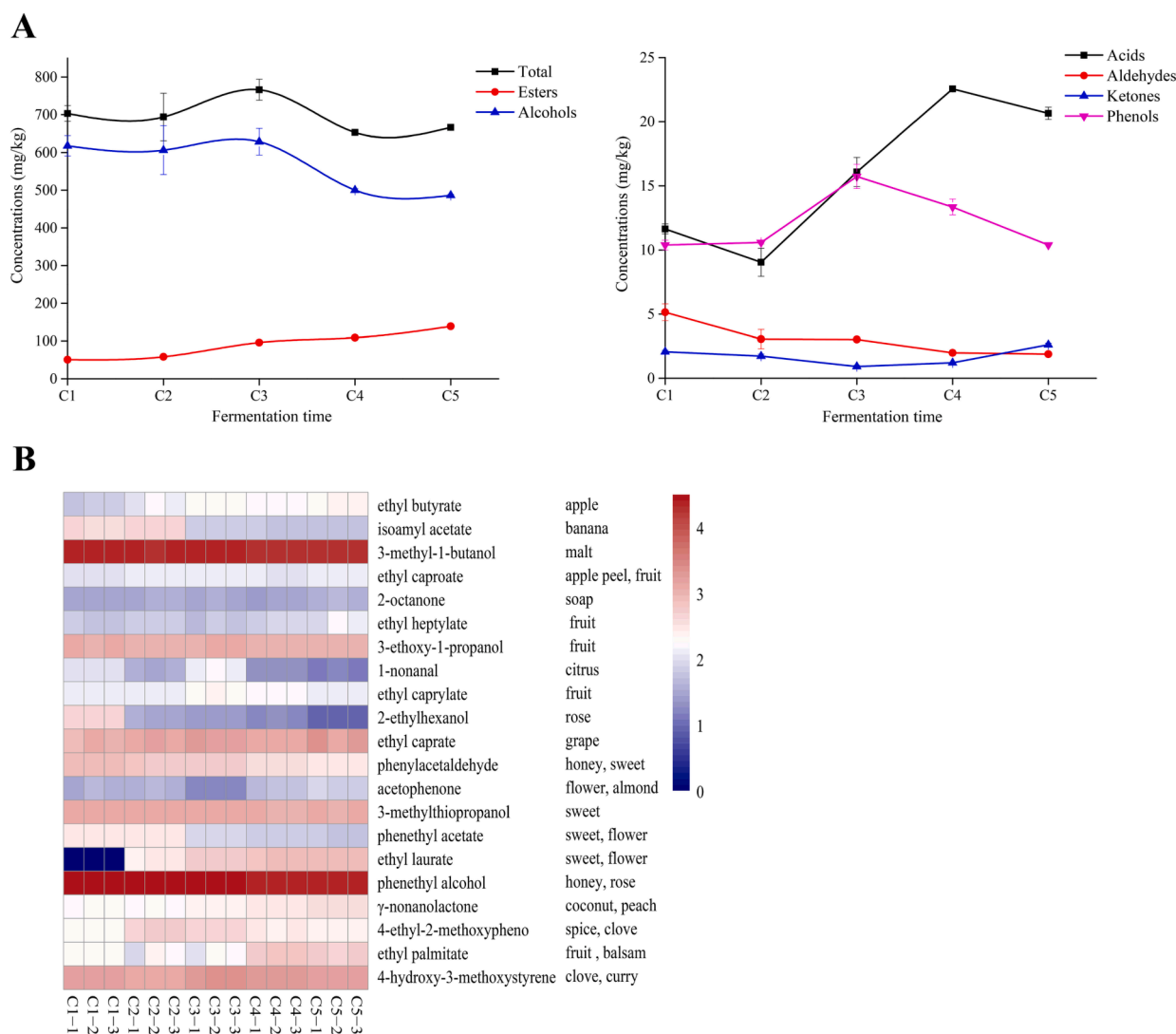


Fig. 2. The total content of aroma compounds and the variation of various compounds in the fermentation process of *Huangjiu* (A); Heatmap of key aroma compounds in traditional *Huangjiu* during fermentation (B) (C1, C2, C3, C4 and C5 correspond to the *Huangjiu* samples fermented for 0d, 22d, 45d, 67d, and 90d, respectively).

with increasing fermentation time, which were similar to those observed in the fermentation process of Chinese rice wine (Liu, Wang, Sun, & Ni, 2020). This result suggested that alcohols were accumulated at the early stage of fermentation and may gradually convert into esters and other flavor compounds as the fermentation process progressed.

3.2. Analysis of the changes in aroma compounds during traditional Huangjiu fermentation by GC-MS

The aroma compounds in the samples collected with different fermentation time points were analyzed by GC-MS, which were listed in Table S1. A total of 54 aroma compounds were detected, including 21 esters, 14 alcohols, 6 acids, 4 aldehydes, 3 ketones and 4 phenols. Among the detected aroma compounds, esters were the most diverse and the contents of alcohols were the highest. The variations of the total content of the aroma compounds including alcohols, esters, acids, aldehydes, ketones and phenols during fermentation are shown in Fig. 2A. On the whole, the total concentrations of aroma compounds increased at C3 period, then slightly declined and tended to stable at the end of fermentation. This trend was also observed in mechanized Huangjiu (Chen et al., 2018). The total content of alcohols (73.01%–87.83%) was the highest, followed by esters, acids, phenols, aldehydes and ketones. Additionally, alcohols and aldehydes at first increased at C3 period and then decreased with increasing fermentation time, acids and phenols decreased slightly at first, then increased and finally decreased as the fermentation process progressed. Moreover, the concentrations of esters gradually increased while ketones decreased slightly and then gradually increased with increasing fermentation time. These results were generally consistent with those described by GC-IMS. According to the result of OAV values, twenty-one compounds with OAV > 1 were identified as characteristic aroma compounds, and their aroma descriptions were used to form a heat map (Fig. 2B) that characterized the distribution of these important aroma compounds during fermentation. And these compounds were basically consistent with the aroma active compounds determined by gas chromatography olfactometry in our previous study of traditional Huangjiu (Yu, Xie, Xie, Ai, & Tian, 2019).

Alcohols in fermented wines are mainly produced by two pathways: sugar metabolism and dehydrogenation decarboxylation of amino acids (Hernandez-Orte et al., 2008). Among the alcohols detected, phenylethanol and 3-methyl butanol had the highest content, followed by isobutanol, *n*-propanol, 2,3-butanediol and 3-methylthiopropanol. The sum of these six alcohols reached 99.69% of the total alcohol content in the sample. The high content of higher alcohols gradually decreased with increasing fermentation time, which might be due to the decrease of metabolic activity of microorganisms to produce higher alcohols, such as phenylethanol, 3-methylbutanol, isobutanol, and 1-propanol (Mou, Mao, Meng, & Liu, 2016). Additionally, phenylethanol had the highest concentration at C1 period (326.21 mg/kg), while that of 3-methylbutanol, isobutanol and *n*-propanol was the highest at C3 stage. Among them, the OAV values of phenylethanol (OAV: 24–33), 3-methylbutanol (3–4) and 3-methylthiopropanol (11–13) were all greater than 1 (Fig. 2B). The OAVs of these three characteristic aroma compounds overall decreased with increasing fermentation time and they had the lowest concentrations at the end of fermentation (C5 period), which may be ascribed to the relatively low temperature slowing down the metabolic activity of yeast cells to produce alcohol (Beltran et al., 2006).

Phenylethanol, with the aroma of rose and honey, was the most important higher alcohol in Huangjiu, which can be mainly produced through the Ehrlich pathway or glycolysis and pentose phosphoric acid pathway (Wang et al., 2020). The compound 3-methylbutanol formed by corresponding leucine contributed to malt aroma. And the compound 3-methylthiopropanol with sweet and potato aromas probably came from the degradation of sulfur-containing amino acids (Mestres, Busto, & Guasch, 2000).

The ester compounds were associated with floral and fruity aroma,

which could be formed by the microbial metabolism or esterification of carboxylic acids and alcohols during fermentation (Li et al., 2022). Among the detected esters, ethyl lactate and ethyl myristate had the highest contents, followed by ethyl 3-hydroxybutyrate, ethyl decanoate, ethyl acetate, ethyl laurate, diethyl succinate and ethyl palmitate. These eight esters accounted for 86.69%–93.95% of the total content of esters, which was consistent with our previous study (Yu et al., 2022). As shown in Fig. 2A, most esters showed an upward trend overall, such as ethyl lactate, ethyl myristate, ethyl acetate and ethyl laurate, which was generally consistent with that of GC-IMS. Among them, ethyl lactate had the largest growth rate, which increased about five times from C1 (12.52 mg/kg) to C5 (61.76 mg/kg). It was also found from Fig. 4 that there were 10 esters with OAV > 1, which were ethyl caprate (OAV: 9–19), ethyl laurate (1–8), ethyl palmitate (1–2), γ -nonanolactone (21–57), ethyl butyrate (8–41), ethyl octanoate (203–370), ethyl hexanoate (58–82), ethyl heptanoate (1–2), phenylethyl acetate (1–5) and isoamyl acetate (5–57), indicating that they had different degrees of aroma contribution including fruit and flower aroma to the samples. The OAVs of these esters (ethyl caproate, isoamyl acetate and phenylethyl acetate) increased at first and then decreased, which can be hypothesized that the microorganism had high diversity and activity at the beginning of fermentation stage while mass microorganisms can not adapt to the environment of high acids and alcohols with the fermentation continuing (Chen et al., 2021). Additionally, the concentration of phenylethyl acetate was highest at the initial stages of fermentation and then decreased, indicating that phenylethyl acetate may derive from the raw materials and metabolites of the microorganisms (Yang et al., 2020). And ethyl octanoate reached the peak at C3 period and its OAV was the highest, which provided abundant fruit aroma for the samples. Followed by ethyl caproate (apple aroma) and γ -nonanolactone (peach aroma), the concentration of the γ -nonanolactone reached the lowest at C2 period, but then increased gradually with the extension of fermentation time.

Aldehydes and ketones could make the aroma incline to be soft and harmonious. Most aldehydes were produced by deamination and decarboxylation of amino acids (Wang et al., 2020). Phenylacetaldehyde had the highest content and it increased first and then decreased continuously during fermentation. However, due to its low threshold concentration, the OAV value still reached 1006 at C5 stage, which endowed the samples with sweet and honey aroma (Qian & Reineccius, 2002). Benzaldehyde with bitter almond flavor was an important aroma compound, which fluctuated but generally increased during fermentation, and reached the highest content at C5 stage. Studies have shown that benzaldehyde mainly came from the oxidation of benzyl alcohol and the metabolic synthesis of various microorganisms using aromatic amino acids (Genovese, Gambuti, Piombino, & Moio, 2007).

Acids are important components contributing to the aroma and taste of Chinese rice wine and they can be produced by the yeasts during fermentation (Ugliano & Moio, 2005). Among them, the content of acetic acid was the highest, and it increased at first and then decreased slightly. Its content reached the highest (19.77 mg/kg) at the C4 stage. Acetic acid strengthened the strong feeling of the wine body and reacted with the corresponding alcohols to esters (Xu et al., 2018). Therefore, acetic acid was an important compound to reconcile the taste and aroma of traditional Huangjiu, which also corresponded to the saying “no acid, no taste”. The content of propionic acid and isobutyric acid followed, which increased during fermentation while declined at the middle of the fermentation. Among the phenols detected, the content of 4-vinyl-2-methoxyphenol was the highest, followed by 4-ethylphenol and 4-ethyl-2-methoxyphenol. The compound 4-ethylphenol with smoke aroma showed an increasing trend during fermentation, which was described as medicinal aroma in Guyue Longshan rice wine (Chen et al., 2013). The compounds 4-ethyl-2-methoxyphenol (OAV: 13–48) and 4-vinyl-2-methoxyphenol (348–642) had higher OAV values, indicating they contributed greatly to the aroma of traditional Huangjiu. With the extension of fermentation time, these substances increased first

bacteria according to the results of alpha diversity analysis during fermentation.

PCoA analysis can be used to study the similarity or difference of sample community composition. Based on the weighted Unifrac distance algorithm, the microbial community structure of the samples during fermentation was analyzed by PCoA (Fig. S1). At the bacterial and fungal level, respectively, the difference between different fermentation time points was obvious, indicating that there were significant indigenous differences in bacterial and fungal community structure among these samples ($p < 0.05$).

3.4. Microbial profile and core microbiota of traditional Huangjiu

To better understand the predominate microbiota composition in the samples during fermentation, LefSe was applied to identify bacterial and fungal taxa that had significantly different effects on sample division. For bacteria composition (Fig. 3A and B), 71 bacterial clades were observed with LDA scores equal to or above 3.0, which consisted of 4 phyla, 5 classes, 15 orders, 20 families, and 27 genera. The evolutionary relationship of the 71 important bacteria is shown in Fig. 3A. According to Fig. 3B, *Actinobacteriota* and *Firmicutes* were the predominant phyla across the entire fermentation process. At genera level, *Saccharopolyspora*, *Lactobacillus*, *Carnobacterium*, *Pediococcus*, and *Staphylococcus* were found to be the most abundant at C1, C2, C3, C4 and C5 period, respectively.

For fungi composition (Fig. 3C and D), 21 fungal clades were found with LDA scores equal or > 3.0 , indicating those were the most important fungal clades to distinguish the samples. *Aspergillus* and *Rhizomucor* were the predominant genera across the entire fermentation process. Samples with at least three differentially abundant fungal clades were day 5 and day 72 samples (Fig. 3D), which suggested that fungal taxa at these two fermentation time points were most likely to explain the differences between the samples. Overall, samples during fermentation had

more diversified bacterial communities than fungal communities (71 vs. 21 clades).

3.5. Dynamics of microbial community in traditional Huangjiu during fermentation

As shown in Fig. 4A, *Actinobacteriota* (49.01%–68.81%), *Firmicutes* (21.04%–38.22%), *Cyanobacteria* (7.03%–13.30%) and *Proteobacteria* (2.89%–5.13%) are major bacterial phyla found in the fermentation process of traditional *Huangjiu* samples with relative abundance $> 1\%$. Levels of *Firmicutes* increased over the period of fermentation, whereas the abundance of *Actinobacteriota* decreased. Additionally, the abundance of *Cyanobacteria* rapidly increased during the first 22 days of fermentation but declined as the fermentation progressed. And the *Proteobacteria* showed an upward trend, but decreased slightly in the middle and late stages of fermentation. At the genus level (Fig. 4B), a total of 13 dominant bacteria were observed, the genus with relative abundance $> 0.1\%$ were *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, *Pediococcus*, *Weissella*, *Streptomyces*, *Pantoea*, *Kocuria*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pseudomonas* and *Chryseobacterium*. This result was generally in agreement with those in Shaoxing mechanized rice wine (Liu et al., 2019; Liu et al., 2015). The absolute dominant genus was *Saccharopolyspora* that accounted for 47.28%–67.71% while slowly decreased with increasing fermentation time. *Saccharopolyspora* was a safe biofunctional bacterium and produced important active substances, such as enzymes, antibiotics and vitamins (Liu et al., 2015). However, most of the initially major genera increased rapidly during the first 22 day of fermentation. Specifically, *Lactobacillus*, *Streptomyces*, *Pantoea*, *Lactococcus* and *Leuconostoc* increased from 14.25%, 0.09%, 0.21%, 0.11% and 0.11% to 15.43%, 1.32%, 0.35%, 0.30% and 0.26%, respectively. In the meantime, these genus started to decline after 22 days and increased from 45 days until the end of fermentation. *Lactobacillus* was a facultative anaerobic acid-tolerant bacteria, which had a

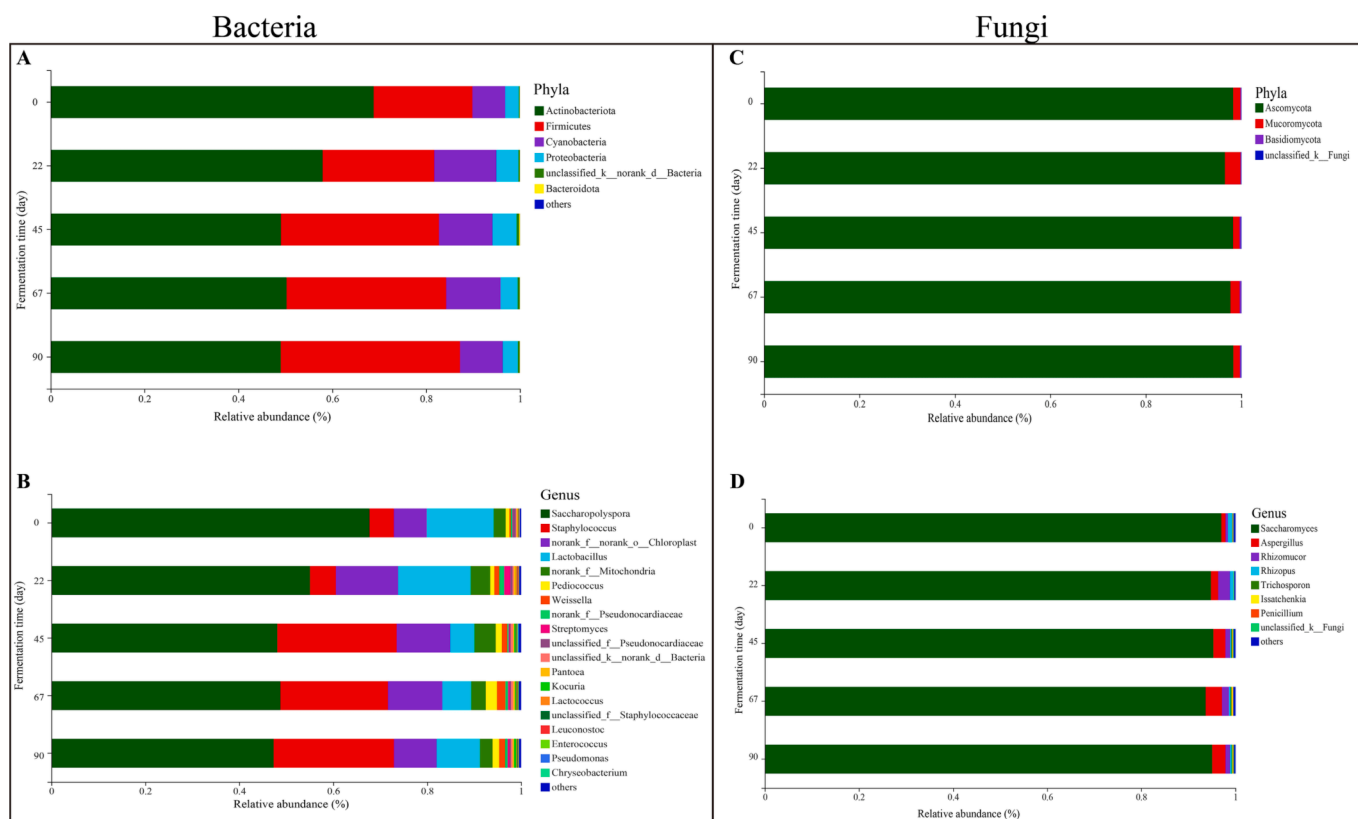


Fig. 4. Relative abundance of microbial at phylum (A and C) and genus level (B and D) in traditional *Huangjiu* samples. (Phylum and genus with relative abundance $< 0.1\%$ were classified as others).

good growth under low oxygen and high acid environment (Zhao et al., 2020). And lactic acid and bacteriocins generated by *Lactobacillus* played a role in inhibiting the spoilage microorganisms (Cappello, Zapparoli, Logrieco, & Bartowsky, 2017). *Pantoea* was a common endophytic bacterium in rice, which widely existed in fermented cereals (Fang, Dong, Chen, & Chen, 2015). The decreasing of *Pantoea* content at the middle stages of fermentation might be related to the enrichment of lactic acid bacteria. Moreover, the abundance of *Staphylococcus*, *Pediococcus*, *Weissella*, *Kocuria* and *Enterococcus* showed an overall upward trend. Among them, the abundance of *Staphylococcus* reached the highest of 25.63% by the end of fermentation. For *Lactococcus*, *Leuconostoc* and *Pseudomonas*, they increased at first and then decreased. *Leuconostoc* generally dominates in the initial stage of fermentation before being succeeded by more acid-resistant *Lactobacillus*, which was consistent with the variation trend of *Lactobacillus*.

Fungi have strong ability to secrete a variety of enzymes, which is conducive to the fermentation of fermented food. As shown in Fig. 4C and D, a total of 4 phyla and 76 genera were identified across all samples. At the phylum level, the fungal community was mainly dominated by *Ascomycota* (96.60%–98.33%) across the whole fermentation process, whereas *Mucoromycota* (1.36%–3.24%) and *Basidiomycota* (0.09%–0.32%) accounted for only a small portion. With regards to genus level, eight fungal genera were detected with relative abundance > 0.1%. *Saccharomyces* (97.03%) was the most abundant genera, followed by *Aspergillus* (0.90%), *Rhizopus* (0.89%) and *Rhizomucor* (0.54%) at the initial stage of fermentation, whereas at the end of fermentation was mainly dominated by *Saccharomyces* (95.09%), *Aspergillus* (2.82%), *Rhizomucor* (1.00%) and *Rhizopus* (0.33%). *Saccharomyces*, *Rhizopus*, and *Aspergillus* were also determined as the core functional microorganisms during Wuyi Hongqu Huangjiu fermentation (Huang et al., 2018). During fermentation, *Saccharomyces* dominated the entire fermentation process. The abundance of *Aspergillus* and *Issatchenkia* increased during the first 67 days of fermentation but declined at the end of fermentation. *Aspergillus* could secrete amylase, protease, peptidase and other enzymes into the environment, which promoted the hydrolysis of residual starch in post-fermentation (Yang et al., 2020) and offered precursors for the synthesis of flavor compounds (Chang et al., 2015). However, the levels of *Rhizopus* generally decreased, and remained at a relatively stable level at the late stage of fermentation. Adequate nutrition favored the formation of fungal communities at the early fermentation while gradually decreased as nutrients expended. *Rhizopus* contributed to saccharification process of Chinese rice wine and could produce some flavor substances, such as ethyl acetate, ethyl lactate, isobutanol, isoamyl alcohol, acetaldehyde, and so on (Londoño-Hernández et al., 2017; Lücke, Fritz, Tannhäuser, & Arya, 2019). During the first 22 days of fermentation, *Rhizomucor* increased quickly but decreased and then remained relatively stable at the middle and late stages of fermentation. Among them, the relative abundance of *Rhizomucor* reached the highest (2.53%) at fermentation periods of 22 days, while that of *Aspergillus* (3.46%), *Trichosporon* (0.24%) and *Issatchenkia* (0.21%) reached the highest on day 67. In addition, the trend of *Trichosporon* and *Penicillium* was similar that decreased at the initial stage of fermentation while increased after 22 days of fermentation.

To summarize, in the fermentation process of traditional Huangjiu produced around Winter Solstice, *Actinobacteriota*, *Firmicutes*, *Cyanobacteria* and *Proteobacteria* were the dominant bacteria at phyla while *Saccharopolyspora*, *Staphylococcus* and *Lactobacillus* were the dominant genus of bacteria. *Saccharomyces*, *Aspergillus*, *Rhizomucor* and *Ascomycota* dominated the fungal community. The greater microbial diversity and complex microbial interactions directly affect the production of flavor compounds (Tian et al., 2022). In this study, winter brewing was beneficial to the growth of low temperature microorganisms (yeast and lactic acid bacteria, etc.), and then promoted the good flavor of Huangjiu in the slow fermentation process.

3.6. Correlations among microbial communities in traditional Huangjiu during fermentation

The interaction between microorganisms is one of the important factors influencing the structure of microbial (Zhao et al., 2020). To identify the relationships between different microbial genera in the microbial community, correlation networks between dominant bacteria and fungi were constructed using Pearson's correlation coefficients and *p* value (Fig. 5). The results of correlation among different bacterial genera showed that *Saccharopolyspora* was positively correlated with *Lactobacillus*, ($|r| > 0.6$, $p < 0.05$) whereas negatively correlated with *Pediococcus*, *Enterococcus*, *Kocuria*, *Staphylococcus* and *Weissella*. And *Staphylococcus* was positively correlated with *Kocuria*, *Enterococcus*, *Weissella*, *Pediococcus*, *Lactococcus*, *Leuconostoc* and *Pantoea*, but negatively correlated with *Leuconostoc* and *Lactobacillus*. Furthermore, *Lactobacillus* was positively correlated with *Leuconostoc*, *Lactococcus* and *Streptomyces*, whereas negatively correlated with *Weissella*, *Pediococcus* and *Enterococcus*. For the fungi, *Saccharomyces* was positively correlated with *Rhizopus*, but negatively correlated with *Aspergillus*, *Issatchenkia* and *Trichosporon*. *Aspergillus* may not be able to adapt to the fermentation environment of high ethanol concentration and acidity caused by *Saccharomyces* (Tian et al., 2022). *Aspergillus* was positively correlated with *Issatchenkia* and *Trichosporon*, whereas negatively correlated with *Rhizopus*. In addition, *Saccharopolyspora* was positively correlated with *Rhizopus* and *Saccharomyces*, but negatively correlated with *Aspergillus*, *Issatchenkia* and *Trichosporon*. Overall, the stronger connection nodes (≥ 10 edges) were mostly distributed in *Lactobacillus*, *Saccharopolyspora*, *Staphylococcus*, *Weissella*, *Aspergillus*, *Rhizopus*, *Trichosporon*, *Pediococcus*, *Issatchenkia*, *Enterococcus* and *Saccharomyces*. The microbes could be mutually coordinated and restricted during fermentation, which had a significant contribution to improving the flavor of Chinese rice wine (Liang et al., 2020).

3.7. Correlations between microorganisms and volatile flavor compounds during fermentation

The Pearson correlation coefficients between 21 kinds of aroma compounds with OAV > 1 and dominant genera including 6 kinds of bacteria and 3 kinds of fungi (relative abundance > 1%) were calculated, and chose the coefficient $|r| > 0.6$ and significance at $p < 0.05$ as indicating strongly correlated nodes of the network (Fig. 6). Six bacterial and three fungal genera chosen were strongly positively correlated ($p < 0.05$) with 18 kinds of characteristic volatile flavor compounds (Fig. 6A), indicating the significance of these nine genera in flavor formation during traditional Huangjiu fermentation. Among them, *Saccharomyces*, the fungi with the highest relative abundance, had a significantly positive correlation with 2-ethylhexanol, phenylacetaldehyde, 3-methylthiopropyl, phenylethanol, nonanal, and phenylethyl acetate. There was also a positive correlation between *Saccharomyces* and phenylethanol in Chinese rice wine (Zheng et al., 2020). *Aspergillus* was positively correlated with ethyl laurate, ethyl butyrate, ethyl palmitate and γ -nonolactone, while *Rhizopus* and *Streptomyces* significantly and positively correlated with the formation of 4-ethyl-2-methoxyphenol alone. The main enzyme producing microorganisms in wheat Qu were *Aspergillus* and *Rhizopus* (Liu et al., 2020), and *Aspergillus* was involved in the production of esterase (Xu et al., 2016). The compound 4-ethyl-2-methoxyphenol with herbal and warm spicy taste has been identified in wines and could be related to various bacterial genera in millet Chinese rice wine (Yan et al., 2022). In bacterial genera, *Saccharopolyspora* had significant positive correlation with 2-ethylhexanol, phenylacetaldehyde, 3-methylthiopropyl, phenylethanol, phenylethyl acetate and isoamyl acetate. The formation of acetate esters was related to the catalytic enzymes present in *Saccharopolyspora* (Liu et al., 2019) and *Saccharopolyspora* was also identified as the dominant bacteria in Shaoxing mechanized rice wine and Moutai starter (Gan et al., 2019). Furthermore, *Staphylococcus* was

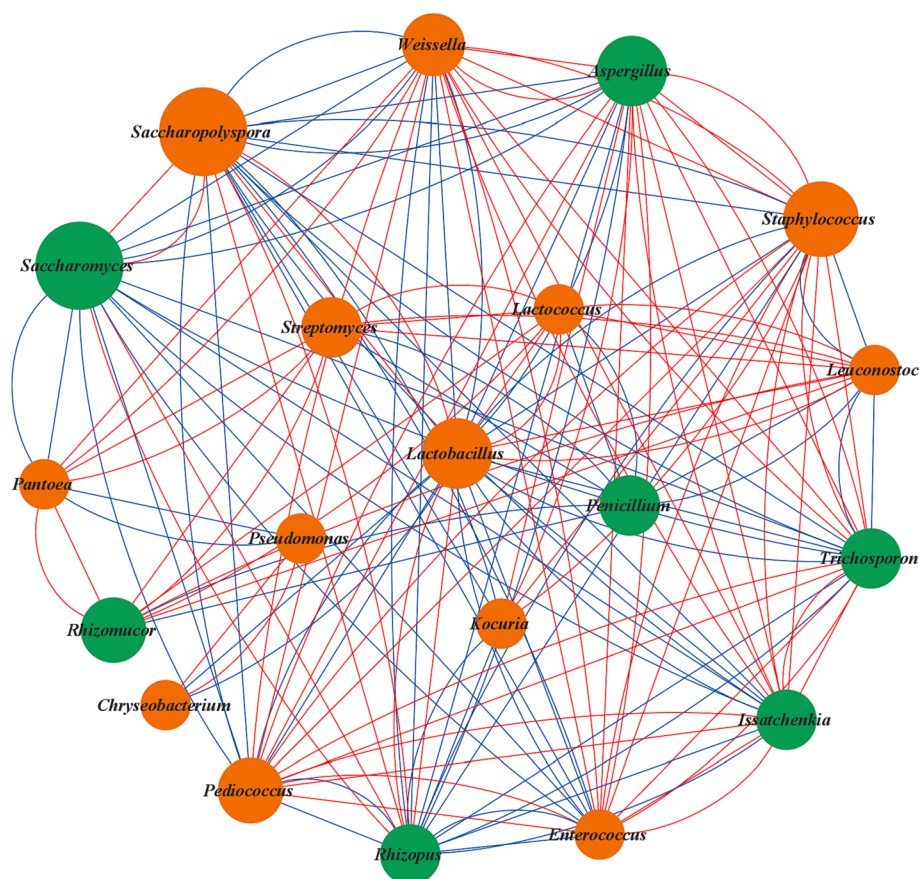


Fig. 5. Association network diagram of fungi and bacteria. The orange and green circles refer to bacteria and fungi, respectively, and the red and blue lines refer to positive correlations and negative correlations, respectively ($|r| > 0.6$, $p < 0.05$).

significantly positively correlated with ethyl butyrate, ethyl laurate, γ -nonlactone, ethyl decanoate and 4-vinyl-2-methoxyphenol. Similar to *Saccharopolyspora*, *Lactobacillus* was also positively correlated with isoamyl acetate, phenylethyl acetate and 3-methylthiopropanol. Moreover, *Pediococcus* and *Weissella* played a positive role in the formation of ethyl laurate and ethyl palmitate, which were consistent with those of Chen et al. (2020). And the *Weissella* was also positively correlated with ethyl butyrate. According to reports, *Weissella* can increase the contents of esters and organic acids during fermentation (Hu et al., 2021). Overall, five genera (*Saccharomyces*, *Aspergillus*, *Saccharopolyspora*, *Staphylococcus* and *Lactobacillus*) were the core functional microorganisms during traditional *Huangjiu* fermentation, which were consistent with those in Shaoxing mechanized rice wine that these genera were most closely related to the synthesis of esters, acids and alcohols (Liu et al., 2019).

Compared to the positive effect, a total of 7 genera showed negative correlations with one or more volatiles of 10 volatiles (Fig. 6B), in which of them there were at least 3 genera correlated with 3-methylbutanol, 2-ethylhexanol, phenylethanol, phenylacetaldehyde, 3-methylthio propanol, ethyl laurate, isoamyl acetate, phenylethyl acetate and the negative effects mainly occurred in 4 genera including *Weissella*, *Aspergillus*, *Staphylococcus* and *Pediococcus*.

4. Conclusions

The dynamic of aroma compounds and microbial communities during traditional *Huangjiu* fermentation around Winter Solstice were studied. The results showed that there were distinct differences in aroma profiles and microbial community among the samples. With increasing fermentation time, alcohols and aldehydes mostly decreased while

esters changed inversely, and the genera *Saccharomyces*, *Aspergillus*, *Staphylococcus* and *Pediococcus* dominated during fermentation. This study found that 11 genera may play a crucial role in maintaining the balance of the complex ecosystem in traditional *Huangjiu*. Furthermore, the correlation network indicated that five core functional microorganisms contributed greatly to the formation of most characteristic aroma compounds. Our study proved that winter brewing was beneficial to the growth of the core microorganisms, promoting the accumulation of characteristic compounds responsible for the harmonious aroma of traditional *Huangjiu*. The comprehensive research provided a theoretical guidance for the flavor regulation of traditional *Huangjiu* via microbial community level and microbial augmentation. However, the exact contributions of the core microorganisms are still unclear, and more in-depth studies are required to explore the specific metabolic pathways.

CRediT authorship contribution statement

Haiyan Yu: Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing. **Qiaowei Li:** Methodology, Formal analysis, Investigation, Writing – original draft. **Wei Guo:** Methodology, Formal analysis, Investigation, Writing – original draft. **Chen Chen:** Methodology. **Lianzhong Ai:** Resources, Supervision. **Huaxiang Tian:** Writing – review & editing, Supervision.

Declaration of Competing Interest

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.

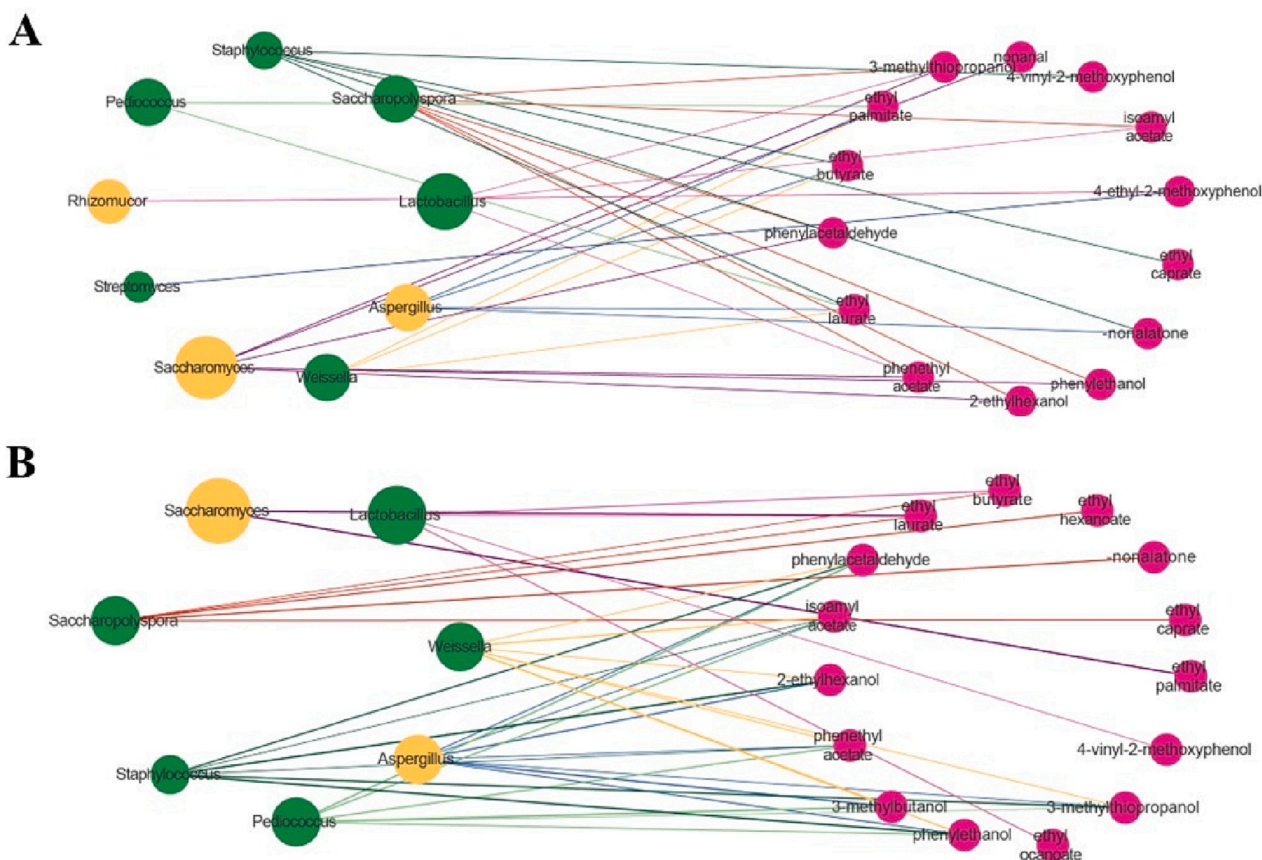


Fig. 6. Positive (A) and negative (B) network of the relationships between dominant genera and characteristic aroma compounds (OAV > 1) of traditional *Huangjiu* samples based on Pearson's correlation ($|r| > 0.6, p < 0.05$). The green, orange and pink nodes represent bacterial genera, fungal genera and characteristic aroma compounds, respectively.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2023.100620>.

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