Comparative analysis of microbial communities and volatile flavor components in the brewing of *Hongqu* rice wines fermented with different starters

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ABSTRACT

As one of the quintessential representatives of Chinese rice wine, *Hongqu* rice wine is brewed with glutinous rice as the main raw material and *Hongqu* (Gutian *Qu* or Wuyi *Qu*) as the fermentation starter. The present study aimed to investigate the impact of *Hongqu* on the volatile compositions and the microbial communities in the traditional production of Gutian *Hongqu* rice wine (GT) and Wuyi *Hongqu* rice wine (WY). Through the OPLS-DA analysis, 3-methylbutan-1-ol, isobutanol, ethyl lactate, ethyl acetate, octanoic acid, diethyl succinate, phenyl-ethyl alcohol, hexanoic acid and n-decanoic acid were identified as the characteristic volatile flavor components between GT and WY. Microbiome analysis revealed significant enrichments of *Lactobacillus*, *Pediococcus*, *Aspergillus* and *Hyphopichia* in WY brewing, whereas *Monascus*, *Saccharomyces*, *Pantoea*, and *Burkholderia-Cab-alleronia-Paraburkholderia* were significantly enriched in GT brewing. Additionally, correlation analysis showed that *Saccharomyces*, *Lactobacillus*, *Weissella* and *Pediococcus* were significantly positively correlated wih most characteristic volatile components. Conversely, *Picha*, *Monascus*, *Franconibacter* and *Kosakonia* showed significant negative correlations with most of the characteristic volatile components. Furthermore, bioinformatical analysis indicated that the gene abundances for enzymes including glucan 1,4-alpha-glucosidase, carboxylesterase, alcohol dehydrogenase, dihydroxy-acid dehydratase and branched-chain-amino-acid transaminase were significantly higher in WY compared to GT. This finding explains the higher content of higher alcohols and characteristic esters in WY relative to GT. Collectively, this study provides a theoretical basis for improving the flavor profile of *Hongqu* rice wine and establishing a solid scientific foundation for the sustainable development of *Hongqu* rice wine industry.

1. Introduction

As a cultural treasure of the Chinese nation, Chinese rice wine is regarded as one of the three ancient wines in the world, and plays an important role in the daily life of Chinese people. According to the different fermentation starter, Chinese rice wine is mainly divided into *Hongqu* rice wine and *Maiqu* rice wine (Liu et al., 2018; Peng et al., 2022, 2023; Xie et al., 2021). *Hongqu* rice wine, a famous and popular traditional fermented alcoholic beverage in Southeast China, is made with glutinous rice and *Hongqu*, which has great influence on the formation of the flavor quality of *Hongqu* rice wine.

As the fermentation starter for *Hongqu* rice wine, traditional *Hongqu* is usually prepared from steamed rice by solid-state fermentation in a non-sterile environment based on empirical knowledge, with the inoculation of starter cultures (mainly *Monascus* spp. and *Aspergillus* spp.) (Zhou et al., 2021). At present, there are two kinds of commonly used fermentation starter for *Hongqu* rice wine brewing, namely Gutian *Qu* and Wuyi *Qu*. The former is produced by inoculating starter cultures of *Qumu* (rich in *Monascus* spp.), the latter is made by a mixed inoculation with *Qumu* and *Quqing* (rich in *Monascus* spp. and *Aspergillus* spp.). Our

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previous study proved that the microbial composition of Gutian *Qu* and Wuyi *Qu* were significant differences (Liu et al., 2018). Microbial composition of traditional fermentation starter has a strong influence on the flavor and quality of rice wine. Compared to the *Hongqu* rice wine made with Gutian *Qu*, Wuyi *Hongqu* rice wine (fermented by Wuyi *Qu*) contained a higher content of bitter and umami amino acids (Liang et al., 2020). According to research findings, the characteristic microbial community (including various molds, yeasts and bacteria) and their metabolic functions in the whole traditional brewing process were closely related to the flavor quality of *Hongqu* rice wine (Ren et al., 2019, 2020). However, the differences in brewing microbiota, volatile flavor components and fermentation parameters between Wuyi *Hongqu* rice wine (WY) and Gutian *Hongqu* rice wine (GT) have not been studied comparatively. The influence of microbiota on the formation of characteristic flavor and potential hazard of *Hongqu* rice wine deserve further exploration, in order to achieve the directional improvement of the flavor quality.

As a major type of volatile flavor components, the content of higher alcohols has a significant effect on the flavor of *Hongqu* rice wine. Higher alcohols, also known as fusel oil, are the main by-products of wine fermentation, one of the main aroma substances of wine, and also the precursors for the formation of some ester aroma compounds. The higher alcohols in rice wine are mainly consist of n-propanol, iso-propanol, n-butanol, isobutanol, isoamyl alcohol, benzyl alcohol, phenylethanol, *etc*. (Zhang et al., 2015; Ren et al., 2016). It was found that proper amount of higher alcohols would give rice wine unique mellow flavor and harmonious taste. However, excessive amounts of higher alcohols can produce a strong taste of fusel oil, make the drinkers suffer from uncomfortable symptoms such as "dizziness" and "hangover" (Valero et al., 2002; Yang et al., 2014). Studies proved that higher alcohols in wine are mainly produced through two pathways: Ehrlich pathway and Harris pathway (Pires et al., 2014; Gonzalez and Morales, 2017). In alcoholic fermentation, higher alcohols are mainly produced by *Saccharomyces cerevisiae* (Ma et al., 2017; Li et al., 2018), in which alpha-ketoacids are decarboxylated and reduced by enzymes encoded by relevant genes in the cytoplasm and mitochondria to form higher alcohols. The effect of microbial community on the production of higher alcohols, i.e., which microorganisms can promote or inhibit the production of higher alcohols in *Hongqu* rice wine brewing, has not been reported so far.

In this study, we systematically compared the composition of volatile components and microbial communities in the traditional production of two representative *Hongqu* rice wines, Gutian *Hongqu* rice wine (GT) and Wuyi *Hongqu* rice wine (WY). Characteristic volatile flavor components in two representative *Hongqu* rice wine were identified through orthogonal PLS-DA analysis. The correlations between the predominant microbial genera and the characteristic volatile components were investigated and visualized through correlation heatmap. Furthermore, bioinformatical analysis based on PICRUSt was conducted to reveal the microbial enzymes related to the metabolism of characteristic volatiles in *Hongqu* rice wine production. This study would provide theoretical basis for improving the quality of *Hongqu* rice wine, and build a solid scientific foundation for the healthy and sustainable development of *Hongqu* rice wine industry.

2. Materials and methods

2.1. Hongqu rice wine brewing and sample collection

In this study, fermentation starters (including GTA, GTB and GTC) for Gutian *Hongqu* rice wine were purchased from Gutian County, Ningde City, Fujian Province, China; fermentation starters (including WYA, WYB and WYC) for Wuyi *Hongqu* rice wine were purchased from Jianou County, Nanping City, Fujian Province, China. Both of Gutian *Hongqu* rice wine and Wuyi *Hongqu* rice wine were brewed with glutinous rice according to the following steps: (1) Preparation: Firstly,

washed and soaked the glutinous rice overnight. Subsequently, steamed the soaked glutinous rice at 100 °C for 45 min, and cooled to room temperature. (2) Fermentation: put the steamed glutinous rice, cold water and *Hongqu* (at the ratio of 10 : 15 : 1) into the wine jar,which was wrapped with 8 layers of gauze, and fermented at 18 °C for 10 days. After the 10th day, the 8 layers of gauze replaced by sterile plastic bags for sealed anaerobic fermentation for 35 days. Fermented samples were collected from three parallel fermentation tanks at ten time points (day 1, 2, 3, 7, 10, 15, 20, 30, 45) during the brewing process, sealed in sterile sample vials for further testing. Subsequently, 10 g fermented sample was taken and put into 90 mL sterile saline and filtered with sterilized gauze after shaking with a vortex shaker for 30 s. The supernatant was collected after being centrifuged at 8000 r/min for 10 min, and then stored at −20 °C for physical and chemical analysis. The precipitate was acquired and immediately stored at −80 °C for full-length 16S/ITS amplicon sequencing analysis.

2.2. Determination of physicochemical parameter

The changes of several physicochemical properties, including alcohol content, total acid, reducing sugar and amino acid nitrogen, of the collected rice wine mash sample were determined. The total acid content and amino acid nitrogen was determined using the method described by a China official method (GB/T 13662-2018). And the determination of reducing sugar was conducted on the basis of 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The determination of alcohol content in mash samples of *Hongqu* rice wine was conducted according to our previous study (Chen et al., 2022) by GC (gas chromatography, 7890A, Agilent, USA) with FID (flame ionization detector) and HP-INNOWAX column (30.0 m × 0.25 mm × 0.25 μm, Agilent, USA). The analysis of the sample was made in triplicate.

2.3. Volatile profiles analyses

Head-space solid-phase microextraction (HS-SPME) combined with GC-MS (Agilent 7890-B/5977A) was employed for volatile compounds profiling. The sample treatment and instrument operation were performed according to our *previous study (*Huang et al., 2019*)* with some modifications. Briefly, sample supernatant (10 times diluted, 6 mL) was added into a 15-mL headspace glass vials with 2 g of sodium chloride and 10 μL internal standard (2-octanol, 10 mg/L). The 50/30 μm divinylbenzene/carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) fiber (2 cm, Supelco, Inc., Bellefonte, PA, USA) in a solid-phase microextraction (SPME) device (Supelco, Bellefonte, PA) was inserted into the 15-mL headspace glass vial, then the vial was equilibrated at 60 °C for 10 min and extracted at 60 °C for 45 min under stirring to extract volatile compounds. The volatile compounds were then desorbed at 250 °C into the GC inlet for 5 min with splitless mode. The GC-MS equipped with HP-INNOWAX capillary column (30.0 m × 0.25 mm × 0.25 μm, Agilent Technology, USA) was used to measure the volatile compounds of each sample. The oven temperature was programmed at 40 °C for 5 min, increased at 5 °C/min to 120 °C, ramped to 240 °C at a rate of 10 °C/min and held for 5 min at this final temperature. The post operating temperature was 250 °C, held for 5 min. The flow rate of carrier gas (helium) was 1 mL/min. The MS interface temperature was set at 280 °C, and the MS quadrupole was 150 °C. The electron ionization (EI) mass spectra mode was used at 70 eV ionization energy. And the temperature of the ion source was 230 °C. The chromatogram was recorded by monitoring the total ion currents in the m/z range of 35–450. Compare with the database of National Institute of Standards and Technology (NIST), record and identify volatile peaks. The analysis of the sample was made in triplicate. The contents of volatile flavor substances in the samples were calculated according to the following formula:

$$C = f \times \frac{m_1 \times A_2}{A_1 \times V}$$

f: the standard material correction factor
A_1: the peak area of internal standard substance
m_1: the weight of internal standard substance
A_2: the peak area of the substance in sample
V: the volume of sample

2.4. Microbial DNA extraction and 16S/ITS amplicon high-throughput sequencing

Total DNA was extracted from the precipitate according to the protocol of rapid DNA extraction kit (MN NucleoSpin 96 Soi, Germany). The extracted DNA was used as a template, and the V3–V4 region of bacterial 16S rDNA were amplified by 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806-R (5'-GGACTACHVGGGTWTCTAAT-3') (Xu et al., 2016). And ITS5-1737-F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2-2043-R (5'-GCTGCGTTC TTCATCGATGC-3') primers were used for internal transcribed spacers (ITS) rRNA amplification from the extracted DNA (Zhang et al., 2016). The PCR amplification conditions of bacteria were according to a previous study (Xu et al., 2016). And the conditions for PCR amplification of fungi refer to a previous study by Zhang et al. (2016). Then, the PCR products were used for the subsequent DNA library construction, and the two libraries were sequenced using Illumina HiSeq 2500 platform (Illumina, USA). After the sequencing data were spliced and filtered according to the overlapping relationship, the quality of the sequences was controlled and screened. Raw sequencing reads were quality-filtered and analyzed using FLASH software (v1.2.7) and QIIME software (v1.8.0). Operational taxonomic unit (OTU) clustering analysis was performed at sequence similarity of 97% based on the UPARSE algorithm using USEARCH software (Version 7.1). The bacterial OTU sequences were annotated using the SILVA/16S rDNA database (Quast et al., 2013) by a QIIME-based wrapper of the RDP-classifier (v.2.2) (Cole et al., 2009). Sequences of fungal ITS were clustered using the UNITE databaseby USEARCH (version 5.2.236) and aligned by the BLAST algorithm (Koljalg et al., 2013). The raw sequencing data of microbial community was deposited at NCBI with the accession number PRJNA1020104.

2.5. Statistical analysis and visualization

The GraphPad Prism 8.0.2 was used to visualize the content of physicochemical parameter. And the heatmap and bubble matrix package in the R software (v.3.5.1) were used to visualize the abundance of volatile components and functional genes, respectively. Additionally, SIMCA-14.1 (UMETRICS, Sweden) software was used for principal component analysis (PCA) to evaluate the variation trend of volatile components in brewing process. Subsequently, the correlation between volatile components and microbial taxa was calculated and visualized based on spearman correlation coefficients, by using R software with Psych, Reshape2 and Pheatmap package.

3. Results and discussion

3.1. Dynamics of physicochemical parameters in Hongqu rice wine brewing

Brewing physicochemical parameters including alcohol, reducing sugar, total acids and amino acid nitrogen are important indexes to estimate the final flavor quality of *Hongqu* rice wine. The changes of physicochemical parameters during fermentation are typically used to reflect the microbial fermentation state in the wine mash (Zhao et al., 2020). In order to explore the influence of fermentation starters on the traditional brewing process of *Hongqu* rice wine, the brewing physicochemical parameters of *Hongqu* rice wine fermented with six kinds of *Hongqu* (3 kinds of Gutian *Qu* and 3 kinds of Wuyi *Qu*) were tracked, and the results were shown in Fig. 1. Compared with the *Hongqu* rice wine
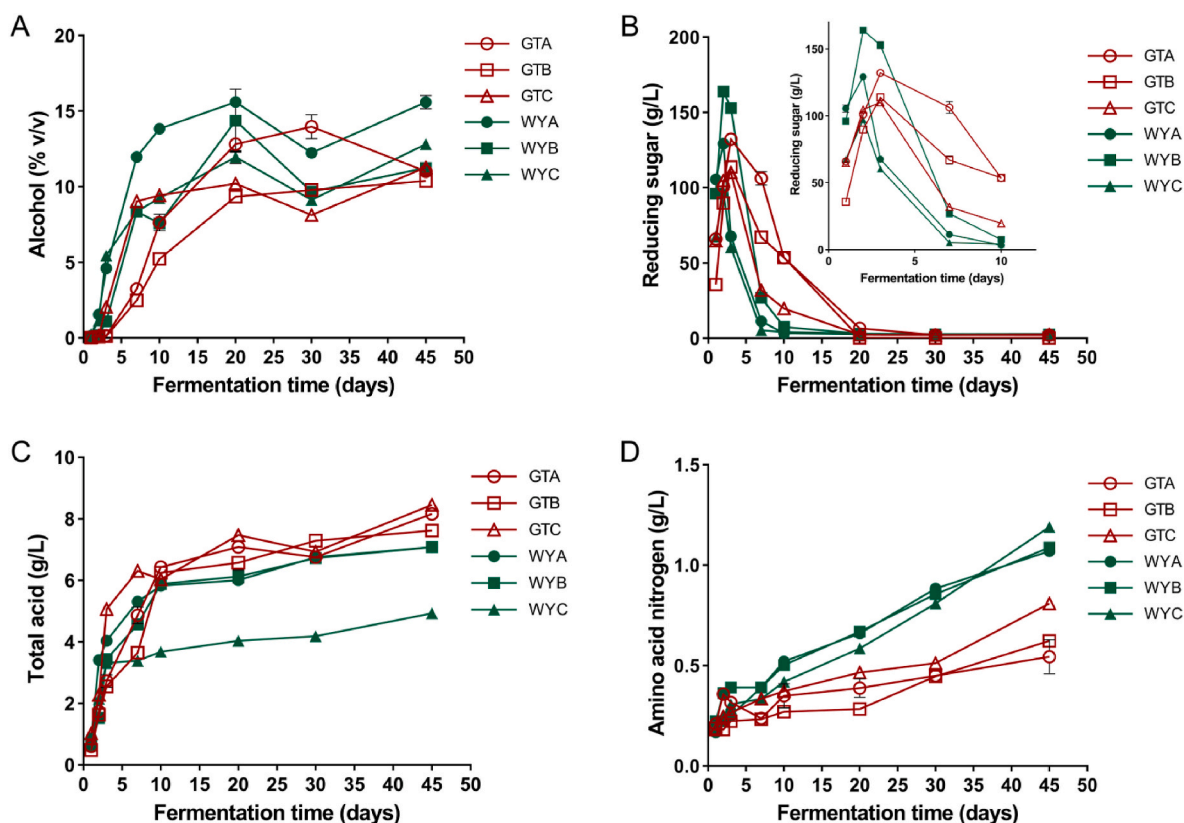

Fig. 1. The dynamic changes of ethanol (A), reducing sugar (B), total acids (C) and amino acid nitrogen (D) during the traditional brewing of *Hongqu* rice wine.

fermented by Gutian *Qu* (GTA, GTB and GTC), the alcohol content of the *Hongqu* rice wine fermented by Wuyi *Qu* (WYA, WYB and WYC) increased more rapidly during the fermentation process, especially in the early fermentation period (days 1–7). That's possibly because WY contained a large amount of *Aspergillus* at this stage, which can provide a higher content of reducing sugars for yeast to produce alcohol. However, the alcohol content of Wuyi *Hongqu* rice wine (WYA, WYB and WYC) showed an obvious downward trend on the 30th day, which possibly because that the yeast consumed a large amount of ethanol to produce ethyl ester compounds. In addition, due to nutrient consumption, ethanol stress, acid stress and other factors affecting the alcohol fermentation of yeasts (Longo et al., 2021), the alcohol content increased slowly and tended to be stable at the late stage of *Hongqu* rice wine fermentation. At 45th day, the alcohol content of WY (15.59 ± 0.44 %, 11.24 ± 0.13 % and 12.82 ± 0.07 % in WYA, WYB and WYC, respectively) was higher than that of GT (11.03 ± 0.09 %, 10.38 ± 0.14 % and 11.28 ± 0.22 % in GTA, GTB and GTC, respectively).

In the early stage of rice wine fermentation, the starch in raw material is rapidly transformed into small sugars such as glucose under the action of liquefaction enzymes and saccharifying enzymes produced by filamentous fungi (Deng et al., 2020), so that the content of reducing sugar increased rapidly and reached a peak in the early stage of *Hongqu* rice wine brewing. Noticeably, the production rates of reducing sugar in Wuyi *Hongqu* rice wine (WYA, WYB and WYC) brewing were faster than those in Gutian *Hongqu* rice wine (GTA, GTB and GTC) brewing. Subsequently, yeasts metabolized the reducing sugar to produce alcohol, and the lactic acid bacteria used the reducing sugar to produce lactic acid, so the reducing sugar content dropped sharply until it was almost exhausted. In terms of reducing sugar content, WYA, WYB and WYC reached the turning point (at 3rd day) faster than GTA, GTB and GTC (at 7th day), indicating that Wuyi *Qu* had stronger saccharification power and alcohol fermentation efficiency than Gutian *Qu*.

As for the content of total acid and amino acid nitrogen, both of them increased during *Hongqu* rice wine fermentation. It is worth noting that at the end of fermentation, the total acid content of *Hongqu* rice wine fermented by Gutian *Qu* (8.16 ± 0.00 g/L, 7.62 ± 0.00 g/L and 8.46 ± 0.10 g/L in GTA, GTB and GTC, respectively) was significantly higher than that of *Hongqu* rice wine fermented by Wuyi *Qu* (7.08 ± 0.00 g/L, 7.08 ± 0.00 g/L and 4.93 ± 0.00 g/L in WYA, WYB and WYC, respectively). A Previous study had shown that the total acid in rice wine is mainly produced by lactic acid bacteria, because most of them have strong alcohol tolerance and excellent ability to produce organic acids, and their abundance was proved to be positively correlated with the total acid content in rice wine (Wang et al., 2014; Xie et al., 2021). The content of amino acid nitrogen can reflect the change of amino acid content in brewing process to some extent. We found that WY contained more genes for enzymes related to amino acid synthesis (such as dihydroxy-acid dehydratase [EC 4.2.1.9] and branched-chain-amino acid transaminase [EC 2.6.1.42]), See Fig. 8 for details), resulting in a higher amino acid content in WY than in GT, so leaded to the amino acid nitrogen content of *Hongqu* rice wine fermented by Gutian *Qu* (0.54 ± 0.08 g/L, 0.62 ± 0.02 g/L and 0.81 ± 0.00 g/L in GTA, GTB and GTC, respectively) lower than that of *Hongqu* rice wine fermented by Wuyi *Qu* (1.07 ± 0.02 g/L, 1.09 ± 0.00 g/L and 1.19 ± 0.02 g/L in WYA, WYB and WYC, respectively). In general, the change trend of brewing physicochemical parameters during *Hongqu* rice wine fermentation is consistent with the results of Liu et al. (2020).

3.2. Dynamics of volatile flavor components in Hongqu rice wine brewing

Volatile components are one of the important factors affecting the flavor quality of *Hongqu* rice wine, and they are mainly produced in the semi-open fermentation process of *Hongqu* rice wine (Liu et al., 2020). In this study, a total of 81 volatile compounds were detected, including 28
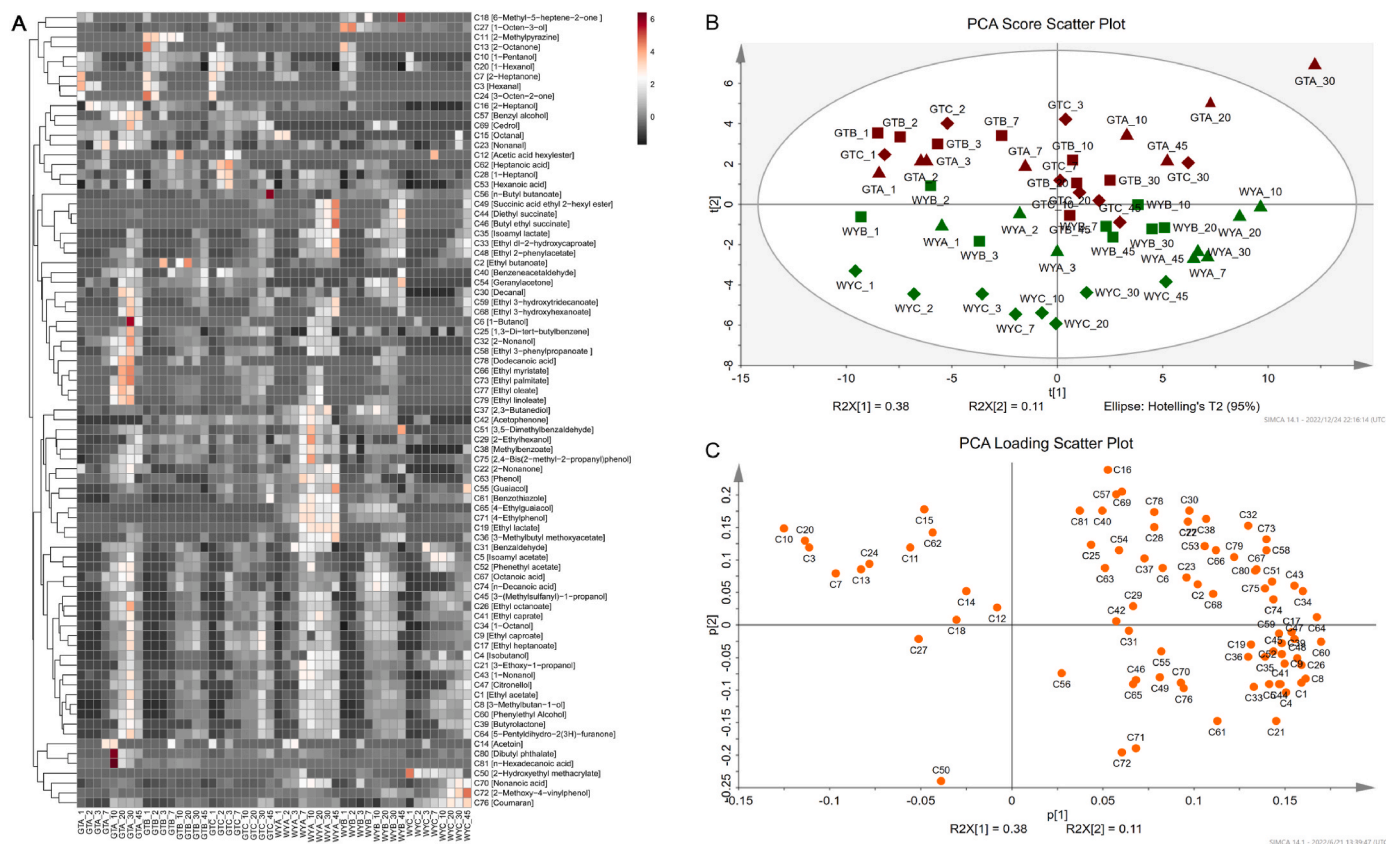

Fig. 2. The dynamic changes of volatile flavor components during the traditional brewing of *Hongqu* rice wine. (A) Heatmap, (B) PCA score plot, (C) PCA loading plot.

esters, 19 alcohols, 7 acids, 6 phenols, 7 aldehydes and 14 others (Fig. 2A). Among them, esters, alcohols and acids were the most abundant volatile compounds. The types and contents of volatile compounds in *Hongqu* rice wines fermented by Gutian *Qu* and Wuyi *Qu* increased gradually with the fermentation time (Fig. 2A). However, the composition of the volatile components produced during the brewing process differed significantly between Gutian *Hongqu* rice wine and Wuyi *Hongqu* rice wine. In addition, the score scatter plot and loading plot obtained from principal component analysis revealed that acetic acid hexylester (C12), octanal (C15), heptanoic acid (C62), 2-heptanol (C16), benzyl alcohol (C57), cedrol (C69), dodecanoic acid (C78), decanal (C30), ethyl myristate (C66), ethyl palmitate (C73), ethyl oleate (C77), ethyl linoleate (C79), dibutyl phthalate (C80) and so on were obviously enriched in Gutian *Hongqu* rice wine (Fig. 2B&C). Otherwise, succinic

acid ethyl 2-hexyl ester (C49), diethyl succinate (C44), butyl ethyl succinate (C46), isoamyl lactate (C35), ethyl dl-2-hydroxycaproate (C33), ethyl 2-phenylacetate (C48), guaiacol (C55), benzothiazole (C61), 4-ethylguaiacol (C65), 4-ethylphenol (C71), ethyl lactate (C19), benzaldehyde (C31), 2-hydroxyethyl methacrylate (C50), nonanoic acid (C70), 2-methoxy-4-vinylphenol (C72) and coumaran (C76) were obviously enriched in Wuyi *Hongqu* rice wine.

In order to further investigate the characteristic volatile flavour components in different types of *Hongqu* rice wine, multivariate statistical analyses of the volatile components in the *Hongqu* rice wine mash samples fermented by Gutian *Qu* and Wuyi *Qu* on the 45th day were carried out by using principal component analysis (PCA), PLS-DA method and OPLS-DA method. As shown in Fig. 3A–C, there were significant differences in the composition of volatile flavor components of
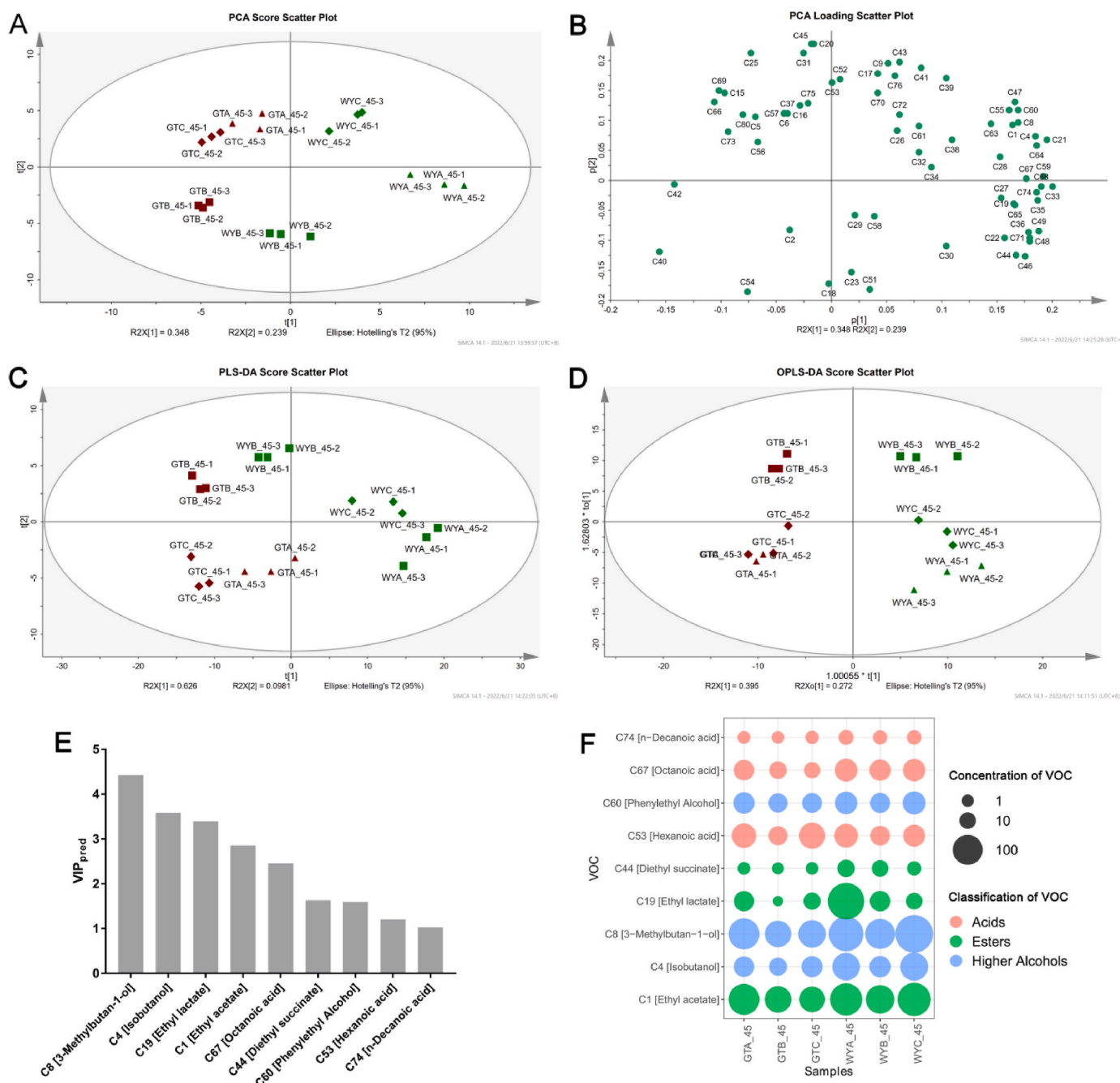

Fig. 3. The analysis of volatile compounds at the 45th day. (A) PCA score scatter plot, (B) PCA loading scatter plot, (C) PLS-DA score scatter plot, (D) OPLS-DA score scatter plot, (E) VIP$_{pred}$ value of differential aroma compounds and (F) bubble chart of differential aroma compounds.

Hongqu rice wines fermented by Gutian *Qu* and Wuyi *Qu*. The OPLS-DA results showed that there were nine volatile components with VIP$_{pred}$ values greater than 1, namely 3-methylbutan-1-ol (C8), isobutanol (C4), ethyl lactate (C19), ethyl acetate (C1), octanoic acid (C67), diethyl succinate (C44), phenylethyl alcohol (C60), hexanoic acid (C53) and n-decanoic acid (C74), which were the characteristic volatile flavour components between Gutian *Hongqu* rice wine and Wuyi *Hongqu* rice wine. It is well known that esters are the key characteristic flavor components in rice wine, which can contribute fruity, flowery and sweet odors to rice wine *(Hu* et al.*, 2018)*. Among them, ethyl acetate, which has rose, honey and fruit flavours, is the main key characteristic aroma substance in rice wine *(Kang* et al.*, 2016; Chen* et al.*, 2021a)*. In this study, we found that ethyl acetate was abundant in all six *Hongqu* rice wines, but Wuyi *Hongqu* rice wine (124.87 ± 6.56 g/L, 87.41 ± 1.15 g/L and 136.36 ± 7.03 g/L in WYA, WYB and WYC, respectively) contained significantly more ethyl acetate than Gutian *Hongqu* rice wine (111.75 ± 20.70 g/L, 69.19 ± 7.96 g/L and 61.44 ± 8.04 g/L in GTA, GTB and GTC, respectively). Diethyl succinate, which was more abundant in Wuyi *Hongqu* rice wine (13.57 ± 2.46 g/L, 10.72 ± 1.33 g/L and 4.19 ± 0.49 g/L in WYA, WYB and WYC, respectively), has been proven to be a key aroma compound *(Yang et al., 2017)* with fruity and sweet aroma characteristics *(Fan and Qian, 2006)*. As a major flavor component in rice-flavor baijiu *(Zhao et al., 2021)* and broomcorn millet *Huangjiu (Wang et al., 2022)*, ethyl lactate is a typical characteristic flavor substance during the fermentation of *Huangjiu* (Waterhouse et al., 2016), which can bring a creamy, buttery, fruity flavour to *Huangjiu* (Jin et al., 2021). In general, esters are important flavor compounds in rice wine, which largely determine the flavor characteristics of rice wine, such as fruit, candy and perfume (Hu et al., 2018). Our study found that the contents of diethyl succinate, ethyl lactate and ethyl acetate were significantly higher in Wuyi *Hongqu* rice wine than those in Gutian *Hongqu* rice wine.

In addition, 3-methylbutan-1-ol, isobutanol and phenylethyl alcohol, known as higher alcohol, are essential factors affecting the quality of rice wine *(Yuan et al., 2021)*, which are by-products of ethanol fermentation by *Saccharomyces cerevisiae* (Hazelwood et al., 2008; Lachenmeier et al., 2008). In this study, we found that the content of higher alcohols in Wuyi *Hongqu* rice wine was higher than those in Gutian *Hongqu* rice

wine, which may be because the strong saccharification ability of Wuyi *Qu* enabled *Saccharomyces cerevisiae* to produce more higher alcohols through Harris pathway (Pires et al., 2014; Gonzalez and Morales, 2017). Moverover, as important precursors of ethyl ester synthesis, fatty acids such as hexanoic acid, octanoic acid and n-decanoic acid are considered to have a rancid or unpleasant fatty odor (Wang et al., 2020), and if the content of these substances exceeds a certain threshold, it may lead to deterioration of rice wine flavor (Chen et al., 2021b). As shown in Fig. 4, there is little difference in the sensory evaluations of WY (WYA, WYB and WYC) and GT (GTA, GTB and GTC). But it is worth noting that GT is superior to WY in flower- and fruit-aroma; And its bitter and astringency are stronger than WY. In general, sensory evaluation results showed that GT has better sensory quality than WY.

3.3. Dynamics of microbial communities in Hongqu rice wine brewing

The traditional brewing process of *Hongqu* rice wine is actually a process of "co-fermentation of multiple microbes", which contains a variety of microorganisms in the brewing system. The dynamic change trends of the key bacterial genera in the brewing of *Hongqu* rice wines fermented with different starters (Gutian *Qu* and Wuyi *Qu*) were shown in Fig. 5, and the PCA analysis results of bacterial and fungal flora in the brewing of *Hongqu* rice wines fermented with different starters (Gutian *Qu* and Wuyi *Qu*) were shown in Fig. 6. It can be found from the PCA score scatter plots that the microbial community composition of different types of *Hongqu* rice wines was obviously different. In general, *Weissella*, *Kosakonia*, *Pantoe* and *Lactococcus* were the dominant bacterial genera in the brewing process of *Hongqu* rice wine. It should be noted that GTA contained more *Acinetobacter* than other *Hongqu* rice wines, especially on the first day, with a whopping proportion of 28.5%, which was consistent with our previous study (Chen et al., 2022). Besides, *Acinetobacter* has also been identified as the dominant bacterial genus in Hakka rice wine fermentation, and is strongly correlated with the production of characteristic flavor metabolites (Qian et al., 2023). It's well known that lactic acid bacteria (LAB) is a functional microorganism of fermented foods and plays a vital role in the process of food fermentation, such as improving the sensory quality and enhancing the nutritional value (Nionelli et al., 2014; Xie et al., 2021). During the
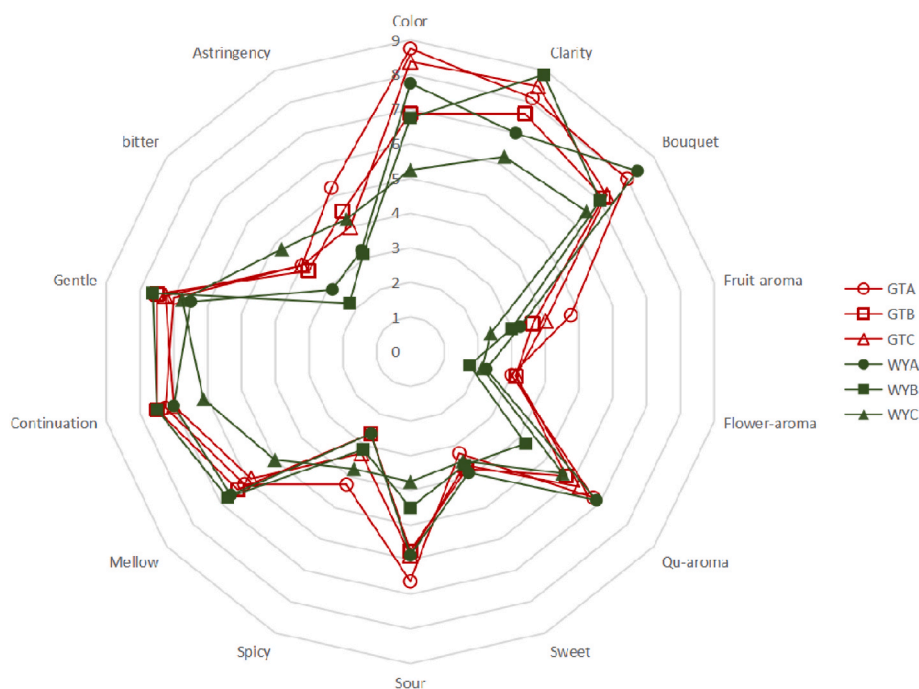

Fig. 4. Sensory profiles of *Hongqu* rice wines (GT and WY) fermented with different starters.
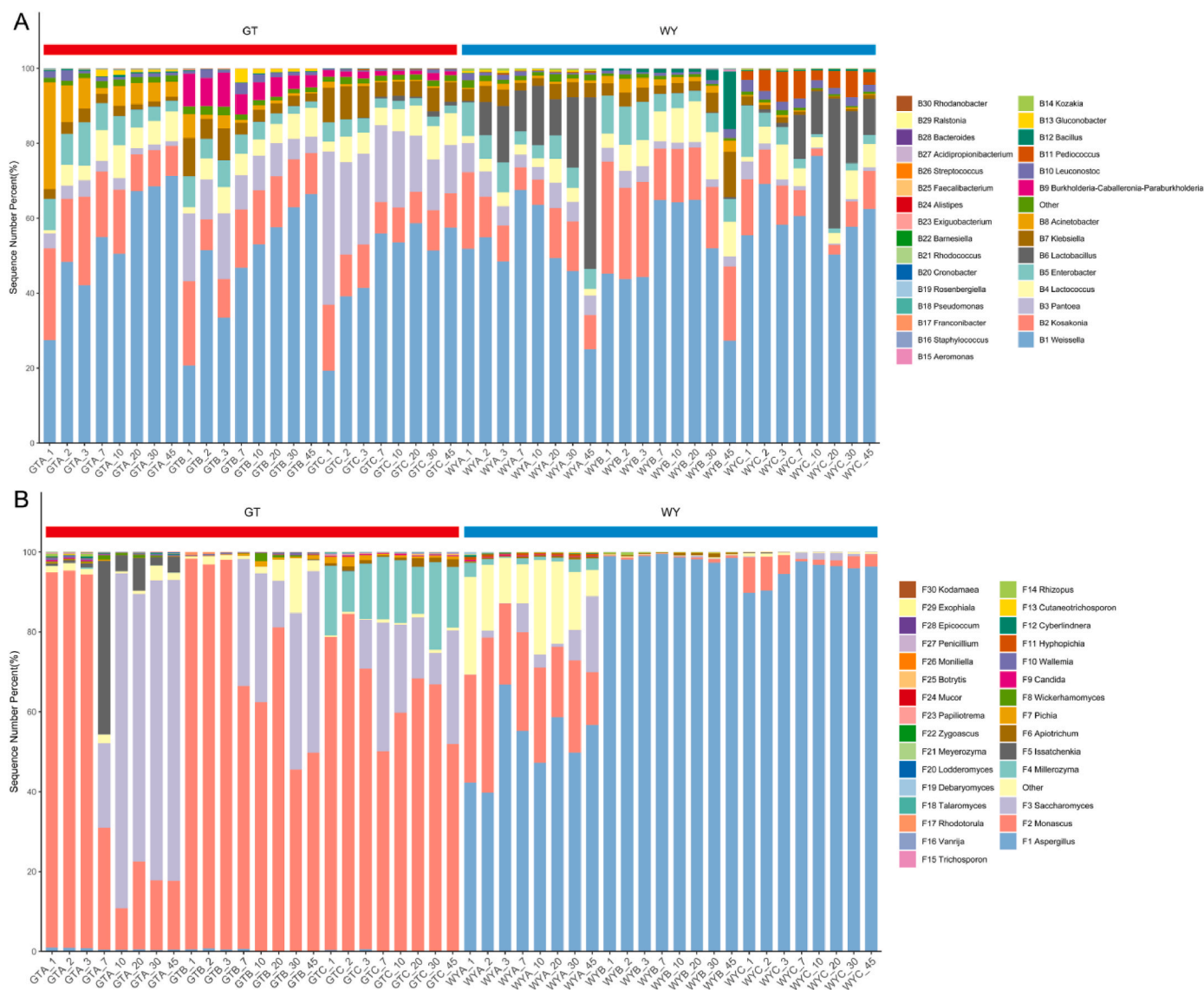
Fig. 5. Stacked histogram of the relative abundance of the predominant microbial phylotypes at the genus level. (A) bacterial genera. (B) fungi genera.

brewing process of Gutian *Hongqu* rice wine, we noticed that the abundance of *Weissella* gradually increased and dominated in the later stage. During the fermentation of WYA, the relative abundance of *Lactobacillus* gradually increased and became the dominant species on the 45th day. Different from WYA, the content of *Lactobacillus* in WYC showed a trend of increasing first, reaching a peak (34.7%) on the 20th day, and then showing a trend of decreasing. *Lactobacillus* gains the competitive edge due to high efficiency of fermentation catabolism and resistance to ethanol during the later period of the fermentation. The advantage of LAB in fermented foods is usually implicated to their ability of rapid environment acidification and tolerance of low pH (Abriouel et al., 2006), as well as inhibitory activity against a large number of microorganisms, especially pathogens and spoilage microorganisms due to producing bacterins in the brewing process (Cappello et al., 2017). Interestingly, WYC contains more *Pediococcus* during brewing compared with other *Hongqu* rice wines. It is worth noting that there was a large amount of *Bacillus* on 45th day of WYB fermentation, *Bacillus* was considered as the primary bacteria in the fermentation process of rice wine (Lv et al., 2015; Liu et al., 2018), due to the biosynthesis of a amount of enzymes related to nutrients metabolism, which can provide adequate supplies to other microorganisms, as well as these primary and secondary metabolites contribute to the formation of

rice wine flavor (Liu et al., 2018).

As shown in Figs. 5B and 6C, the dynamic succession process of fungal communities at the genus level during the brewing process of six kinds of *Hongqu* rice wine. It indicated that the structure of fungal floras in six *Hongqu* rice wines at genus level were significantly different. In general, *Monascus* and *Saccharomyces* were the main fungi in the brewing process of Gutian *Hongqu* rice wine. It was worth noting that the content of *Issatchenkia* in GTA showed a trend of first increase and then decrease, and reached the peak value (43.4%) on the 7th day, which was consistent with our previous study (Chen et al., 2022). In addition, GTC contained more *Millerozyma*, *Apiotrichum* and *Pichia*. The fungal flora structure of WYA was significantly different from that of the other two kinds of Wuyi *Hongqu* rice wine. Specifically, the main fungi of WYA were *Aspergillus*, *Monascus* and *Saccharomyces*, while the fungi of WYB and WYC are mainly *Aspergillus*. Especially in WYB, the relative abundance of *Aspergillus* was always greater than 97% in the brewing process. However, compared to WYB, WYC contains more *Monascus* and *Saccharomyces*. Previous studies suggested that *Saccharomyces* can use the sugar which produced by the saccharification of mold such as *Aspegillus* as raw material of its metabolism to produce higher alcohol. Due to the fact that Wuyi *Hongqu* rice wine (WY) contains more *Aspergillus* in the fermentation process, which conduced to the accelerated
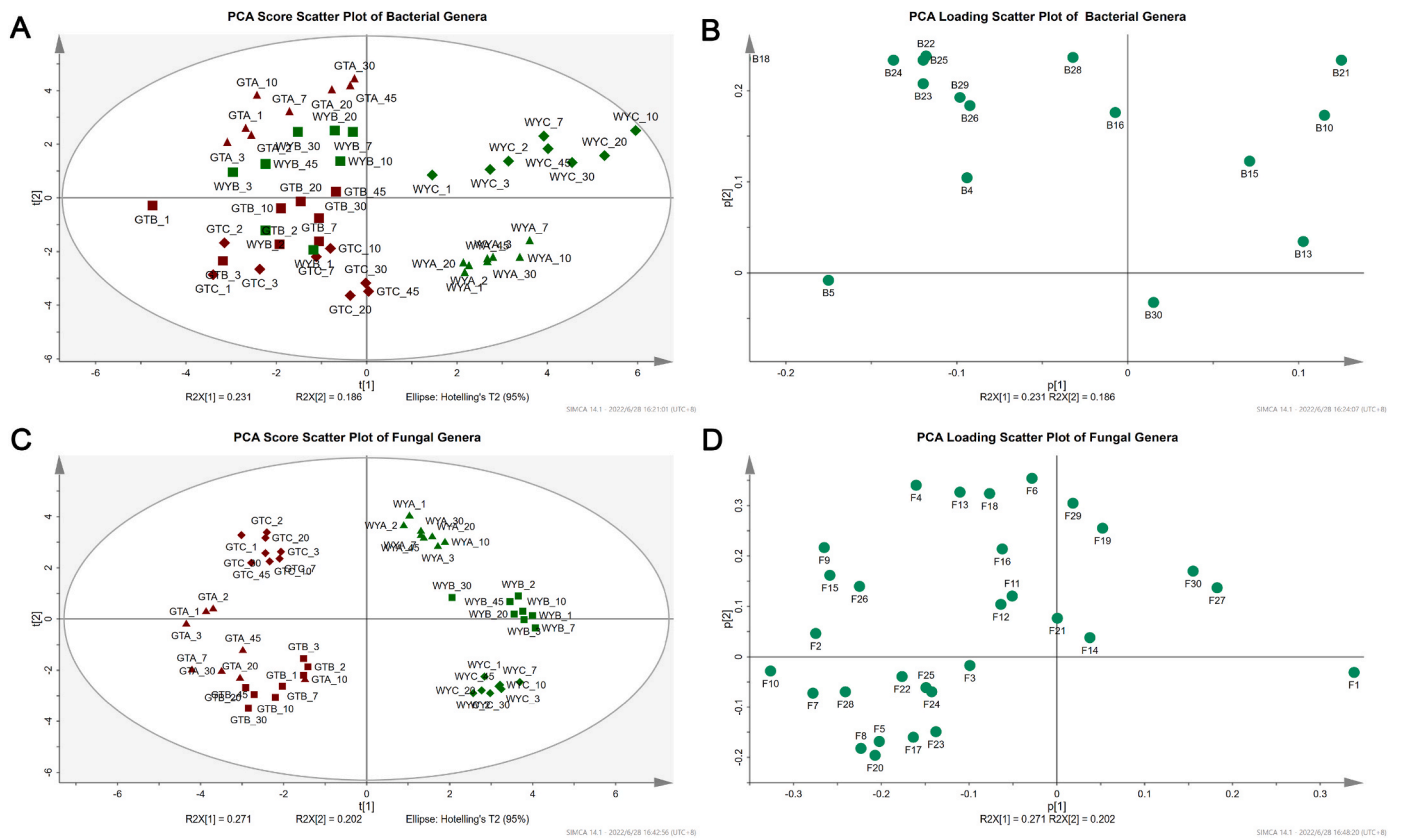
Fig. 6. PCA analysis of the relative abundance of the predominant microbial phylotypes at the genus level. (A) PCA score plot of the predominant bacterial community. (B) PCA loading plot of the predominant bacterial community. (C) PCA score plot of the predominant fungal community. (D) PCA loading plot of the predominant fungal community.
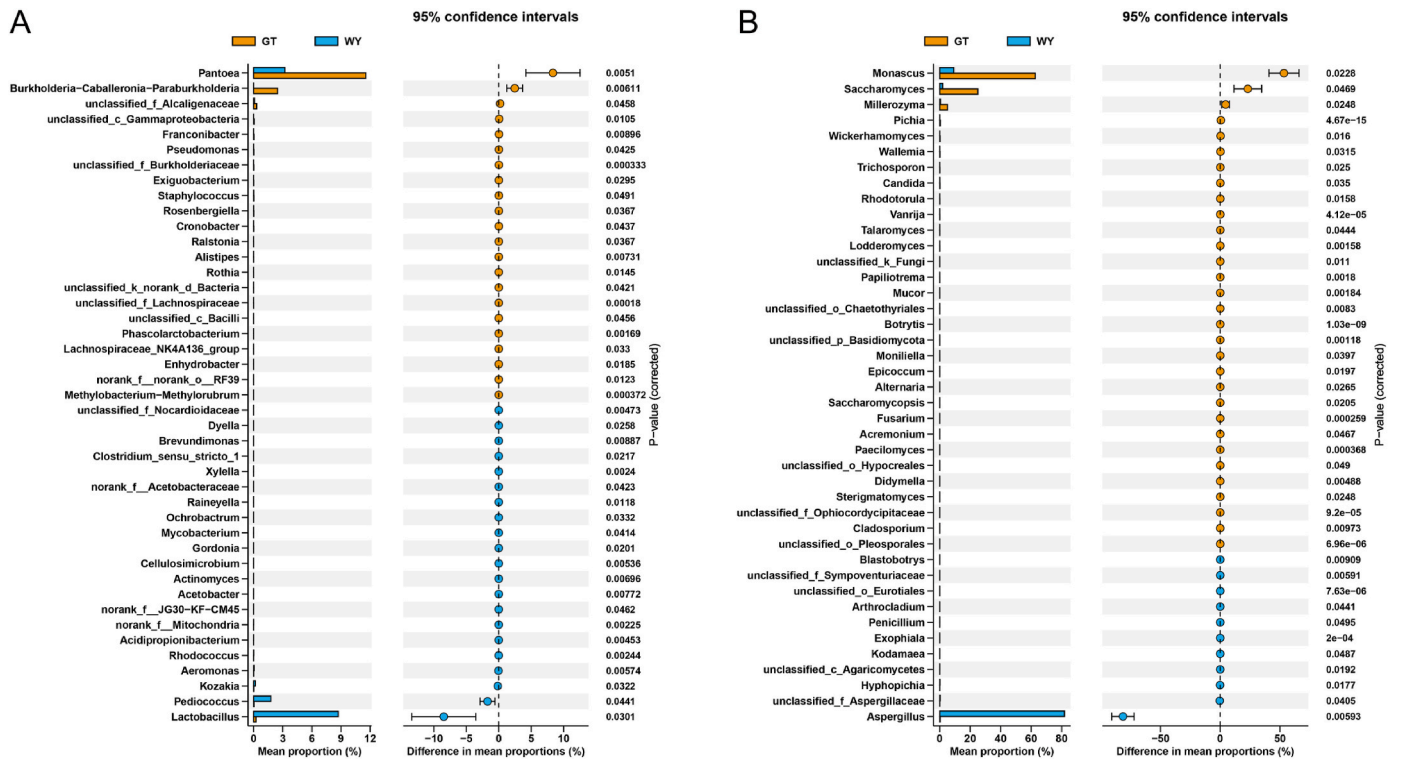

Fig. 7. Analysis and visualization of the differences in the relative abundance of bacterial (A) and fungal (B) genera between *Hongqu* rice wines fermented with Gutian *Qu* and Wuyi *Qu* (GT and WY) using STAMP software. Microbial genera with significant differences between GT and WY were determined using a Welsh's *t*-test, and the Benjamini-Hochberg procedure was used to control the false-discovery rate.

saccharification rate of Wuyi *Hongqu* rice wine (WY), hence the content of isobutanol, 3-methylbutan-1-ol and phenylethyl alcohol in Wuyi *Hongqu* rice wine (WY) was higher than Gutian *Hongqu* rice wine (GT).

Subsequently, the differences in the relative abundance of microbial genera between the two kinds of *Hongqu* rice wine (GT and WY) were analyzed and visualized through by STAMP (http://kiwi.cs.dal.ca/Software/STAMP) (Fig. 7A and B). 43 bacterial and 42 fungal genera with significant differences between the Gutian *Hongqu* rice wine (GT) and Wuyi *Hongqu* rice wine (WY) were revealed. We found that the relative abundance of *Lactobacillus, Pediococcus, Kozakia, Aeromonas, Rhodococcus, Acidipropionibacterium,* norank_f_*Mitochondria,* norank_f_JG30-KF-CM45, *Acetobacter, Actinomyces, Aspergillus,* unclassified_c_ *Aspergillaceae, Hyphopichia, Kodamaea, Exophiala, Penicillium* and *Arthrocladium* were significantly higher in WY brewing than those in GT brewing (corrected *P*-value <0.05). Compared with WY brewing, *Pantoea, Burkholderia-Caballeronia-Paraburkholderia,* unclassified_f_*Alcaligenaceae, Franconibacter, Pseudomonas, Exiguobacterium, Staphylococcus, Rosenbergiella, Cronobacter, Ralstonia, Alistipes, Rothia, Monascus, Saccharomyces, Millerozyma, Pichia, Wickerhamomyces, Wallemia, Trichosporon, Candida* and *Rhodotorula* were significantly enriched in GT brewing, which were consisted with previous study (Liu et al., 2018; Liang et al., 2020).

3.4. Correlation analysis of microbiota and metabolites in Hongqu rice wine

To a large extent, the microbial communities in wine-making are closely related to the flavor characteristics of rice wine (Jin et al., 2017). In this study, Spearman's correlations between the predominant microbial phenotypes at the genus level and the characteristic volatile components with significant differences between the WY and GT groups were visualized through correlation heatmap (Fig. 8). The results showed that hexanoic acid was negatively correlated with *Leuconostoc* and *Bacillus,* but positively correlated with *Talaromyces, Millerozyma, Apiotrichum* and *Saccharomyces.* Octanoic acid production was significantly positively correlated with the abundances of *Rosenbergiella, Pseudomonas, Lactococcus, Kodamaea* and *Saccharomyces,* but significantly negatively correlated with the abundances of *Franconibacter.* The production of n-decanoic acid in *Hongqu* rice wine brewing was significantly positively correlated with the abundance of microorganisms such as *Lactococcus, Kodamaea* and *Saccharomyces,* while significantly negatively correlated with *Franconibacter* and *Monascus.* In addition, ethyl lactate, diethyl succinate, isobutanol, 3-methylbutan-1-ol, ethyl acetate and phenylethyl alcohol were positively correlated with *Lactobacillus, Rhodococcus* and *Saccharomyces,* while significantly negatively correlated with *Franconibacter, Kosakonia, Pichia* and *Rhodotorula.* Furthermore, diethyl succinate, isobutanol, 3-methylbutan-1-ol, ethyl acetate and phenylethyl alcohol were associated with *Weissella,* while
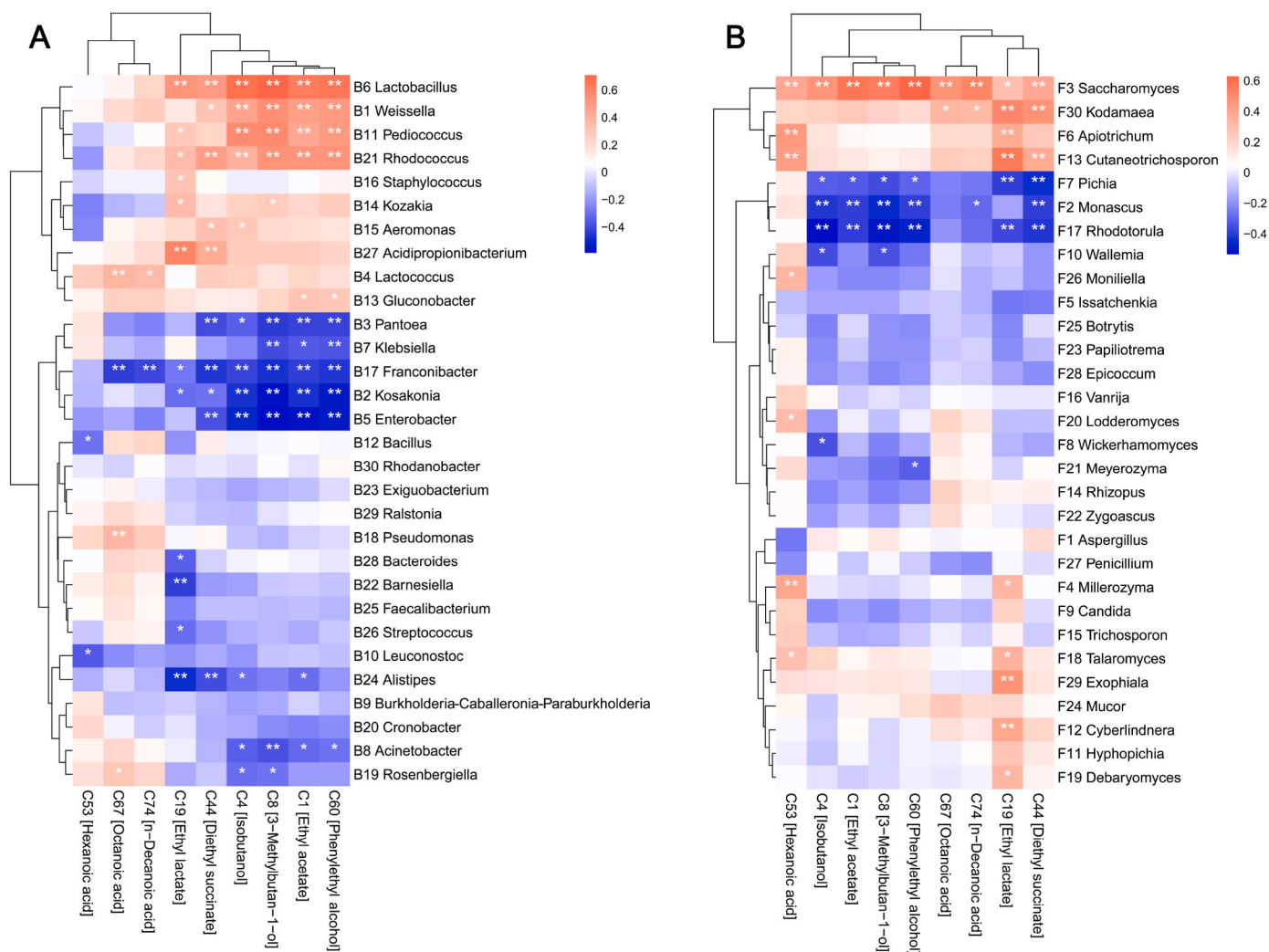

Fig. 8. Correlation heatmap analysis between the characteristic volatile components and the predominant microbial phylotypes at the genus level in the fermentation of *Hongqu* rice wine. (A) Characteristic volatile components-bacteria, (B) Characteristic volatile components-fungi.

negatively correlated with *Pantoea*, *Enterobacter* and *Monascus*. In fact, *Weissella* can increase the contents of organic acids, short-chain fatty acids and esters during the fermentation of food (Kamboj et al., 2015), so we can conjecture that it can increase the yield of volatile acids, alcohols and esters in *Hongqu* rice wine. Interestingly, *Pediococcus* was only positively correlated with a few substances such as ethyl lactate, isobutanol, 3-methylbutan-1-ol, ethyl acetate and phenylethyl alcohol. And previous study claimed that *Pediococcus* played a key role in the flavor formation and fermentation activity of rice wine (Chen et al., 2020), which was consisted with our results. Our previous study had shown that *Gluconacetobacter, Lactobacillus, Lactococcus, Pichia, Wickerhamomyces* and *Saccharomyces* were the core microbiota producing the main volatile compounds in the traditional brewing process of *Hongqu* rice wine, and *Saccharomyces cerevisiae, Lactobacillus plantarum* and *Aspergillus niger* were the main contributors to esters, alcohols and acids (Huang et al., 2018). And *Lactococcus lactis, Burkholderia gladioli, Cronter helveticus, Wickerhamomyces anomalus, Saccharomyces cerevisiae, Aspergillus vitricola, Aspergillus penicillioides*, and *Monascus purpureus* were

closely related to the production of a variety of volatile flavor substances, and may play a key role in aroma formation of Gutian *Hongqu* rice wine (Liu et al., 2020).

3.5. Functional prediction of microbial enzymes for the metabolism of characteristic volatiles

The metabolic function profiles of microbial communities during *Hongqu* rice wine brewing were predicted using PICRUSt2 analysis based on the sequencing data (Fig. 9). The metabolic network of the characteristic volatile components of *Hongqu* rice wine was constructed through the KEGG metabolic pathway database (Fig. 10), which clearly demonstrated the metabolic pathways involved in the formation of characteristic volatile flavor components. Combined with Figs. 7 and 9, we found that the bacterial community composition of WY and GT have significant differences at genus level, but they have similar metabolic function profiles (Fig. 9A). The results showed that there was no significant difference in the abundance of enzymes produced by the vast
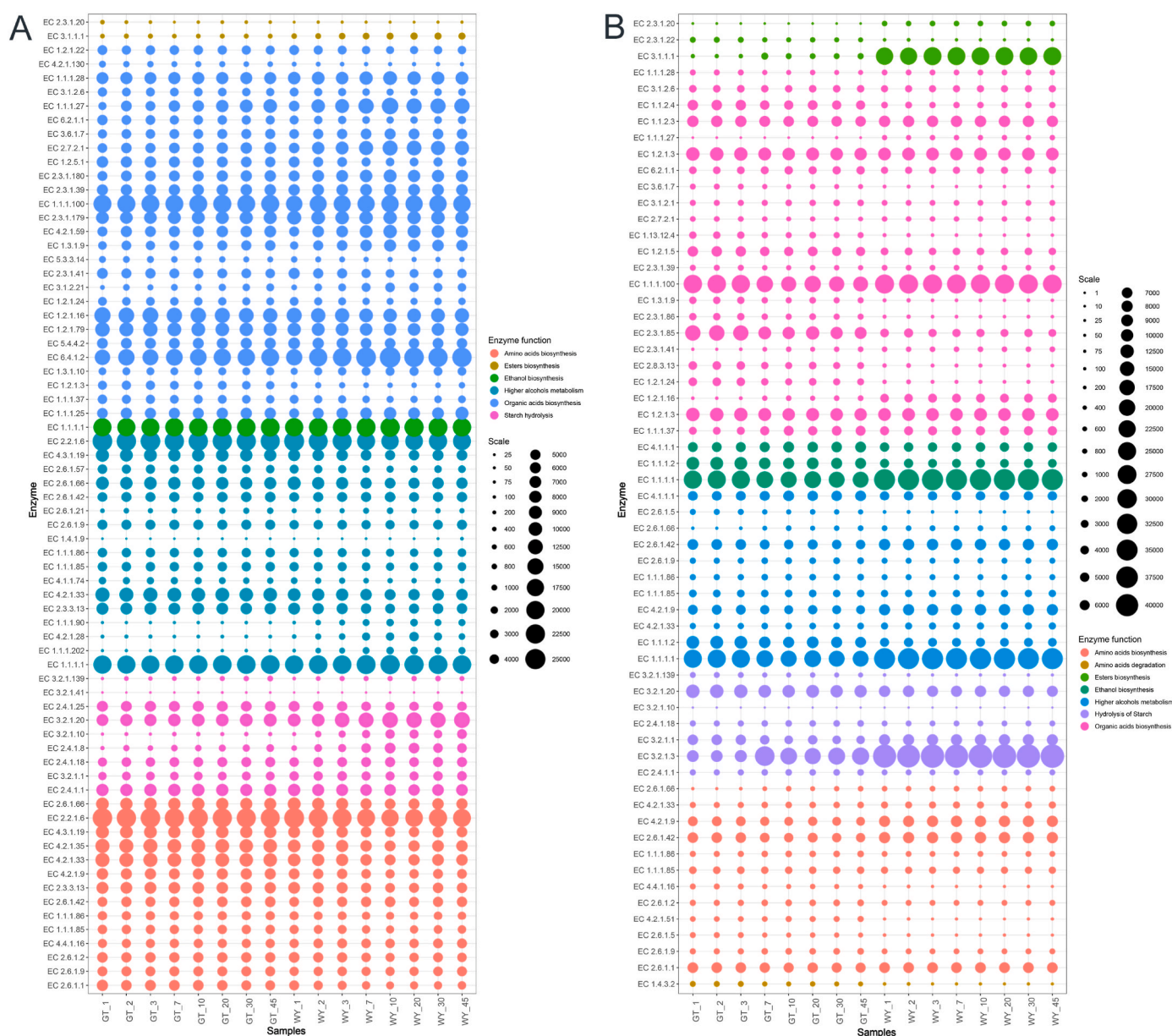

Fig. 9. Bubble chart of the abundance of microbial enzymes closely related to the metabolism of characteristic volatile flavor components during the traditional brewing of *Hongqu* rice wine fermented with Gutian *Qu* and Wuyi *Qu*. (A) Bacterial enzymes (B) Fungal enzymes.
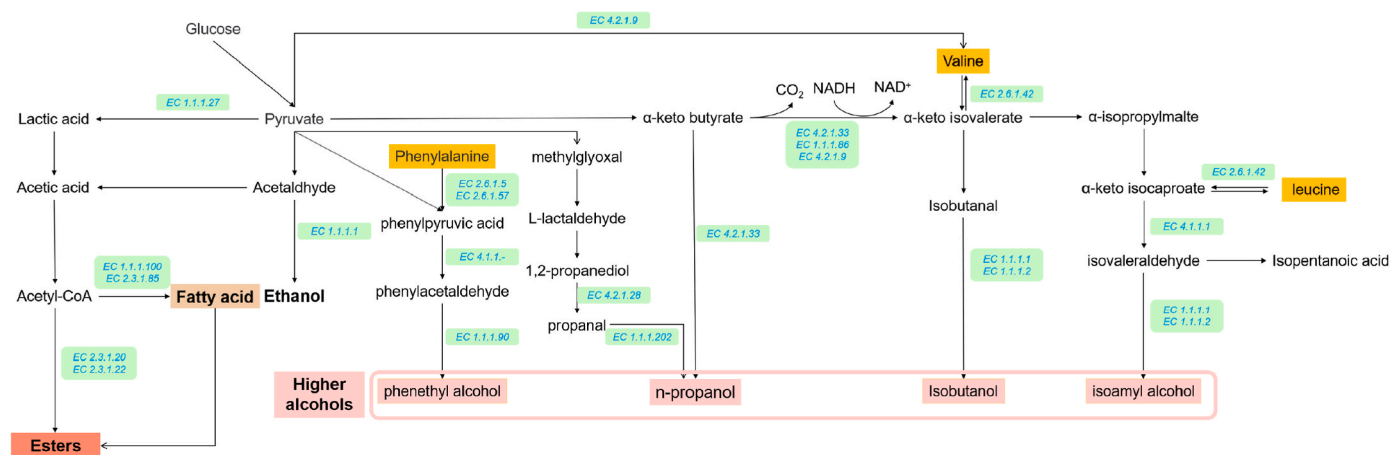
Fig. 10. The metabolic network of the characteristic volatile components in *Hongqu* rice wine.

majority of bacteria in GT and WY brewing, but there were a few exceptions. For example, L-lactate dehydrogenase [EC 1.1.1.27], aryl-alcohol dehydrogenase [EC 1.1.1.90], propanediol dehydratase [EC 4.2.1.28], 1,3-propanediol dehydrogenase [EC 1.1.1.202] and alpha-glucosidase [EC 3.2.1.20] were more abundant in WY brewing than those in GT. L-lactate dehydrogenase [EC 1.1.1.27] is closely related to the synthesis of organic acids. Aryl-alcohol dehydrogenase [EC 1.1.1.90], propanediol dehydratase [EC 4.2.1.28], 1,3-propanediol dehydrogenase [EC 1.1.1.202] are related to the synthesis of higher alcohols, and alpha-glucosidase [EC 3.2.1.20] is a key enzyme involved in starch hydrolysis. Predictive analyses of fungal enzyme-producing potential indicated higher abundance of glucan 1,4-alpha-glucosidase (EC 3.2.1.3, an enzyme related to starch hydrolysis) in WY brewing compared to GT (Fig. 9B). Owing to the higher relative abundance of *Aspergillus* in WY brewing, which has the excellent capacity to produce 1,4-alpha-glucosidase (Lambre et al., 2022), the saccharification rate during the early stages of Wuyi *Hongqu* rice wine (WY) brewing was significantly faster than that of Gutian *Hongqu* rice wine (GT). The glucan 1,4-α-glucosidase produced by *Aspergillus* contributes to the rapid hydrolysis of starch to produce large quantities of glucose, and *Saccharomyces cerevisiae* uses the starch hydrolysis products to produce large quantities of alcohol, as well as higher alcohols and other by-products through glycolysis in the brewing process of Wuyi *Hongqu* rice wine (WY). The results showed that the abundance of alcohol dehydrogenase [EC 1.1.1.1], which is closely related to the anabolism of alcohol and higher alcohols (Xu et al., 2022), differed between WY and GT brewing. It has been shown that alcohol dehydrogenase can reduce aldehydes to alcohol in *Saccharomyces* (Hummel, 1999; Hernandez-Tobias et al., 2011). Since Wuyi *Hongqu* rice wine (WY) contains abundant reducing sugars in the early stage of brewing, and the abundance of alcohol dehydrogenase produced by the microbial flora of Wuyi *Hongqu* rice wine (WY) is higher than that of Gutian *Hongqu* rice wine (GT), the production rate of ethanol and higher alcohols in Wuyi *Hongqu* rice wine (WY) is significantly higher than that of Gutian *Hongqu* rice wine (GT). The content of amino acids as precursors of higher alcohols is also closely related to the yield of higher alcohols. Dihydroxy-acid dehydratase [EC 4.2.1.9] is one of the key enzymes in the biosynthesis pathway of branched-chain amino acids, including valine, leucine and isoleucine (Bayaraa et al., 2022), and branched-chain-amino-acid transaminase [EC 2.6.1.42] catalyses the bidirectional transamination reaction between α-keto acids and branched-chain amino acids (Hammer and Avalos, 2017). The abundances of dihydroxy-acid dehydratase [EC 4.2.1.9] and branched-chain-amino-acid transaminase [EC 2.6.1.42] are closely related to the yield of higher alcohols. In this study, we found that Wuyi *Hongqu* rice wine (WY) brewing contained more dihydroxy-acid dehydratase [EC 4.2.1.9] and

branched-chain-amino-acid transaminase [EC 2.6.1.42] than those in Gutian *Hongqu* rice wine (GT) brewing, which may be one of the important reasons why the content of higher alcohols in Wuyi *Hongqu* rice wine (WY) was significantly higher than that in Gutian *Hongqu* rice wine (GT). At different stages of brewing, bacteria, molds and yeasts play different roles in the formation of aroma and taste of rice wine. In the traditional brewing process, mold plays a key role in the production of liquefaction and glycosylation enzymes, while yeast plays an important role in the production of alcohols and esters. Bacteria, especially lactic acid bacteria, produce a large number of organic acids in the traditional brewing process of rice wine, some of which can degrade macromolecules, and most of the organic acids can react with alcohols to produce ester aroma components such as ethyl lactate. Therefore, lactic acid bacteria not only promotes the aroma formation in rice wine brewing, but also plays an important contribution to the production of sour taste of rice wine.

4. Conclusion

This study was the first to compare the microbial communities and volatile flavor components in brewing of *Hongqu* rice wines fermented with different starters, Gutian *Qu* (GT) and Wuyi *Qu* (WY). Multivariate statistical analysis showed that fermentation starter has a great influence on the composition of microbial community and the formation of volatile components in *Hongqu* rice wine brewing. Characteristic volatile flavor components (including 3-methylbutan-1-ol, isobutanol and phenylethyl alcohol, *etc.*) in the two representative *Hongqu* rice wine (GT and WY) were identified through orthogonal PLS-DA analysis. Microbiome analysis based on 16S/ITS amplicon sequencing found that *Lactobacillus* and *Aspergillus* were enriched in WY brewing, while *Pantoea*, and *Monascus* were significantly enriched in GT brewing. Correlation analysis was performed to reveal the potential relationship between the predominant microbial genera and characteristic volatile flavor components, and found the positive correlation between LAB/*Saccharomyces* and most of the characteristic volatile components, while *Picha* and *Monascus* were significantly negatively correlated with most of the characteristic volatile components. Furthermore, bioinformatical analysis based on PICRUSt indicated the reason of the differences between GT and WY. This study is helpful to understand the difference of flavor quality of *Hongqu* rice wine and its microbiological principle, and provides an important scientific basis for reducing the content of higher alcohols in *Hongqu* rice wine. Nevertheless, high-throughput sequencing of 16S/ITS amplicon can only reveal the microbial taxonomic composition, but not the real metabolic function information. The microbial communities and their metabolic functions at the species level in *Hongqu* rice wine brewing should be further investigated through shotgun

metagenomic sequencing combined with metabolomics technology.

CRediT authorship contribution statement

Guimei Chen: Methodology, Investigation, Writing – original draft, Validation. **Yujie Yuan:** Methodology, Investigation, Writing – original draft, Validation. **Suwen Tang:** Investigation, Writing – original draft, Validation. **Ziyi Yang:** Methodology, Investigation, Writing – original draft, Validation. **Qi Wu:** Investigation, Software, Writing – original draft, Validation. **Zihua Liang:** Software, Investigation, Writing – original draft. **Shiyun Chen:** Investigation, Writing – original draft. **Wenlong Li:** Investigation, Writing – original draft. **Xucong Lv:** Funding acquisition, Writing – review & editing, Supervision, Project administration. **Li Ni:** Supervision, Conceptualization, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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