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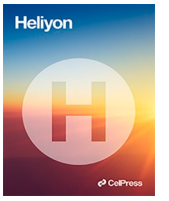

Effects of six commercially available koji (Chinese Xiaoqu) on the production of ethyl acetate, ethyl lactate, and higher alcohols in Chinese Baijiu (distilled spirit) brewing

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ABSTRACT

Commercial koji has been increasingly used in Chinese Baijiu brewing; however, there are only few studies comparing different koji and their relationship with key components of Chinese Baijiu such as ethyl acetate, ethyl lactate, and higher alcohols. Here, we studied six commercially available koji and showed that the microbial communities in the individual koji varied in composition, with *Rhizopus*, *Aspergillus*, and *Bacillus* primarily associated with starch hydrolysis and *Saccharomyces* mainly associated with alcohol production. In the brewing processes using the six koji, *Saccharomyces* was undoubtedly the most abundant fungus and *Weissella*, *Bacillus*, and *Acinetobacter* were the predominant bacterial groups. The levels of ethyl acetate, ethyl lactate, and higher alcohols in all brewing processes using the koji exhibited rapid increase in the early stages of fermentation, which stabilized in the later stages, followed by substantial increase after distillation. The results of metagenomic and redundancy analyses of samples taken during the brewing processes indicated that *Saccharomyces* from the koji was closely related to the production of ethyl acetate, ethyl lactate, and higher alcohols. This study provides a basis for the quality improvement and application of commercial koji.

1. Introduction

Chinese Xiaoqu (small koji) is prepared using rice flour, rice bran, or wheat bran (some also add Chinese herbs) that is inoculated with a koji starter culture, mixed with water, and incubated at the appropriate temperature and humidity [1]. The mature Xiaoqu is used for Xiaoqu Baijiu brewing with sorghum or rice as the raw material. Traditional Xiaoqu Baijiu brewing typically uses Xiaoqu made by natural inoculation with fungi such as *Rhizopus* and yeast and bacteria including *Lactobacillus* and *Bacillus* involved in saccharification and fermentation [2]. With the advancement and expansion of technology for koji preparation, modern brewing processes have increasingly adopted the use of commercial koji prepared by artificial inoculation of pure strains like *Rhizopus* and yeasts [3].

The microbial composition of koji affects the brewing process and quality of the final product to a great extent. For example, *Rhizopus* and *Bacillus* secrete amylase for saccharification, *Saccharomyces* promotes alcoholization, and bacteria are responsible for the production of various flavor compounds [4]. Traditional microbial research on koji was mainly based on strain isolation, culturing, and

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Table 1
Information on six commercially available koji.

Koji brand	Code	Origin	Main ingredients
An Qi Bai Jiu Qu	AB	Yichang, Hubei, China	Yeast, *Rhizopus*, α-amylase, glucoamylase, phytase
An Qi Fu Qu	AF	Yichang, Hubei, China	Yeast, *Rhizopus*, wheat bran
Hua Xi	HX	Pengzhou, Sichuan, China	Yeast, wheat bran, rice
Liu Er	LE	Kunming, Yunnan, China	Yeast, *Rhizopus*, wheat bran, rice bran
Xiang Jiu Zhong	XJ	Nanning, Guangxi, China	Yeast, *Rhizopus*, water, spices, wheat bran, corn
Ya Da	YD	Yongzhou, Hunan, China	Enhanced yeast, wheat bran, cornmeal

identification. However, this approach is not suitable for effectively analyzing complex microbial systems, especially those containing non-culturable microorganisms. In recent years, high-throughput sequencing and metagenomics sequencing have been increasingly applied to the study of microorganisms in koji. For example, Gou et al. [5] identified *Weissella* and *Enterobacter* as the dominant bacterial genera and *Rhizopus* and *Saccharomyces* as the dominant fungal genera in koji using high-throughput sequencing. Tang et al. [6] showed that *Weissella* and *Staphylococcus* were the predominant bacteria, and *Rhizopus* and *Candida* were the dominant fungi. Zhao et al. [7] concluded that *Lactobacillus*, *Bacillus*, *Acinetobacter*, and *Weissella* were the principal bacterial genera and *Aspergillus*, *Saccharomyces*, *Pichia*, and *Rhizopus* were the major fungal genera.

The amounts of ethyl acetate, ethyl lactate, and higher alcohols in Xiaoqu Baijiu are closely linked to its flavor and quality. Ethyl acetate is the main aromatic component of Xiaoqu Baijiu, and the appropriate ratio of ethyl lactate to ethyl acetate produces an elegant and soft or fragrant and pure flavor, which increases the aroma and richness of the liquor [8]. Studies have shown that *Saccharomyces cerevisiae*, *Hansenula*, *Candida*, and mold can secrete esterases that promote the synthesis of these two esters [9]. In addition, *S. cerevisiae* can also produce alcohol acyltransferase to synthesize ethyl acetate [10]. Higher alcohols contribute to Xiaoqu Baijiu's flavor; however, while a moderate amount of higher alcohols in a liquor will render it a rich flavor, excessive amounts will have adverse effects on taste and cause neurotoxicity [11]. Previous research suggested that the primary source of higher alcohols comes from the metabolism of *S. cerevisiae* during the brewing process [12].

Although a comparison of several commercially available koji for rice wine brewing were reported recently [13], there is no comparative study about commercially available koji for distilled spirit making so fa as we know. As there are considerable differences in making process and concerning points between rice wine and distilled spirit, we compared six commercially available koji for Xiaoqu Baijiu in this study. We also used metagenomic analysis to annotate key enzyme genes in the koji related to some target metabolites in Xiaoqu Baijiu. We expect that this study will provide a reference for the quality improvement and practical application of commercial koji.

2. Materials and methods

2.1. Raw materials and koji

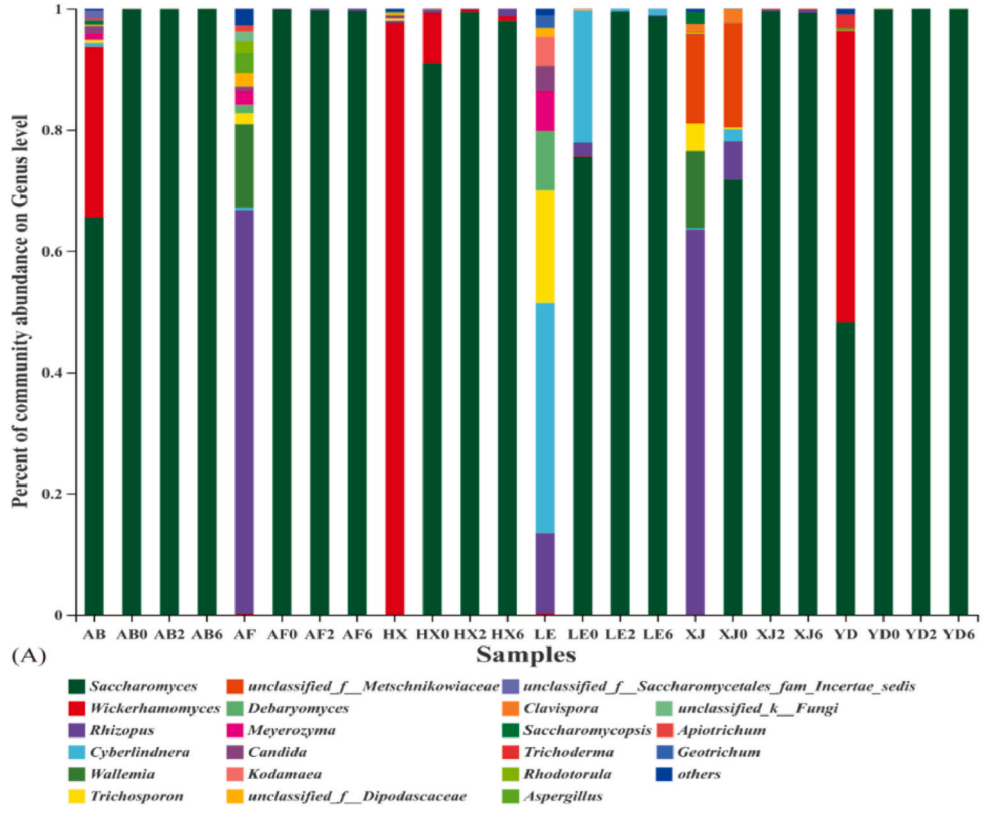
The rice used in this study was purchased from Walmart (Asia Pacific Branch), Chengdu. Information about the six commercially available koji is shown in Table 1. The samples were divided into two sets: one group was stored at −80 °C and used for high-throughput sequencing and metagenomic analysis; the other set was stored at −20 °C and used for physicochemical and flavor compound analyses and brewing tests.

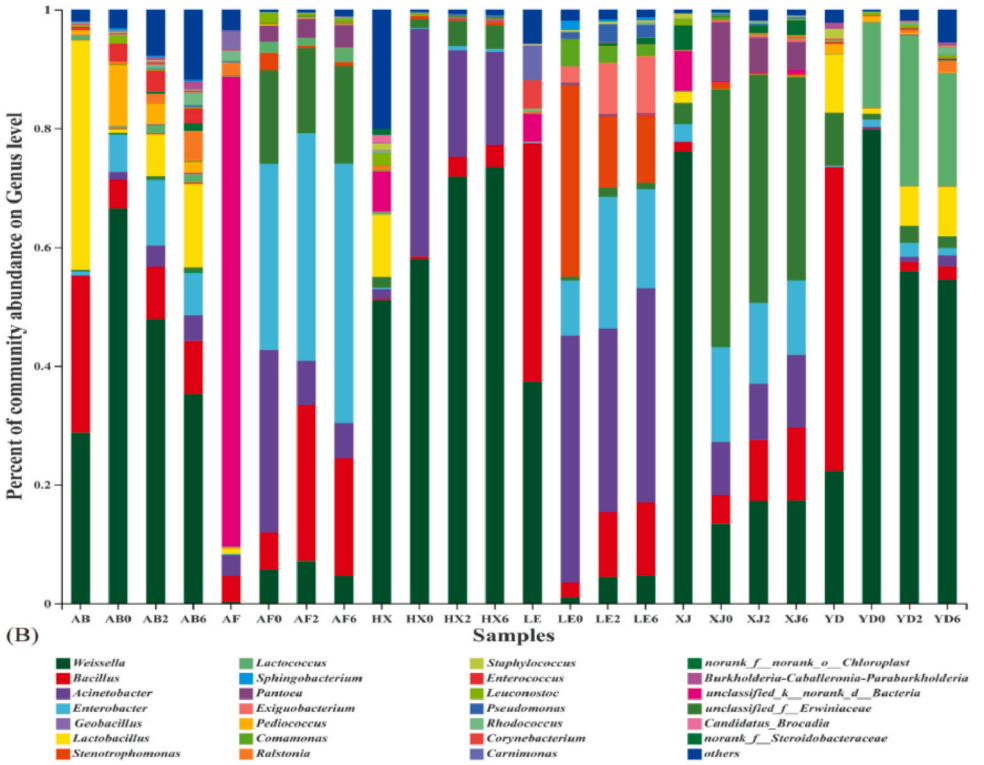
2.2. Fermentation

One thousand grams of rice was rinsed, soaked, steam cooked, and then cooled for later use. About 5 g of activated koji was added to the cooked rice. The mixture was thoroughly mixed and transferred to a 5 L fermenter, which was then nested, sealed with four layers of sterile gauze, and incubated at 32 °C for 24 h for saccharification [14,15]. Afterward, sterile water was added in a 1:2 rice: water ratio and the mixture was left for fermentation at 28 °C. Samples were taken on days 0, 2, 4, 6, and 8 during fermentation, of which 50 mL was stored at −20 °C and later used for physicochemical and flavor compound analyses, and another 50 mL was stored at −80 °C and later used for high-throughput sequencing and metagenomic analysis. These samples were named according to the koji used and fermentation time (e.g., AB8), and for samples of the liquors the suffix "-J" was added after the koji codes (e.g., AB-J).

2.3. High-throughput sequencing and metagenomic analysis

For high-throughput sequencing, total DNA was extracted from the microbial community in the koji and fermented samples using the Fast DNA SPIN extraction kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The quality of DNA extraction was assessed using 1% agarose gel electrophoresis. The concentration and purity of DNA were measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification of the V3–V4 variable regions of bacterial genes was done using the 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers, while ITS1 (CTTGGTCATTTAGAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) primers were used for PCR amplification of the fungal ITS1 region. Afterward, the amplification products were sequentially subjected to purification and recovery, fluorescence





(caption on next page)

Fig. 1. Composition of the fungal (A) and bacterial (B) communities at the genus level of the six koji and their brewing processes.

quantification, library construction and sequencing, and data preprocessing. High-throughput sequencing was performed by Shanghai Major Biomedical Technology Co., Ltd (Shanghai, China).

For metagenomic analysis, the DNA extraction of the koji was fragmented, then linked. Beads were applied to screen to remove the linker self-linked fragments. Library templates were enriched by PCR amplification then the products were recovered by beads to construct the final library. The raw data is optimized for splitting, mass shearing and decontamination, and the optimized sequence is used for splicing assembly and gene prediction. The obtained genes are annotated and classified into species and functions. Metagenomics analysis was performed by Shanghai Major Biomedical Technology Co., Ltd (Shanghai, China).

2.4. Analysis of physicochemical properties

Moisture, acidity, saccharification, and liquefaction performance of the koji were determined according to published methods [16]. Total acidity and the alcohol content of the finished Baijiu were determined and calculated following the method used by Chang [17].

2.5. Analysis of volatile compounds

Volatile compounds were analyzed using headspace solid-phase microextraction (HS-SPME) combined with gas chromatography mass spectrometry (GC-MS) using a GCMS-QP2010SE (Shimadzu, Japan), following the conditions used by Ding et al. [18]. The results were analyzed against relevant database libraries to select compounds with a match factor above 85 as the target compound, and the compound types were identified. The amount of the target compounds in the sample was calculated according to the ratio of the peak area of the target compound to that of the internal standard.

Higher alcohols were quantified using gas chromatography flame ionization detector (GC-FID) (GC-9790, Fuli Analytical Instruments Co., Zhejiang, China) with a KB-ALCOHOL column (40 m $\times$ 0.53 mm), following the GC temperature ramping program by Tavasoli et al. [19]. The higher alcohols in the samples were identified and quantified based on their retention time and the peak area ratio of the specific compound to the internal standard.

2.6. Sensory evaluation

A comprehensive sensory evaluation of the six finished base liquors was carried out by 10 professionally trained liquor tasters (5 males and 5 females) following previously the relevant standards [20]. These sensorial experiments were conducted according to established ethical guidelines, and informed consent obtained from the participants.

2.7. Data analysis

The data obtained were subjected to variance analysis and Duncan multiple test, and the significance was considered at the $p < 0.05$ level. SIMCA-P v14.1 was used for principal component and correlation analyses, and Canoco 5.0 was used for redundancy analysis.

3. Results and discussion

3.1. Changes in the microbial community structure of six koji and their respective brewing processes (Fig. 1)

A total of 55 fungal genera were detected in the six koji. The dominant fungi in the AB and YD koji were *Saccharomyces* and *Wickerhamomyces*, and *Wickerhamomyces* accounted for more than 95% of fungual abandance in the HX koji. The predominant fungi in the AF and XJ koji were *Rhizopus* and *Wallemia*, followed by *Aspergillus* and unclassified *Metschnikowiaceae,* respectively. *Cyberlindnera* was relatively abundant in LE koji, followed by *Trichosporon*, *Rhizopus*, and *Debaryomyces*. Among these microorganisms, *Rhizopus*, *Saccharomyces*, and *Aspergillus* are commonly reported as the dominant fungi in previous studies of other small koji [4,5,21]. *Rhizopus* and *Aspergillus* possess relatively high amylase activity [22,23], *Saccharomyces* is an important ethanol producer, and *Candida* is associated with formation of flavor compounds such as acids and esters [24]. In addition, *Wickerhamomyces* can produce ethyl acetate and 2-phenylethanol [25]; *Wallemia* is widely distributed in the brewing environment of Maotai-flavor Baijiu [26] and is one of the dominant bacteria present during the fermentation process of Luzhou-flavor Baijiu [1]. Among the other identified fungal genera, *Cyberlindnera* is a non-*Saccharomyces* yeast with high ester-producing capacity [27], *Trichosporon* can produce esters and β-glucosidase during ethanol production [28], and *Debaryomyces* is a non-*Saccharomyces* yeast associated with the formation of Chinese Baijiu flavors [29,30]. The unclassified *Metschnikowiaceae* detected in the AF and XJ koji in this study have not been previously reported in liquor production, and their significance needs to be explored in the future.

A total of 444 bacterial genera were detected in the six koji. The dominant bacteria in the AB and YD koji were *Weissella*, *Bacillus*, and *Lactobacillus* and YD koji also contained relatively high abandance of unclassified *Erwiniaceae*. *Weissella* was overwhelmingly predominant in the HX, LE, and XJ koji. In addition, the HX koji had a high abundance of *Lactobacillus* and unclassified bacteria, and the LE koji had relatively high abandance of *Bacillus*, *Carnimonas*, *Stenotrophomonas*, and unclassified bacteria. Unclassified bacteria also prevailed in the XJ and AF koji. Altogether, the bacterial composition of different koji varied considerably. Some of these

Table 2
The alpha diversity of the microbial community within six commercially available koji.

Species	Koji	OTU	Chao1	Ace	Simpson	Shannon
Fungi	AB	28	61.500	85.304	0.510	0.948
	AF	35	56.000	56.000	0.454	1.548
	HX	17	32.000	36.907	0.952	0.171
	LE	24	41.000	43.948	0.212	1.926
	XJ	32	62.000	62.452	0.428	1.357
	YD	23	34.250	33.365	0.465	0.864
Bacteria	AB	194	253.182	307.021	0.245	1.846
	AF	193	207.667	212.235	0.632	1.182
	HX	495	517.813	523.607	0.271	2.813
	LE	214	254.227	272.328	0.222	2.038
	XJ	97	164.364	192.228	0.592	1.097
	YD	161	205.200	219.996	0.311	1.760

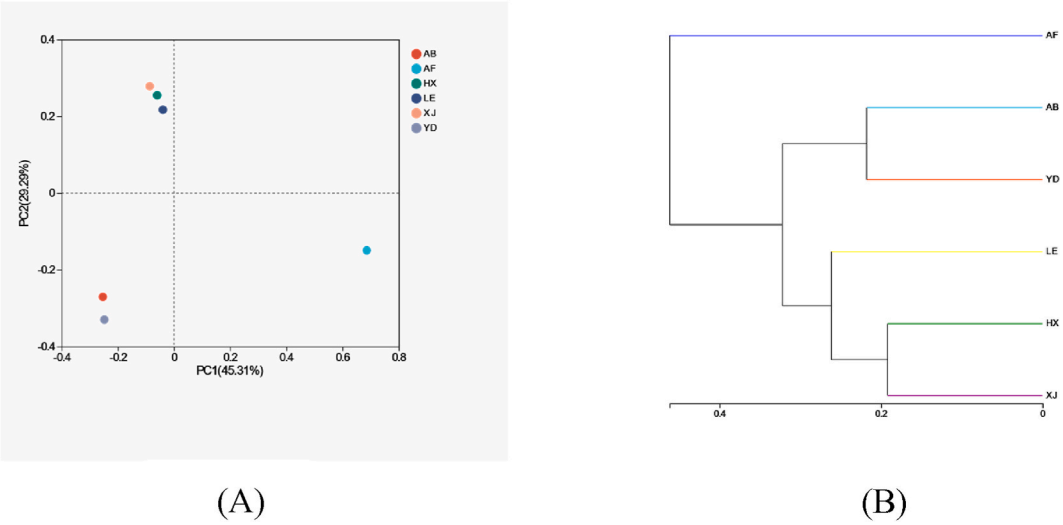


Fig. 2. Beta diversity of the microbial community within the six koji. (A: PCoA; B: Hierarchical clustering).

dominant bacteria such as *Lactobacillus, Weissella*, *Enterobacter, Leuconostoc*, and *Staphylococcus* have been previously reported [4,5,21]. *Lactobacillus* and *Weissella* were highly abundant in AB and YD koji. *Bacillus* is commonly found in brewing process and is capable of producing high levels of enzymes such as protease and amylase, as well as flavor compounds such as 4-methylpyrazine and acetoin during metabolism [31]. The unclassified *Erwiniaceae* and unclassified bacteria in this study have not been previously reported and their function in brewing is unknown. These bacteria were not listed in the corresponding products' ingredient list and may have arisen from contamination during koji preparation.

Overall, microorganisms associated with starch hydrolysis in the six koji in this study included *Rhizopus*, *Aspergillus* (AF, XJ, and LE koji) and *Bacillus* (AB, YD, and LE koji). *Saccharomyces* was the main alcohol-producing microorganism in all koji and the other detected microorganisms may be involved in the development of the liquor's flavor.

In the samples taken during the brewing processes using the six koji, 26 fungal genera were detected. *Saccharomyces* was the dominant fungus in all samples and is primarily responsible for ethanol fermentation. In addition, *Wickerhamomyces, Cyberlindnera*, and unclassified *Metschnikowiaceae* were also highly abundant in the brewing processes using HX, LE, and XJ koji, respectively. In these samples, 378 bacterial genera were also detected, with *Weissella* as the dominant bacteria in the AB, HX, XJ, and YD koji brewing processes. Additionally, *Acinetobacter* and *Lactococcus* had relatively high abundance in the HX and YD koji brewing processes, respectively. The brewing processes using XJ koji included relatively high levels of unclassified *Erwiniaceae* and *Bacillus* whereas *Bacillus* and *Enterobacter* were the dominant bacteria in the brewing processes using AF and LE koji. In addition, *Acinetobacter* and *Stenotrophomonas* were also present in some abundance in the brewing processes using LE koji. *Enterobacter* was also reported in the brewing process by Wang et al. [32]. *Acinetobacter* has not been previously reported in liquor production and its significance needs to be further investigated.

Some of the dominant fungal genera in the koji such as *Rhizopus*, *Aspergillus*, *Wallemia*, *Trichosporon*, and *Debaryomyces* were not detected during the brewing processes. Among them, *Aspergillus*, *Trichosporon*, and *Debaryomyces* are all aerobic microorganisms and *Rhizopus* is a facultative anaerobic fungi. Thus, their absence may be due to the anaerobic environment during fermentation. Most of the dominant bacterial genera present during fermentation such as *Weissella* (AB, HX, XJ, and YD koji), *Bacillus*, and *Stenotrophomonas*

Table 3
Physicochemical properties of the six koji and sensory evaluation of corresponding liquors.

Index	Moisture content (g/100 g)	Total acidity (mmol/10 g)	Liquefying power (g/g·h)	Saccharifying power (mg/g·h)	Alcohol yield (55% vol)	sensory scores of the liquors				
						Appearance	Aroma	Taste	Mouth feel	Total score
AB	5.20 ± 0.004^{b}	9.93 ± 0.80^{a}	15.20 ± 4.71^{a}	795.00 ± 68.74^{b}	68.5 ± 0.62^{b}	8.71	32.71	33.43	7.86	82.71
AF	6.60 ± 0.20^{b}	6.27 ± 0.34^{b}	0.88 ± 0.17^{b}	150.50 ± 51.96^{d}	66.5 ± 0.47^{b}	8.86	32.57	32.71	7.71	81.86
HX	9.73 ± 0.12^{a}	6.83 ± 0.53^{b}	1.11 ± 0.31^{b}	447.00 ± 82.87^{c}	67.8 ± 0.41^{b}	8.86	33.43	33.43	7.14	82.86
LE	10.87 ± 0.12^{a}	2.57 ± 0.20^{c}	0.61 ± 0.19^{b}	160.00 ± 52.67^{d}	70.2 ± 0.71^{b}	9	32.71	33.19	7.71	82.61
XJ	7.00 ± 0.00^{b}	3.58 ± 0.54^{c}	1.18 ± 0.09^{b}	677.00 ± 78.24^{b}	62.3 ± 0.85^{c}	8.86	29.86	29.29	6.86	74.86
YD	3.93 ± 0.12^{c}	9.73 ± 0.57^{a}	12.33 ± 0.86^{a}	1044.00 ± 55.07^{a}	75.2 ± 0.81^{a}	8.86	34	33.29	8.14	84.29

Note: Values are mean ± standard deviation (n = 3), $^{a\text{-}d}$ different letters in the same column represent significant differences ($p < 0.05$).

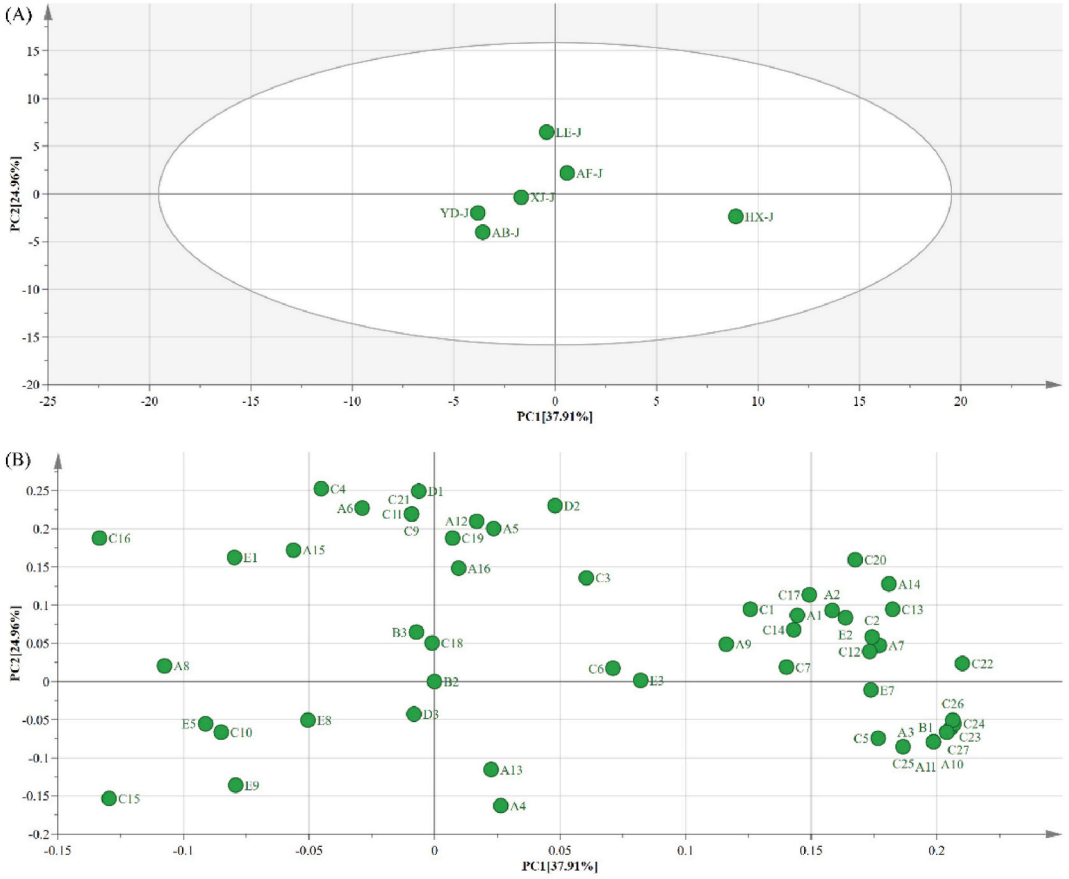


Fig. 3. Principal component analysis score chart (A) and load map (B) of the flavor substances of the liquors brewed with different koji.
Note: A1: Methanol; A2: 1-Propanol; A3: 1-Butanol; A4: 2,3-Butanediol; A5: 2-Butanol; A6: Isobutanol; A7: Tert-Butanol; A8: Isoamyl alcohol; A9: 1-Pentanol; A10: 2-Methyl-1-butanol; A11: 3-Ethoxy-1-propanol; A12: 1-Hexanol; A13: Phenethyl alcohol; A14: 1-Octanol; A15: 1-hendecanol; A16: 1-Dodecanol; B1: Acetic acid glacial; B2: Octanoic acid; B3: Linoleic acid; C1: Ethyl formate; C2: Ethyl acetate; C3: Ethyl lactate; C4: Ethyl butyrate; C5: Isoamyl acetate; C6: Ethyl valerate; C7: Ethyl caproate; C8: Ethyl 2-hydroxyhexanoate; C9: Diethyl succinate; C10: Ethyl heptanoate; C11: Isobutyl hexanoate; C12: Ethyl caprylate; C13: Octyl acetate; C14: Phenethyl acetate; C15: Ethyl nonanoate; C16: Octanoic acid,2-methylpropyl; C17: Ethyl caprate; C18: 3-Methylbutyl octanoate; C19: Isobutyl caprate; C20: Ethyl laurate; C21: Isoamyl decanoate; C22: Ethyl myristate; C23: Ethyl hexadec-9-enoate; C24: Ethy l Palmitate; C25: Ethyl elaidate; C26: Ethyl stearate; C27: Ethyl linoleate.

(LE koji) were derived from the koji, while the remaining dominant bacterial genera such as *Enterobacter* (AF and LE koji) were probably derived from the raw materials or the brewing environment.

The alpha and beta diversity of the microbial community within the six koji are shown in Table 2 and Fig. 2, respectively. Based on the scatter plots and clustering results (Fig. 2A&B), the six koji can roughly be divided into three categories.

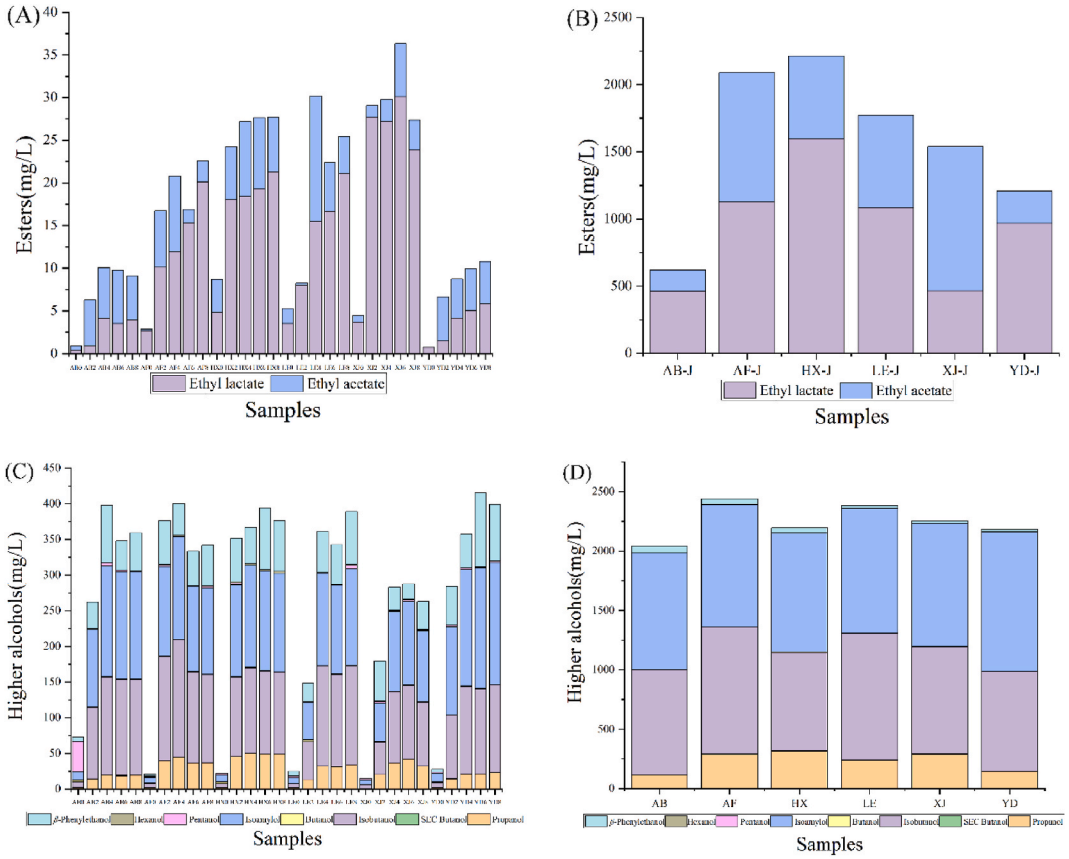


Fig. 4. The content of ethyl acetate, ethyl lactate and higher alcohols in the six koji brewing processes and corresponding liquors (A: esters in the brewing processes; B: esters in the liquors; C: higher alcohols in the brewing processes; D: higher alcohols in the liquors).

3.2. Physicochemical properties and volatile fractions of the six koji and their brewing products

The physicochemical properties of the six koji were measured (Table 3) and they are within the ranges required in the relevant Chinese national standards [33]. Overall, the liquefaction and saccharification performance varied widely between the tested koji. According to the ingredient lists of the koji (Table 1), compound enzyme preparations (α-amylase, glucoamylase, and phytase) were added to the AB koji and the YD koji was enhanced with selected functional strains, which corresponded to their higher liquefaction and saccharification performance than the other koji. YD koji exhibited a relatively high alcohol yield, which seems related to its high liquefaction and saccharification performance.

The sensory scores of the liquors made with the six koji (Table 3) showed that the LE liquor had the highest Appearance score, the YD liquor had the highest Aroma and Mouth feel scores, and the AB and HX liquors scored the highest in Taste. Upon totaling the scores, YD liquor scored the highest, XJ liquor scored the lowest, and the other four liquors achieved similar scores in between.

A total of 60 volatile flavor compounds, including 16 alcohols, 27 esters, 3 acids, 4 aldehydes, and 10 hydrocarbons, were detected in the liquors brewed using the six koji. The results of PCA analysis (Fig. 3A) showed that the flavor compound composition of HX-J liquor significantly differed from those of the other five liquors. The HX-J liquor contained relatively high levels of flavor components (Fig. 3B) such as 1-butanol (A3), 2-methyl-1-butanol (A10), 3-ethoxy-1-propanol (A11), glacial acetic acid (B1), isoamyl acetate (C5), ethyl 9-hexadecenoate (C23), ethyl palmitate (C24), ethyl elaidate (C25), ethyl stearate (C26), and ethyl linoleate (C27).

The ethyl acetate and ethyl lactate contents in the brewing processes increased initially and stabilized in the later stages (Fig. 4A), which was similar to the results reported by Liu et al. [34]. After concentrating by distillation, the levels of the two esters in the liquors were significantly elevated, and the levels roughly corresponded to those in the late fermentation stages (Fig. 4B). The higher alcohols in both the brewing processes and the liquors were dominated by isobutanol and isoamyl alcohol, followed by n-propanol and β-phenylethanol, which was consistent with that reported by Hu et al. [35]. The total amount of higher alcohols in the brewing processes also increased mainly in the early stage (Fig. 4C), which agrees with a previous report [36]. The contents of the higher alcohols in the liquors also increased greatly after being concentrated by distillation (Fig. 4D). The contents of higher alcohols in Xiaoqu Baijiu usually ranges from 600 to 2500 mg/L [36], and the six liquors produced in our study contained relatively high level of higher alcohols.

The correlation analysis showed that significant positive correlation existed between the aroma score of the liquors and the ratio of

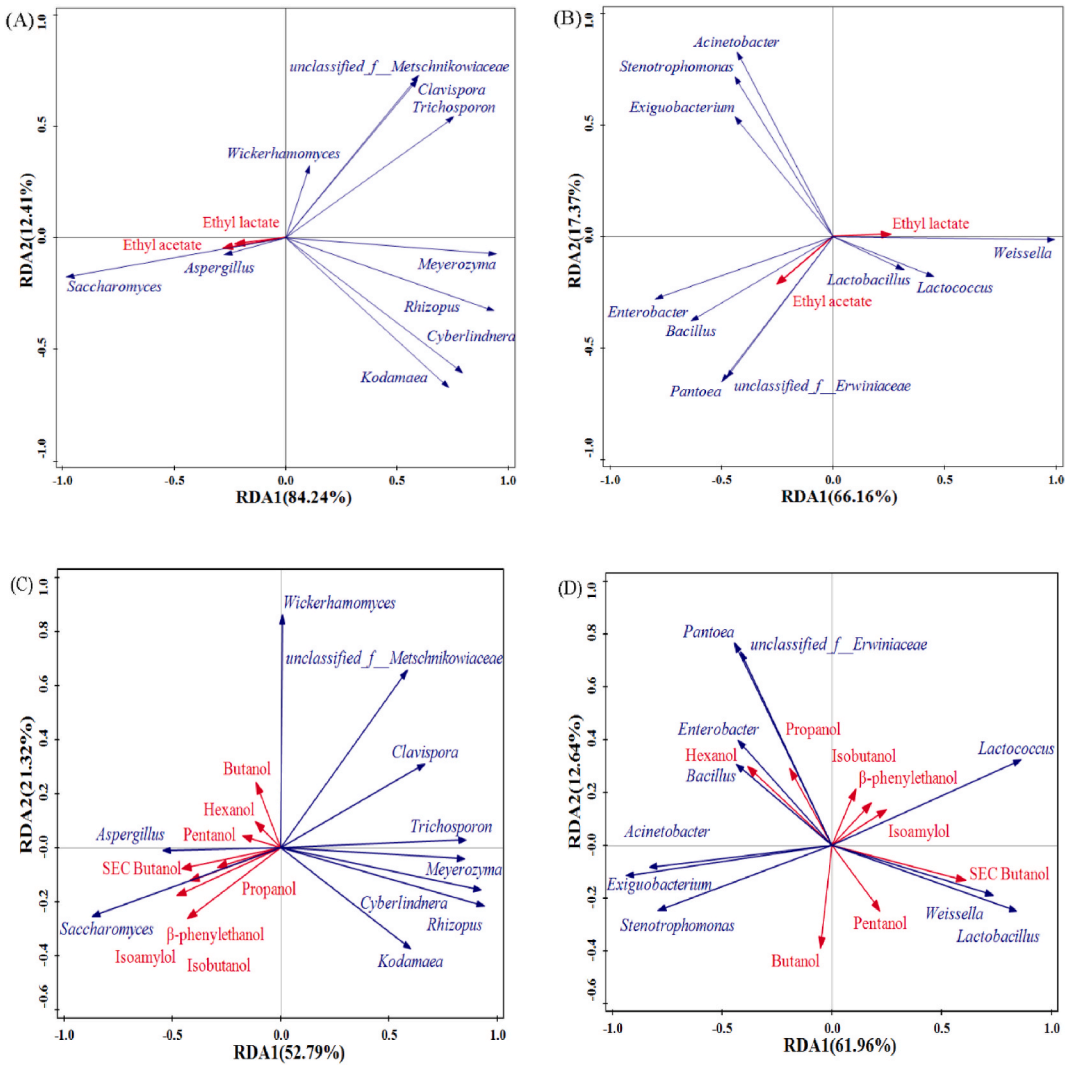


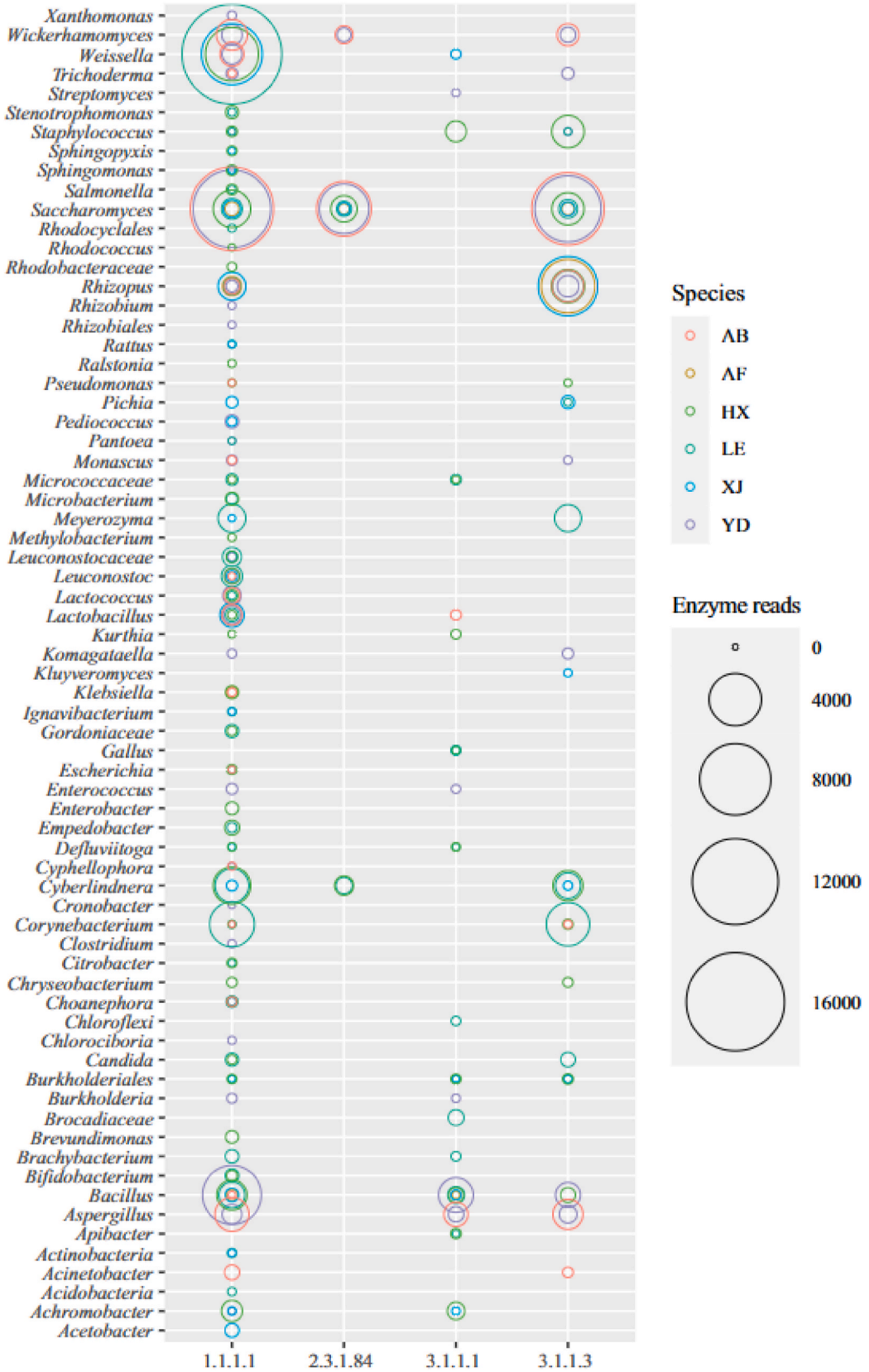
Fig. 5. Redundant analysis of ethyl acetate, ethyl lactate and higher alcohols with top ten microorganisms in abundance during winemaking (A: Acetate/Fungal; B: Acetate/Bacterial; C: Higher alcohols/Fungal; D: Higher alcohols/Bacterial).

ethyl acetate and ethyl lactate (correlation coefficient = 0.831, $p < 0.05$). The ethyl acetate and ethyl lactate contents of the liquors made from the six koji ranged from 461 to 1,598 mg/L and 159 to 1,074 mg/L, respectively, and the ratio of ethyl acetate and ethyl lactate was 0.4–4.0. It was previously reported that the ethyl acetate and ethyl lactate contents in most of the Xiaoqu Baijiu were 497–1500 mg/L and 263–1400 mg/L, respectively, and the ratio of ethyl acetate and ethyl lactate was 1.1–2.3 [3,37–39]. While the contents of ethyl acetate and ethyl lactate in the liquors in our study were close to those previously reported, the ratios of ethyl acetate and ethyl lactate range much wider, and the higher ratios corresponds to higher aroma scores of the liquor.

3.3. Relationships between ethyl acetate, ethyl lactate and higher alcohols and microorganisms in the koji-mediated brewing processes

As mentioned above, ethyl acetate and ethyl lactate are closely related to the aroma of the Xiaoqu Baijiu, and the higher alcohols are related to both the flavor and safety of the product. The formation of these components is largely determined by the metabolism of the koji microbes and the brewing process. Redundancy analysis was performed on the ethyl acetate and ethyl lactate in the six brewing processes and the top ten abundant fungal and bacterial genera. *Saccharomyces* and *Aspergillus* were positively correlated with these two esters; the bacterial genera *Weissella, Lactobacillus*, and *Lactococcus* were positively correlated with ethyl lactate while *Enterobacter*, *Bacillus*, *Pantoea*, and unclassified *Erwiniaceae* were positively correlated with ethyl acetate (Fig. 5A&B).

The annotation of Kyoto Encyclopedia of Genes and Genomes metabolic pathways from the metagenomics data of the six koji indicated that the abundance of genes encoding esterases was the highest in *Saccharomyces*, followed by *Weissella*, *Rhizopus*, *Aspergillus*, *Wickerhamomyces*, *Lactobacillus*, *Bacillus*, and *Cyberlindnera* (Fig. 6). In summary, *Saccharomyces* made a substantial contribution to the production of both esters during the brewing process, which was consistent with that reported in previous study [10]. The lactic acid



(caption on next page)

Fig. 6. Enzyme genes and microbial distribution associated with the formation of ethyl acetate, ethyl lactate in six koji.
Note: Ethanol and organic acids can be catalyzed by alcohol dehydrogenase (EC1.1.1.1), alcohol acetyltransferase (EC2.3.1.84), carboxylesterase (EC3.1.1.1), and lipase (EC3.1.1.3) to produce the ethyl esters of the corresponding organic acids. Branched-chain-amino-acid transaminase (EC2.6.1.42) can catalyze the conversion of valine and leucine to their respective α-keto acids, and valine-pyruvate transaminase (EC2.6.1.6) can also convert valine to its α-keto acid. The conversion of phenylalanine to its respective α-keto acid is catalyzed by aromatic-amino-acid transaminase I (EC2.6.1.27, EC2.6.1.57), aromatic-amino-acid transaminase II (EC2.6.1.58), and D-amino-acid IAP transaminase (EC2.6.1.9). IAP transaminase (EC2.6.1.9) and threonine deaminase (EC4.3.1.19) are also involved in the transamination reaction in the Ehrlich pathway. α-Keto acids are converted to their corresponding aldehydes by the decarboxylation reaction, which is catalyzed by decarboxylase (EC4.1.1.-). Aldehydes are further converted to higher alcohols by alcohol dehydrogenase (EC1.1.1.1), aryl-alcohol dehydrogenase (EC1.1.1.90) and lactaldehyde dehydrogenase (EC1.1.1.283).

production capacity and esterases of lactic acid bacteria such as *Weissella*, and *Lactobacillus* were largely correlated with ethyl lactate synthesis while the acetic acid production capacity and esterases of *Bacillus* favored ethyl acetate synthesis [40]. Aerobic microorganisms such as *Aspergillus* and *Rhizopus* were gradually eliminated during the brewing process due to anaerobic environment and their roles in ester formation have yet to be investigated.

The redundancy analysis of higher alcohols in the liquors brewed with the six koji and the top ten fungal and bacterial genera in abundance showed that the fungi *Saccharomyces* and *Aspergillus* were positively correlated with most of the higher alcohols such as pentanol, propanol, isoamylol, β-phenylethanol, and sec-butanol. Among the bacterial genera, *Bacillus*, *Enterobacter*, *Pantoea* and

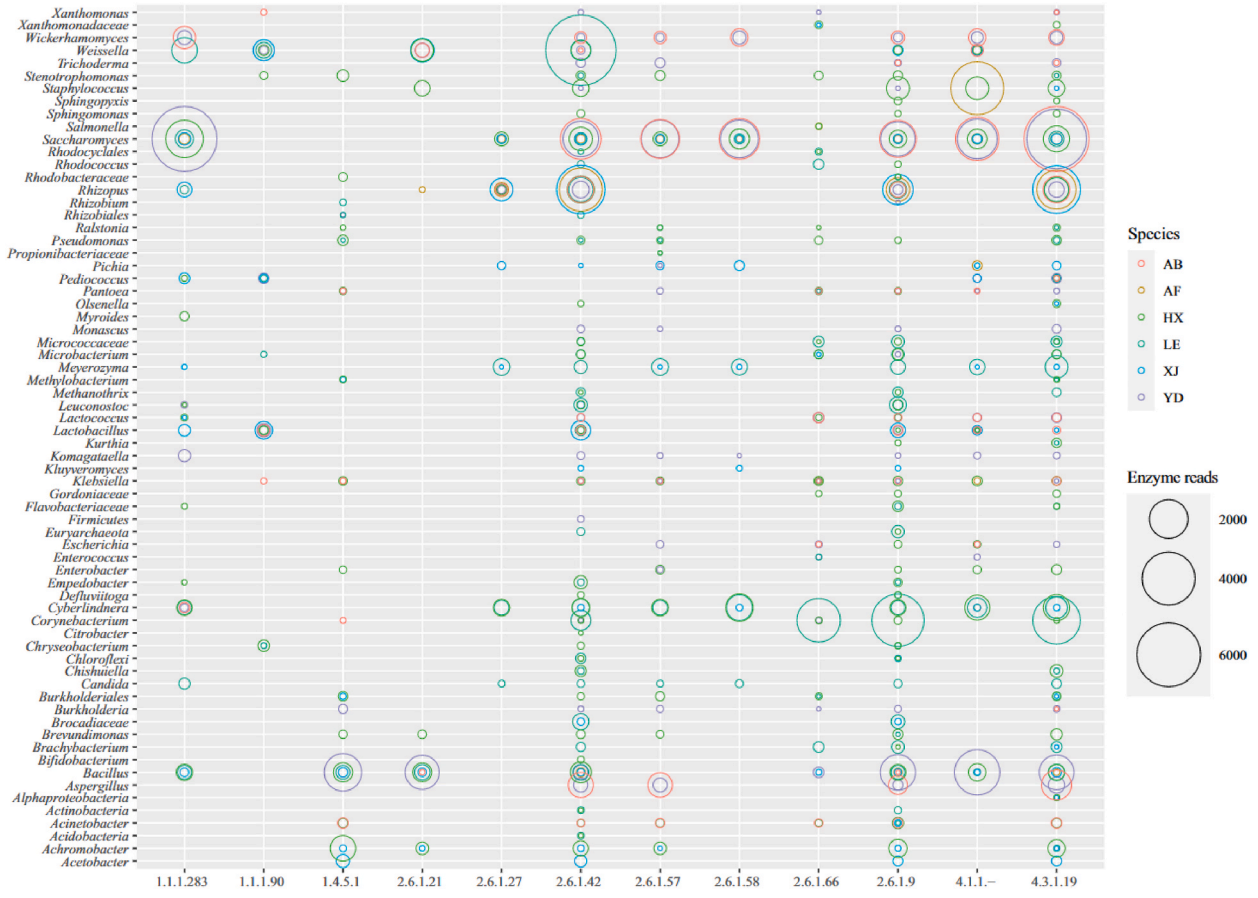


Fig. 7. Enzyme genes and microbial distribution associated with the formation of ethyl acetate, higher alcohol in six koji.
Note: Ethanol and organic acids can be catalyzed by alcohol dehydrogenase (EC1.1.1.1), alcohol acetyltransferase (EC2.3.1.84), carboxylesterase (EC3.1.1.1), and lipase (EC3.1.1.3) to produce the ethyl esters of the corresponding organic acids. Branched-chain-amino-acid transaminase (EC2.6.1.42) can catalyze the conversion of valine and leucine to their respective α-keto acids, and valine-pyruvate transaminase (EC2.6.1.6) can also convert valine to its α-keto acid. The conversion of phenylalanine to its respective α-keto acid is catalyzed by aromatic-amino-acid transaminase I (EC2.6.1.27, EC2.6.1.57), aromatic-amino-acid transaminase II (EC2.6.1.58), and D-amino-acid IAP transaminase (EC2.6.1.9). IAP transaminase (EC2.6.1.9) and threonine deaminase (EC4.3.1.19) are also involved in the transamination reaction in the Ehrlich pathway. α-Keto acids are converted to their corresponding aldehydes by the decarboxylation reaction, which is catalyzed by decarboxylase (EC4.1.1.-). Aldehydes are further converted to higher alcohols by alcohol dehydrogenase (EC1.1.1.1), aryl-alcohol dehydrogenase (EC1.1.1.90) and lactaldehyde dehydrogenase (EC1.1.1.283).

unclassified *Erwiniaceae* were positively correlated with hexanol and propanol; *Lactococcus* was positively correlated with isobutanol, β-phenylethanol, and isoamylol; *Weissella* and *Lactobacillus* were positively correlated with sec-butanol and negatively correlated with hexanol, and propanol (Fig. 5 C&D).

The annotated data from the metagenomics analysis of the koji showed that the microorganisms associated with higher alcohol synthesis were mainly *Saccharomyces*, followed by *Rhizopus*, *Aspergillus*, *Weissella*, *Bacillus*, *Staphylococcus*, *Wickerhamomyces*, *Cyberlindnera*, *Corynebacterium,* and *Lactobacillus* (Fig. 7). In brief, *Saccharomyces* plays a major role in higher alcohol formation, which is similar to previous reports [41]. The participation of *Bacillus* and *Lactobacillus* in higher alcohol production has also been reported [42–44]. The relationship between molds such as *Aspergillus* and higher alcohol production has not been previously reported. The annotation data from the metagenomic analysis in this study indicated that *Saccharomyces* is associated with a rather comprehensive range of enzymes associated with higher alcohol synthesis. Considering the absolute dominance of *Saccharomyces* in the brewing process (Fig. 1), its performance and metabolic regulation are closely related to the content of higher alcohols in the liquor. Other microorganisms in the koji (e.g., *Weissella*) were also annotated with genes related to the production of the higher alcohols, but only limited enzymes (e.g., EC1.1.1.1). Their role in higher alcohol production needs to be further investigated.

Although only a few are commercially available, many kinds of Xiaoqu are used in liquor making in China. For further understanding the influence of Xiaoqu on liquor making, more samples may be required. Compared with metagenomic analysis, metatranscriptomic analysis or RT-qPCR, which can provide quantitative information about gene transcription related with key enzymes, will be helpful for find the real roles that the microbes from koji played in liquor brewing.

4. Conclusions

The composition of microbial communities in the six commercially available koji varied, with *Rhizopus*, *Aspergillus* (AF, XJ, and LE koji), and *Bacillus* (AB, YD, and LE koji) primarily associated with starch hydrolysis, while *Saccharomyces* was the major alcohol producer. In the brewing processes using the six koji, *Saccharomyces* was the overwhelmingly dominant fungal genera and *Weissella*, *Bacillus,* and *Acinetobacter* were the predominant bacterial genera. There were variations in the brewing indicators and volatile fractions between the brewing process of the six koji. Ethyl acetate, ethyl lactate, and higher alcohols were primarily produced in the early fermentation stage, and their concentrations were substantially elevated after distillation. The results of metagenomic and redundancy analyses indicated that *Saccharomyces* in the koji was closely related to the production of ethyl acetate, ethyl lactate and higher alcohols during the brewing process while *Weissella* and *Lactobacillus* were closely related to the production of ethyl lactate, and *Bacillus* was related to the formation of ethyl acetate. Among the six commercially available koji, the HX koji has better performance due to its high ester content, low higher alcohol content, excellent level of sensory score and physicochemical indexes for liquor making.

Author contribution statement

Yu Xiaoyang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Huang Tingting: Performed the experiments; Contributed reagents, materials, analysis tools or data. Huang Zhijiu: Analyzed and interpreted the data. Wu Zhengyun: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. Che Jingwei: Analyzed and interpreted the data. Qin Fengyang: Performed the experiments. Zhang Wenxue: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

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.

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References

[1] M. Liu, Y. Tang, K. Zhao, Y. Liu, X. Guo, D. Ren, X. Zhang, Determination of the fungal community of pit mud in fermentation cellars for Chinese strong-flavor liquor, using DGGE and Illumina MiSeq sequencing, Food Res. Int. 91 (2017) 80–87, https://doi.org/10.1016/j.foodres.2016.11.037.

[2] B. Lin, J. Tang, Q. Yang, Z.X. Su, L.P. Zhu, Q. Li, S.X. Chen, Microbial succession and its effect on key aroma components during light-aroma-type Xiaoqu Baijiu brewing process, World J. Microbiol. Biotechnol. 38 (10) (2022), https://doi.org/10.1007/s11274-022-03353-x.

[3] S.X. Chen, G. Perez-Samper, B. Herrera-Malaver, L.P. Zhu, Y.C. Liu, J. Steensels, K.J. Verstrepen, Breeding of new Saccharomyces cerevisiae hybrids with reduced higher alcohol production for light-aroma-type-xiaoqu Baijiu production, J. Am. Soc. Brew. Chem. (2022), https://doi.org/10.1080/03610470.2022.2033608.

[4] W. Ma, X. Geng, F. Jia, X. Zhang, Y. Zhang, J. Xue, Investigation of microbial composition and functional characterization of Zangqu using high throughput sequencing, LWT–Food Sci. Technol. 136 (2021), 110342, https://doi.org/10.1016/j.lwt.2020.110342.

[5] M. Gou, H.Z. Wang, H.W. Yuan, W.X. Zhang, Y.Q. Tang, K. Kida, Characterization of the microbial community in three types of fermentation starters used for Chinese liquor production, J. Inst. Brew. 121 (4) (2015) 620–627, https://doi.org/10.1002/jib.272.

[6] Q.X. Tang, G.Q. He, J. Huang, C.D. Wu, Y. Jin, R.Q. Zhou, Characterizing relationship of microbial diversity and metabolite in Sichuan Xiaoqu, Front. Microbiol. 10 (2019), https://doi.org/10.3389/fmicb.2019.0069.

[7] C. Zhao, W. Su, Y. Mu, Y.C. Mu, L. Jiang, Integrative metagenomics-metabolomics for analyzing the relationship between microorganisms and non-volatile profiles of traditional Xiaoqu, Front. Microbiol. 11 (2021), https://doi.org/10.3389/fmicb.2020.617030.

[8] T.Q. Pan, W. Cheng, J. Zhang, N. Li, B. Peng, Z.L. Gong, L.H. Wu, H.P. Wu, Application of angel Rhizopus starter in the production of Xiaoqu Qingxiang Baijiu, liquor-making, Sci. Technol. (10) (2019) 116–123, https://doi.org/10.13746/j.njkj.2019206.

[9] H.A. Sakandar, R. Hussain, Q. Farid Khan, H. Zhang, Functional microbiota in Chinese traditional Baijiu and Mijiu Qu (starters): a review, Food Res. Int. 138 (2020), 109830, https://doi.org/10.1016/j.foodres.2020.109830.

[10] F.J. Reyes-Sanchez, J.B. Paez-Lerma, J.A. Rojas-Contreras, J. Lopez-Miranda, N.O. Soto-Cruz, M. Reinhart-Kirchmayr, Study of the enzymatic capacity of *Kluyveromyces marxianus* for the synthesis of esters, J. Mol. Microbiol. Biotechnol. 29 (1–6) (2020) 1–9, https://doi.org/10.1159/000507551.

[11] Y.P. Wang, Z.G. Sun, C.Y. Zhang, Q.Z. Zhang, X.W. Guo, D.G. Xiao, Comparative transcriptome analysis reveals the key regulatory genes for higher alcohol formation by yeast at different α-amino nitrogen concentrations, Food Microbiol. 95 (2021), 103713, https://doi.org/10.1016/j.fm.2020.103713.

[12] J. Jiang, Y.C. Liu, H.H. Li, Q. Yang, Q. Wu, S.X. Chen, Y. Xu, Modeling and regulation of higher alcohol production through the combined effects of the C/N ratio and microbial interaction, J. Agric. Food Chem. 67 (38) (2019) 10694–10701, https://doi.org/10.1021/acs.jafc.9b04545.

[13] F.Y. Qin, Z.Y. Wu, W.X. Zhang, Evaluation of six commercial koji on the formation of biogenic amines and higher alcohols in rice wine, J. Inst. Brew. 128 (2022) 124–132, https://doi.org/10.1002/jib.696.

[14] S. Liu, L. Yang, Y. Zhou, S.D. He, J.L. Li, H.J. Sun, S.F. Yao, S.Y. Xu, Effect of mixed moulds starters on volatile flavor compounds in rice wine, LWT–Food Sci. Technol. 112 (2019), 108215, https://doi.org/10.1016/j.lwt.2019.05.113.

[15] C. Zhao, W. Su, Y.C. Mu, L. Jiang, Y. Mu, Correlations between microbiota with physicochemical properties and volatile flavor components in black glutinous rice wine fermentation, Food Res. Int. 138 (2020), 109800, https://doi.org/10.1016/j.foodres.2020.109800.

[16] S.H. Gan, F. Yang, S.K. Sahu, R.Y. Luo, S.L. Liao, H.Y. Wang, H. Liu, Deciphering the composition and functional profile of the microbial communities in Chinese moutai liquor starters, Front. Microbiol. 10 (2019), https://doi.org/10.3389/fmicb.2019.01540.

[17] Y. Chang, Y.J. Luo, C.J. Qian, Analysis of relationship between flavor and quality of fuyu flavor Baijiu by two comprehensive dimensional gas chromatography-mass spectrometry, J. Food Sci. Technol. 37 (6) (2019) 64–70.

[18] X.F. Ding, C.D. Wu, J. Huang, R.Q. Zhou, Characterization of interphase volatile compounds in Chinese Luzhou-flavor liquor fermentation cellar analyzed by head space-solid phase micro extraction coupled with gas chromatography mass spectrometry (HS-SPME/GC/MS), LWT–Food Sci. Technol. 66 (2016) 124–133, https://doi.org/10.1016/j.lwt.2015.10.024.

[19] A. Tavasoli, S. Karimi, M. Davari, N. Nasrollahi, T. Nematian, Enhancement of MoO3–K2O/CNTs nanocatalyst activity and selectivity in higher alcohols synthesis using microemulsion technique, J. Ind. Eng. Chem. 20 (2) (2014) 674–681, https://doi.org/10.1016/j.jiec.2013.05.032.

[20] QB/T 4259-2011, Strong flavor Daqu, Beijing, China, 2011.

[21] H.C. Wu, S.Y. Zhang, Y.Y. Ma, J. Zhou, H.B. Luo, J.G. Yang, Comparison of microbial communities in the fermentation starter used to brew Xiaoqu liquor, J. Inst. Brew. 123 (1) (2017) 113–120, https://doi.org/10.1002/jib.388.

[22] B. Chen, Q. Wu, Y. Xu, Filamentous fungal diversity and community structure associated with the solid state fermentation of Chinese Maotai-flavor liquor, Int. J. Food Microbiol. 179 (2014) 80–84, https://doi.org/10.1016/j.ijfoodmicro.2014.03.011.

[23] Y. Jin, D. Li, M. Ai, Q. Tang, J. Huang, X. Ding, R. Zhou, Correlation between volatile profiles and microbial communities: a metabonomic approach to study Jiang-flavor liquor Daqu, Food Res. Int. 121 (2019) 422–432, https://doi.org/10.1016/j.foodres.2019.03.021.

[24] L. De Vuyst, H. Harth, S. Van Kerrebroeck, F. Leroy, Yeast diversity of sourdoughs and associated metabolic properties and functionalities, Int. J. Food Microbiol. 239 (2016) 26–34, https://doi.org/10.1016/j.ijfoodmicro.2016.07.018.

[25] M.S. Zha, B.G. Sun, Y.P. Wu, S. Yin, C.T. Wang, Improving flavor metabolism of Saccharomyces cerevisiae by mixed culture with *Wickerhamomyces anomalus* for Chinese Baijiu making, J. Biosci. Bioeng. 126 (2) (2018) 189–195, https://doi.org/10.1016/j.jbiosc.2018.02.010.

[26] Y.Y. Li, Y.X. Cheng, H. Wang, X.X. Hu, L. Wang, Y.G. Huang, Diverse structure and characteristics of the fungal community during the different rounds of Jiang-flavoured Baijiu production in Moutai town, LWT–Food Sci. Technol. 161 (2022), https://doi.org/10.1016/j.lwt.2022.113313.

[27] K. Bellut, M. Michel, M. Zarnkow, M. Hutzler, F. Jacob, J.J. Atzler, E.K. Arendt, Screening and application of Cyberlindnera yeasts to produce a fruity, non-alcoholic beer, Fermentation 5 (4) (2019), https://doi.org/10.3390/fermentation5040103.

[28] N. Isono, H. Hayakawa, A. Usami, T. Mishima, M. Hisamatsu, A comparative study of ethanol production by Issatchenkia orientalis strains under stress conditions, J. Biosci. Bioeng. 113 (1) (2012) 76–78, https://doi.org/10.1016/j.jbiosc.2011.09.004.

[29] Y.L. Li, S.P. Liu, S.Y. Zhang, T.T. Liu, H. Qin, C.H. Shen, J. Mao, Spatiotemporal distribution of environmental microbiota in spontaneous fermentation workshop: the case of Chinese Baijiu, Food Res. Int. 156 (2022), https://doi.org/10.1016/j.foodres.2022.111126.

[30] C.T. Zhu, Y.X. Cheng, Q.C. Zuo, Y.G. Huang, L. Wang, Exploring the impacts of traditional crafts on microbial community succession in Jiang-flavored Daqu, Food Res. Int. 158 (2022), https://doi.org/10.1016/j.foodres.2022.111568.

[31] H. Du, X. Wang, Y. Zhang, Y. Xu, Exploring the impacts of raw materials and environments on the microbiota in Chinese Daqu starter, Int. J. Food Microbiol. 297 (2019) 32–40, https://doi.org/10.1016/j.ijfoodmicro.2019.02.020.

[32] L. Wang, Y.G. Huang, X.X. Hu, Y.Y. Li, The impact of environmental factors on the environmental bacterial diversity and composition in the Jiang-flavoured Baijiu production region, LWT–Food Sci. Technol. 149 (2021), 111784, https://doi.org/10.1016/j.lwt.2021.111784.

[33] GB/T 10781.3-2006, Rice-flavored liquor, Beijing, China, 2006, 2006.

[34] J.J. Liu, Y. Chen, G. Fu, Y. Chen, Y. Wan, M. Deng, M. Li, Improvement of the flavor of major ethyl ester compounds during Chinese Te-flavor Baijiu brewing by *Wickerhamomyces anomalus*, Food Biosci. 50 (2022), 102022, https://doi.org/10.1016/j.fbio.2022.102022.

[35] Y.L. Hu, Q. Yang, D. Chen, B. Fu, Y. Zhang, Y. Zhang, S. Zhao, Study on microbial communities and higher alcohol formations in the fermentation of Chinese Xiaoqu Baijiu produced by traditional and new mechanical technologies, Food Res. Int. 140 (2021), 109876, https://doi.org/10.1016/j.foodres.2020.109876.

[36] Z.G. Sun, L. Liu, Y.P. Wang, X.S. Wang, D.G. Xiao, Higher alcohols metabolism by Saccharomyces cerevisiae: a mini review, Chin. J. Biotechnol. 37 (2) (2021) 429–447, https://doi.org/10.13345/j.cjb.200302.

[37] S. Chen, J. Tang, S.S. Fan, J. Zhang, S.X. Chen, Y.C. Liu, Y. Xu, Comparison of potent odorants in traditional and modern types of Chinese Xiaoqu liquor (Baijiu) based on odor activity Values and multivariate analyses, Foods 10 (10) (2021), https://doi.org/10.3390/foods10102392.

[38] Y.W. Niu, Z.M. Yao, Z.B. Xiao, G.Y. Zhu, J.C. Zhu, J.Y. Chen, Sensory evaluation of the synergism among ester odorants in light aroma type liquor by odor threshold, aroma intensity and flash GC electronic nose, Food Res. Int. 113 (2018) 102–114, https://doi.org/10.1016/j.foodres.2018.01.018.

[39] X.Z. Sun, Q.Q. Qian, Y.Q. Xiong, Q.Q. Xie, X.X. Yue, J.H. Liu, Q. Yang, Characterization of the key aroma compounds in aged Chinese Xiaoqu Baijiu by means of the sensomics approach, Food Chem. 384 (2022), https://doi.org/10.1016/j.foodchem.2022.132452.

[40] J.L. Wang, Dynamic Changes of Microbial Communities and Screening of Dominant Mold during the Preparation of Daqu of Shanxi Aged Vinegar, 2019, https://doi.org/10.27285/d.cnki.gsxnu.

[41] Y.P. Wang, X.Q. Wei, X.W. Guo, D.G. Xiao, Effect of the deletion of genes related to amino acid metabolism on the production of higher alcohols by Saccharomyces cerevisiae, BioMed Res. Int. (2020), https://doi.org/10.1155/2020/6802512.

[42] J.H. Hu, J.Y. Yu, Y.G. Liu, W. Li, M.G. Zhang, Y.Q. Li, S. Chen, N. Li, X.L. Han, Correlation between sensory quality and flavor composition of Qingxiang Baijiu of different distillates, liquor-making, Sci. Technol. (5) (2020) 32–37, https://doi.org/10.13746/j.njkj.2019338.
[43] G.Y. Qu, J. Tang, J. Jiang, Q. Yang, Y.C. Liu, Q. Wu, S.X. Chen, Y. Xu, Metabolism characteristics of higher alcohols synthesized by microbiota in the fermentation process of light aroma type Baijiu started by Xiaoqu, Food Ferment. Ind. 47 (11) (2021) 32–37, https://doi.org/10.13995/j.cnki.11-1802/ts.025697.
[44] H.D. Zhang, J. Han, X.Q. Wei, L. Liu, X.W. Guo, D.G. Xiao, Effects of Lactobacillus plantarum on alcohol fermentation and ester-alcohol metabolism of Saccharomyces cerevisiae with high-yield ester, China Brew. 38 (11) (2019) 85–89.