



A quality comparison for Xiecun Huangjiu with different aging stages based on chemical profile, aroma composition and microbial succession

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ABSTRACT

The Xiecun Huangjiu (XCHJ), an exemplary representation of North Huangjiu, exhibits a distinct and invigorating aroma predominantly formed during its aging period. In this study, we observed dynamic changes in 16 key aroma compounds by gas chromatography-mass spectrometry (GC-MS) during the aging of XCHJ, with compounds such as phenethylalcohol, isoamylalcohol, benzaldehyde, and ethylbenzoate initially increasing and then decreasing. Ultra-Performance Liquid Chromatography (UPLC) detected nineteen amino acids, with total content ranging from 1901.45 to 3764.45 mg/L. High-throughput sequencing indicated that *Pseudomonas*, *Ochrobactrum*, *Moesziomyces* and *Aspergillus* et al. were abundant in aged XCHJ. Totally, 4 bacteria and 8 fungi exhibited strong associations with aroma compounds production. Physicochemical properties were primarily interacted with *Pseudomonas*, *Aspergillus*, *Pseudeurotium*, *Thermomyces*, *Bacteroides* and *Blautia*. Furthermore, co-occurrence network analysis highlighted significant interactions between *Pantoea*, *Rhodotorula*, *Monascus*, and amino acids. These findings provide valuable insights for the regulation of aroma in aged XCHJ.

Introduction

Huangjiu, the fermented alcoholic beverage often referred to as Chinese rice wine, has garnered significant popularity among consumers in East Asia. Its appeal lies in its unique flavor profile and rich nutritional heritage, boasting a history that extends back over 5000 years (Yang et al., 2022). In China alone, its annual consumption surpasses 2 million kiloliters (Yu et al., 2020). Xiecun Huangjiu (XCHJ), representing one of the most characteristic examples of North Huangjiu with recognized health benefits, is traditionally crafted from glutinous rice, Wheat Qu, and yeast, in addition to 18 kinds of Chinese herbs (including *Angelica dahurica*, *Asarum sieboldii* Miq and *Gardenia jasminoides* Ellis and so on) in an open environment. These raw materials need to undergo fermentation and extended aging periods (exceeding 3 years) to yield high-quality products suitable for the market.

The evaluation of XCHJ involves various criteria, among which flavor holds significant importance. Fermentation is responsible for the generation of numerous flavor-related compounds derived from sugars, proteins, and various other constituents. Nonetheless, it is essential to recognize that aging also plays a pivotal role in the continued enhancement of XCHJ's overall quality. As widely recognized, wines

tend to develop more pronounced fragrances with extended aging. In general, fresh Huangjiu often presents a pronounced astringency, subtle aroma, and occasionally even a bitter taste. However, these inherent deficiencies could be alleviated through the aging process, which instigates physiochemical reactions leading to alterations in the flavor profile of matured Chinese rice wine (Huangjiu) (Tao, García, & Sun, 2014). Currently, over 900 volatile compounds have been detected in Huangjiu, including esters, alcohols, and aldehydes etc. (Chen, Xu, & Qian, 2018). Volatile compounds could undergo dynamic changes during the aging process. The certain compounds generated by fermentation have the potential to undergo further transformations through aging. For instance, alcohols can undergo oxidation to yield aldehydes (Cao, Xie, Wu, & Lu, 2010). Additionally, condensation reactions between acids and alcohols may lead to the formation of esters (Yang et al., 2020). Moreover, the metabolism of bacteria or fungi such as enzymes and organic acid could cause the variation of some aroma precursor substances through the degradation of raw materials or alterations in environmental condition (Liu, Chen, Fan, Huang, & Han, 2018; Yu et al., 2023). Among the plethora of volatiles, only a subset known as aromatic compounds play a pivotal role in shaping the perception of aroma, thereby partially reflecting the aromatic characteristics inherent to food.

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By tracking these key aroma compounds, it becomes possible to gain insights into the aromatic profile of matured Huangjiu, establishing valuable benchmarks for quality control in preserving the aromatic characteristics of XCHJ. In previous studies, our research team has selected the 16 key aroma-active compounds in the aged XCHJ through gas chromatography-mass spectrometry-olfactometry/detection frequency (GC-MS-O/DF) coupled with odor activity values (OAV) analysis (Li et al., 2023). Based on this foundation, the present study further elucidated the alterations in 16 compounds during the aging process.

Microorganisms play a pivotal role in the formation of aroma compounds in Huangjiu, and their composition could directly or indirectly influence the flavor profile of the beverage. Each microbe within this ecosystem can exert its dominant function in the fermented products. For example, *Saccharomyces* played a crucial role in the generation of alcohol (Tian, Zeng, Fang, Zhou, & Du, 2022). *Bacillus* was responsible for the formation of esters and pyrazines (Huang et al., 2018). Throughout the aging process, microbial community succession may occur due to fluctuations in environmental factors such as water content, acidity, and oxygen levels. Nowadays, many studies paid close attention to microbiota composition during fermentation of Huangjiu. Wang, Wu, Wang, and Ren (2023) found *Pediococcus*, *Pantoea*, *Weissella*, *Saccharomyces* and *Rhizopus* were the dominant genera when broomcorn millet Huangjiu was brewing. *Lactococcus* and *Saccharomyces* played the primary role on fermentation of northern Huangjiu using the high-throughput sequencing (Yan et al., 2022). Moreover, Ren et al. (2020) identified four bacteria, namely *Lactococcus*, *Virgibacillus*, *Sphingobacterium* and *Sporolactobacillus*, as the key contributors to flavor generation during the fermentation of corn-based Huangjiu. In regard to the aging process, researchers have already turned their focus towards the microbial flora structure of Huangjiu during this stage. An investigation pointed that *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were abundant in aged Huangjiu (Yu et al., 2023). Nevertheless, limited information exists regarding the dynamic succession of microorganisms, particularly the scarce studies that uncover the potential relationships between microorganisms, physicochemical properties, and flavor during the aging process of Huangjiu. Revealing these intrinsic interactions is crucial for achieving precise control over the specific quality changes that occur during the aging process of Huangjiu.

Thus, the objectives of this study were: (1) to monitor the changes of 16 key aroma compounds via headspace solid phase microextraction and gas chromatography mass spectrometer (HS-SPME/GC-MS), determine the characteristic physicochemical, and reveal amino acids variation by Ultra-Performance Liquid Chromatography (UPLC) in XCHJ during aging period; (2) to elucidate the bacteria and fungi community structures of different aged samples by 16S rDNA and ITS sequencing; (3) to assess the relationships between core microbiota and physicochemical, amino acids and key aroma compounds through redundancy correlation analysis (RDA), occurrence network analysis and Pearson correlation analysis, respectively.

Materials and methods

Preparation of Huangjiu samples

XCHJ samples were obtained from Qinyang Changsheng Liquor Co., Ltd., a reputable Huangjiu producer located in Hanzhong, Shaanxi Province, China. Based on common Huangjiu age on the market, selected five XCHJ samples aged for 3, 4, 6, 7, and 9 years, were named as Y3, Y4, Y6, Y7, and Y9, respectively. Each vintage Huangjiu were sampled for three batches and each batch was collected three samples. Totally, one group with the same age contained 9 samples, promptly sealed in sample vials, and preserved at -40°C until subsequent analysis.

Determination of physicochemical properties

The total acid, pH, and amino nitrogen were measured according to the Chinese national standards for Huangjiu (GB/T 13662–2018). The ethanol content was measured according to the Chinese national standards for wine (GB5009.225–2016). Total sugar content was determined by the 3,5-dinitrosalicylic acid (DNS) method as described by Zohri et al. (2017) with minor modifications (Zohri, Abdelazim, & Ibrahim, 2018). The hydrolysate sample (2 mL) was mixed with DNS reagent (2.5 mL) and incubated in boiling water for 8 min. Subsequently, the mixture was rapidly cooled to room temperature and maintain for 20 min. The absorbance intensity was subsequently measured using a Microplate Reader (SP754PC, Shanghai Spectrum Co., LTD) at 540 nm, and the calibration curve was established with glucose as the standard reagent.

Quantitative analysis of amino acids

The amino acids were extracted according to the research with some modification (Shen et al., 2022). The 1 mL sample was placed into a 2 mL centrifuge tube, followed by adding 400 μL solution (methanol:water 1:1, v/v) containing 10 % formic acid, then centrifuged at 12000 rpm for 5 min. The supernate was diluted 100 times by above same solution, and then 100 μL prepared sample along with 100 μL Trp-d3 (internal standard substance, 0.10 $\mu\text{g}/\text{mL}$) was filtrated by 0.22 mm microporous membrane. The contents of amino acids in XCHJ were determined via an Ultra-Performance Liquid Chromatography (UPLC, ACQUITY Arc, Waters, USA) system equipped with a C18 column (2.1 mm \times 100 mm, 1.7 μm particle size) according to our pre-optimized program conditions. The mobile phase consisted of 10 % methanol water solution containing 0.1 % formic acid (solvent A) and 50 % methanol water solution with 0.1 % formic acid (solvent B). The temperature of the column was set at 40°C . The gradient procedure was as follows: 0 min, 90 % A; 6.5 min, 70 % A; 7 min–14 min, 0 % A; 17.5 min, 90 % A. The flow rate was set 0.3 mL/min (0–8.0 min) and 0.4 mL/min (8.0–17.5 min). The injection volume was 5 μL . Each sample was detected in triplicates. Pure amino acid standards were employed as the external standard for qualitative and quantitative analysis. The pure amino acid standards were diluted into a series of different concentrations of standard liquids using 10 % formic acid methanol-H₂O (1:1 v/v) solution. The standard liquids were detected as described aforementioned condition. Finally, the standard curve was established with the standard solution concentration as the horizontal coordinate and the peak area ratio as the vertical coordinate.

Volatile profiles analyses

In our published research (Li et al., 2023), we have identified 16 key aroma compounds in XCHJ, so we focused on monitoring their changes during aging, and volatile compounds of XCHJ samples were analyzed by HS-SPME-GC-MS with the same experimental conditions. The XCHJ sample was diluted with ultrapure water to a final concentration of 6 % ethanol (v:v). A total of 5.0 g of the diluted Huangjiu sample and 1 μL of 1,2-dichlorobenzene (internal standard, dissolved in ethanol at a concentration of 1.306 $\mu\text{g}/\mu\text{L}$) were transferred into a 20 mL preheated Teflon/Silicon-sealed headspace vial with screw cap. Subsequently, the vial was equilibrated at 40°C in a water bath for duration of 15 min. Following this, a 50/30 μm DVB/CAR/PDMS fiber (1 cm, Bellefonte, PA, USA) was gently inserted into the headspace of the vial for the extraction of the volatile compounds at 50°C for 35 min and was rapidly desorbed in the GC injector port for 7 min at 250°C .

GC/MS analyses were performed on an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled with an Agilent 5977 mass spectrometer (7890B-5977B; Agilent Technologies, Santa Clara, CA). The capillary column of DB-WAX (30 m \times 0.25 mm \times 0.25 μm ; Agilent 122-7032) and HP-5 (30 m \times 0.25 mm \times 0.25 μm ; Agilent 19091S-433UI) were used to separate the volatile compounds of XCHJ

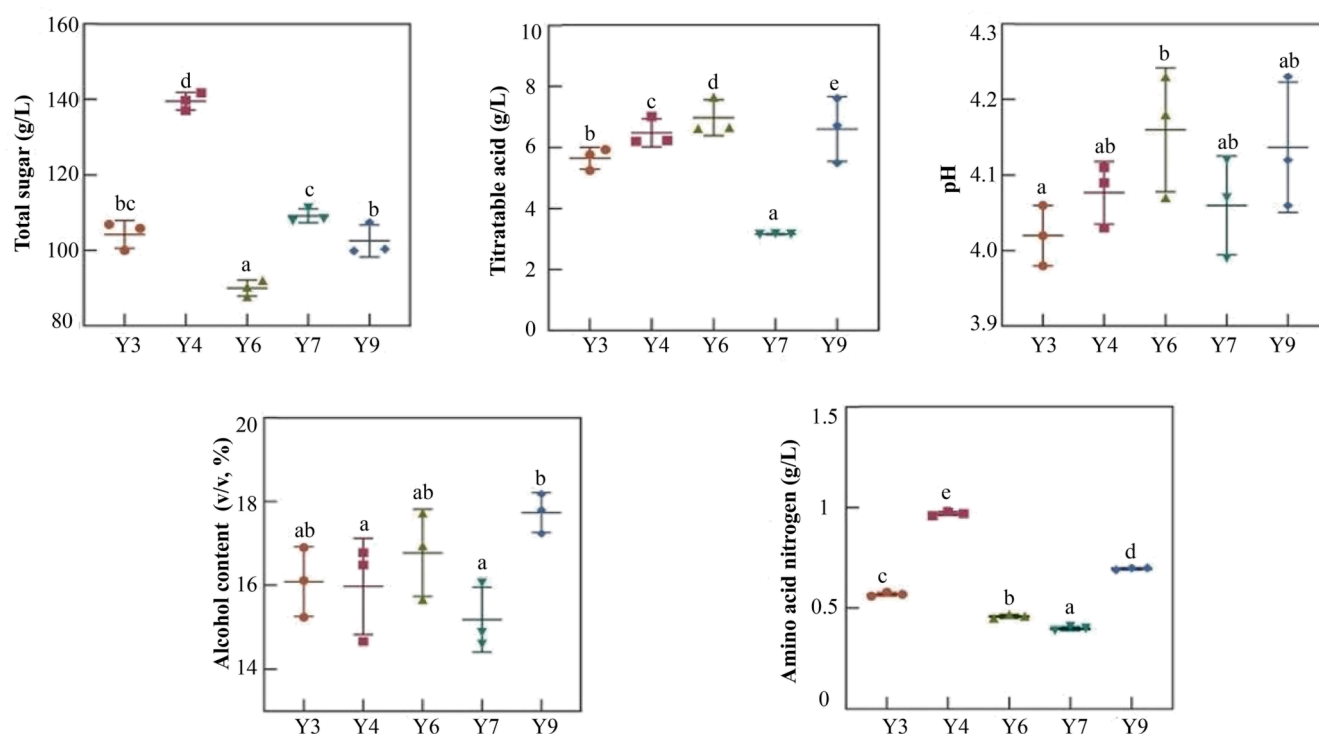


Fig. 1. Changes of physicochemical properties among samples.

samples. The main purposes of employing the two capillary columns in this study were twofold. Firstly, certain volatiles could not be detected using a single capillary column due to their varying polarities. Secondly, the utilization of double capillary columns ensured the accuracy of volatile compound identification. As carrier gas, helium (99.999 %) flowed at the rate of 1.5 mL/min. The oven temperature program for the two capillary columns commenced at 50 °C for 3 min, followed by a ramping rate of 5 °C/min to 80 °C (for a duration of 5 min). Subsequently, the temperature was increased at a rate of 5 °C/min to attain 100 °C (maintained for 5 min), further increased at the same rate to achieve 150 °C (held for another 5 min), and then elevated again at a rate of 5 °C/min until reaching 180 °C (maintained for an additional period of three minutes). Finally, the temperature was raised rapidly at a rate of 8 °C per minute until it reached at 230 °C and remained this temperature for three minutes. The electron impact energy was 70 eV, and the mass scanning range was 35–400 *m/z*. The temperature of the ion source was maintained 230 °C. For qualitative analysis, volatile compounds were identified based on MS database with NIST 14 Library, the Kovats retention index (KI) and comparison of authentic standards. Volatiles were quantified using standard curves constructed in our previous research (Li et al., 2023).

DNA extraction, amplification and sequencing

The microbiome preprocessing and sequencing were determined according to the research with some modification (Liu, Wang, Sun, & Ni, 2020). The total genome DNA of XCHJ samples was extracted via cetyltrimethyl-ammonium-bromide (CTAB) method, and the purity and concentration of DNA were assessed through 1 % agarose gel electrophoresis. The V3-V4 region of bacterial 16S rDNA was amplified with forward primer 341F (5'-CCTACGGGNGGCWGCAG-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'), while the Internal Transcribed Spacer ITS1 region of fungal was amplified using the forward primer ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS1R (5'-GCTGCGTTCTTCATCGATGC-3'). All PCR reactions were carried out in High-Fidelity PCR Master Mix (New England Biolabs). The PCR

products were resolved by 2 % agarose gel electrophoresis in the presence of loading buffer, followed by purification using a Gel Extraction Kit (Axygen). Finally, the DNA amplicons were subjected to sequencing on an Illumina MiSeq/HiSeq2500 platform, with each sample being replicated three times. The paired-end reads obtained from the initial DNA fragments were merged using FLASH software, and subsequent sequence analysis was performed utilizing the UPARSE software package. Sequences with ≥ 97 % similarity were assigned to the same operational taxonomic units (OTUs), and each OTU representative sequence was analyzed and annotated via the RDP classifier.

Statistical analysis

The differences among samples were analyzed using SPSS 25.0 (SPSS Inc., Chicago, IL), and the results are expressed as mean \pm standard deviation. All column diagrams were drawn by GraphPad Prism 8. Heatmap plots were visualized based on relative coefficients between content of key aroma compounds and abundances of microorganisms using TBtools. The network analysis about the correlation of amino acids and microorganisms was conducted in Cytoscape software. The redundancy analysis (RDA) was performed to reveal the relationship of microorganisms and physicochemical indexes.

Results and discussion

Analysis of physicochemical properties at different aging periods of XCHJ

The complex reactions could occur during the aging process of Huangjiu, such as the Maillard reaction between carbohydrates and nitrogenous compounds, as well as esterification reactions between organic acids and alcohols, which contribute to the development of desirable organoleptic properties (Wei, Zhang, Shao, & Wang, 2022). Consequently, the physicochemical properties might fluctuate dynamically throughout the aging process.

The total sugar content serves as an indicator that, to some extent, reflects changes in the carbohydrate substrate. As illustrated in Fig. 1, its

Table 1
Amino acids content in XCHJ samples from five different aging stages.

Amino acid	Code	Standard curve	R ²	Concentration (mg/L)				
				Y3	Y4	Y6	Y7	Y9
Glycine	Gly	Y = 0.0009X + 0.0010	0.9954	8.75 ± 0.43a	80.22 ± 7.05c	56.73 ± 1.61b	5.14 ± 0.36a	63.11 ± 4.44b
Alanine	Ala	Y = 0.0042X + 0.0188	0.9910	217.40 ± 1.78a	335.69 ± 7.38b	211.53 ± 8.27a	212.45 ± 7.77a	398.29 ± 26.51c
γ-Aminobutyric acid	GABA	Y = 0.0070X + 0.0027	0.9943	22.36 ± 2.19b	32.66 ± 0.65c	54.13 ± 1.21e	44.16 ± 1.33d	18.77 ± 1.25a
Serine	Ser	Y = 0.0072X + 0.0414	0.9911	158.74 ± 4.70c	256.92 ± 6.70e	140.55 ± 6.38b	67.68 ± 5.56a	237.52 ± 12.10d
Proline	Pro	Y = 0.0594X + 0.0299	0.9901	136.54 ± 4.03a	248.34 ± 9.54d	161.06 ± 8.16b	133.00 ± 3.01a	174.74 ± 1.63c
Valine	Val	Y = 0.0314X + 0.0249	0.9934	136.66 ± 5.86c	192.96 ± 16.63d	99.10 ± 7.00b	59.81 ± 3.21a	182.59 ± 6.24d
Threonine	Thr	Y = 0.0026X + 0.0121	0.9921	87.92 ± 1.28c	133.04 ± 8.25e	74.67 ± 1.08b	51.69 ± 2.66a	114.64 ± 6.78d
Isoleucine	Ile	Y = 0.0043X + 0.0047	0.9921	91.58 ± 7.06b	135.17 ± 3.27c	79.86 ± 5.52b	43.86 ± 0.86a	141.21 ± 8.20c
Leucine	Leu	Y = 0.0351X + 0.0509	0.9939	222.00 ± 19.29c	326.46 ± 12.33e	149.95 ± 6.22b	106.31 ± 6.57a	281.44 ± 13.29d
Asparagine	Asn	Y = 0.0001X + 0.0020	0.9902	71.93 ± 3.67b	23.98 ± 1.21a	69.22 ± 3.41b	73.46 ± 2.97b	163.62 ± 10.09c
Ornithine	Orn	Y = 0.0143X + 0.0306	0.9908	94.43 ± 9.75c	12.88 ± 1.04a	64.83 ± 1.59b	83.21 ± 3.58c	86.14 ± 3.34c
Aspartic acid	Asp	Y = 0.0086X + 0.0044	0.9929	113.38 ± 1.74c	232.15 ± 9.10d	91.77 ± 1.15b	52.09 ± 2.12a	121.84 ± 4.71c
Lysine	Lys	Y = 0.0095X + 0.0108	0.9901	473.81 ± 8.12d	500.29 ± 20.10d	286.33 ± 16.62a	339.61 ± 14.96b	420.99 ± 2.51c
Glutamic acid	Glu	Y = 0.0136X + 0.0338	0.9929	208.25 ± 2.62c	210.56 ± 4.56c	189.83 ± 2.65b	119.56 ± 2.49a	122.03 ± 0.09a
Methionine	Met	Y = 0.0086X + 0.0063	0.9913	61.73 ± 3.09c	75.03 ± 3.30e	28.14 ± 1.11b	12.81 ± 0.98a	69.08 ± 3.12d
Histidine	His	Y = 0.0287X + 0.0503	0.9900	83.68 ± 1.44e	71.31 ± 2.35d	63.56 ± 2.00b	46.23 ± 0.85a	67.34 ± 0.19c
Phenylalanine	Phe	Y = 0.0465X + 0.0349	0.9905	194.81 ± 16.05d	247.93 ± 9.37e	51.90 ± 1.53a	96.03 ± 6.88b	134.87 ± 6.08c
Arginine	Arg	Y = 0.0281X + 0.0246	0.9929	355.73 ± 15.83b	405.84 ± 15.75c	237.83 ± 6.19a	262.21 ± 12.84a	397.31 ± 12.97c
Tyrosine	Tyr	Y = 0.0116X + 0.0188	0.9949	174.16 ± 9.94b	243.02 ± 16.93c	88.46 ± 2.01a	92.14 ± 5.10a	190.67 ± 7.34b
Total				2913.86 ± 118.87	3764.45 ± 155.51	2199.45 ± 83.71	1901.45 ± 84.10	3386.20 ± 130.88

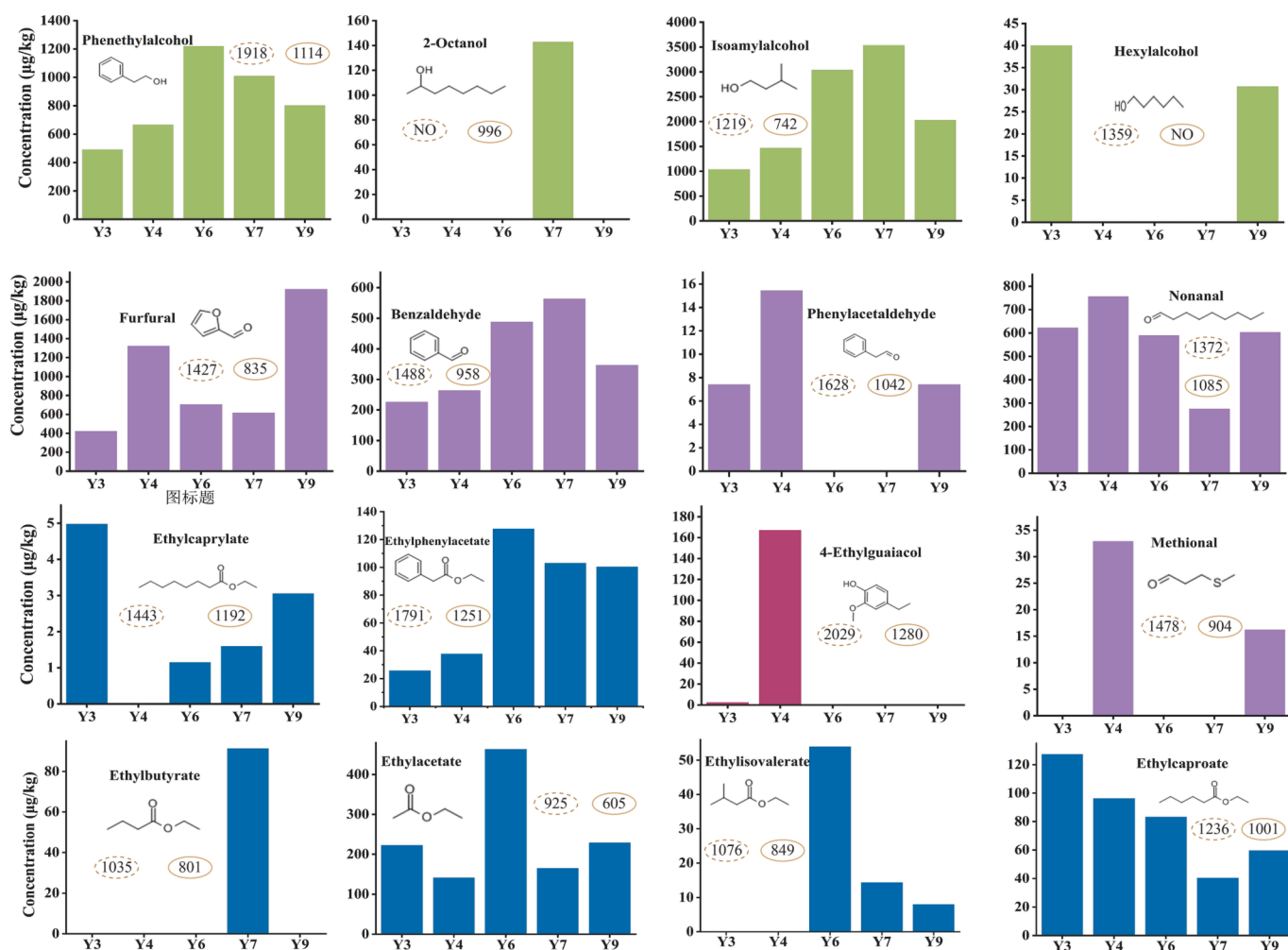


Fig. 2. The concentration changes of 16 key aroma compounds during the aging period (The number enclosed by dotted lines is the KI value on the DB-WAX column; The number framed by solid lines is the KI value on the HP-5 column; "NO" means the compound can't be detected on corresponding gas chromatographic column. The more details of these compounds about identification and quantification were referred to our previous research (Li et al., 2023)).

content exhibited its lowest level in Y6, followed by Y9 and Y3, with the highest concentration observed in Y4 (139.50 g/L). The fluctuation in total sugar levels could be attributed to variations in starch degradation and microbial consumption, resulting in differential accumulation (Hao et al., 2021). The acidity, as a pivotal environmental parameter, directly influences the succession of essential microbial groups, leading to noticeable flavor modifications within the system (Luo et al., 2023). It was observed that the titratable acid exhibited a steady increase tendency during the aging of XCHJ from three years to four years and six years, followed by a significant decrease in the Y7 sample (with the lowest content recorded at 3.16 g/L), and ultimately, another increase in the Y9 sample. Similarly, the pH exhibited a corresponding pattern within the range of 3.95 to 4.25. Typically, Huangjiu contains an alcohol content ranging from 8 % to 18 % (v/v) (Wang et al., 2022). This alcohol content serves as a key indicator for monitoring the fermentation state and degree in the system, and it is also a direct reflection of wine quality (Qian et al., 2023). In our study, we observed that the average alcohol content in each sample fell within this established range. Notably, even after aging for nine years, XCHJ managed to maintain its exceptional quality. Furthermore, the levels of amino acid nitrogen exhibited a fluctuating pattern, with the highest recorded value (0.97 g/L) occurring in Y4.

Variations in amino acid profiles among samples

Amino acids could serve as essential nitrogen sources for microbial growth and metabolism while also participating in reactions with other compounds, leading to the formation of flavor compounds such as alcohols and esters (Gambetta, Cozzolino, Bastian, & Jeffery, 2017). These compounds primarily originate from the degradation of proteins present in the raw materials, facilitated by proteases derived from both secretory and autolytic microorganisms (Zhou et al., 2023). Nineteen amino acids in XCHJ were identified using UPLC, with their total concentrations falling within the range of 1901.45 to 3764.45 mg/L (Table 1).

The amino acids leucine, lysine, arginine, isoleucine, valine, and phenylalanine contribute to enhancing flavor harmony and impart bitterness to Huangjiu through their interactions with alcohols, esters, and acids (Wu et al., 2021). Among these amino acids, lysine had the highest content, reaching its peak value (500.29 mg/L) in Y4 but reaching its lowest level (286.33 mg/L) in Y6. The aforementioned six amino acids displayed dynamic changes over the course of aging, initially showing a notable increase, followed by a decline, and ultimately another increase. Only one sulfur-containing amino acid, methionine, was detected in this study. We found methionine played a vital role on the aroma of XCHJ, and methionine can undergo further degradation, resulting in the production of methional (Fan, Shen, & Xu, 2011). On the basis, it is plausible that the alteration in methional, stemming from changes in this sulfur-containing amino acid, contributed to the diverse aroma perceptions experienced during the aging of Huangjiu. As shown in Table 1, methionine was the most dominant in Y4 (75.03 mg/L) and the lowest content in Y7 (12.81 mg/L). GABA (γ -aminobutyric acid) exhibits various biological activities, including the regulation of blood sugar levels, enhancement of memory function, and alleviation of anxiety symptoms (Xia et al., 2023). In our study, its concentration rose from 22.36 mg/L in Y3 to 54.13 mg/L in Y6 before declining to 18.77 mg/L in Y9. The contents of other amino acids (such as glycine, alanine, serine, etc.) also displayed fluctuations in their levels. These results imply that the aging process of Huangjiu could lead to substantial alterations in amino acid content rather than composition.

Variations in key aroma compounds during XCHJ aging period

The content variations of 16 key aroma compounds, comprising 6 eaters, 5 aldehydes, 4 alcohol and 1 additional compound, were analyzed by GC-MS. These variations were observed to varying extents, as illustrated in Fig. 2. Among them, 2-octanol and ethylbutyrate were

Table 2

OTU number and Alpha diversity index of microorganism in XCHJ samples with different aging stages.

Sample		Y3	Y4	Y6	Y7	Y9
OTU	bacteria	369	254	101	347	173
	fungi	241	437	239	268	476
Shannon	bacteria	3.69 ± 1.52	3.54 ± 0.66	2.82 ± 0.43	3.41 ± 1.19	2.17 ± 0.56
	fungi	4.17 ± 1.44	6.07 ± 0.38	5.55 ± 0.12	5.42 ± 0.25	5.23 ± 0.86
Simpson	bacteria	0.61 ± 0.22	0.66 ± 0.17	0.52 ± 0.08	0.57 ± 0.15	0.40 ± 0.08
	fungi	0.77 ± 0.19	0.95 ± 0.02	0.95 ± 0.01	0.94 ± 0.01	0.88 ± 0.10
Chao1	bacteria	586.75 ± 87.55	465.01 ± 67.10	451.04 ± 48.37	583.34 ± 69.92	397.95 ± 58.13
	fungi	181.79 ± 19.02	257.75 ± 41.89	168.99 ± 6.48	193.33 ± 10.00	262.13 ± 16.47
Goods coverage	bacteria	1.00	1.00	1.00	1.00	1.00
	fungi	1.00	1.00	1.00	1.00	1.00

exclusively detected in Y7, with concentrations of 143.08 $\mu\text{g}/\text{kg}$ and 91.36 $\mu\text{g}/\text{kg}$, respectively. Benzaldehyde, phenethylalcohol, isoamylalcohol, and ethylphenylacetate initially showed an ascending trend, followed by a subsequent descent. Benzaldehyde, responsible for imparting a bitter almond aroma, displayed a concentration range from 226.24 $\mu\text{g}/\text{kg}$ in Y3 to 564.34 $\mu\text{g}/\text{kg}$ in Y7. This variation in benzaldehyde concentration potentially contributes to the fluctuations observed in the caramel aroma of Huangjiu since it is a contributing factor to this aroma profile (Yu, Xie, Xie, Ai, & Tian, 2019). The alcohols were generated via amino acid metabolism, and subsequent reduction reactions and degradation of fatty acid (Okuda et al., 2016). Among these alcohols, isoamylalcohol (ranging from 1039.02 to 3537.02 $\mu\text{g}/\text{kg}$), the most abundant aroma compound, played a pivotal role in shaping the aroma profile across all samples. Additionally, the concentration of phenethylalcohol, a byproduct of phenylalanine degradation (Zhou et al., 2023), reached its highest point at 1220.8 $\mu\text{g}/\text{kg}$ after six years of aging for XCHJ. 4-Ethylguaiaicol, with its roasted and smoky odors, played a crucial role in the formation of the distinctive smoky aroma found in Huangjiu (Chen et al., 2018). As a representative of phenolic compounds, this compound exhibited potent antioxidant activities in cells, thus being considered one of the functional components in baijiu (Hong, Zhao, & Sun, 2023). Based on these findings, we speculate that the health benefits associated with rice wine may also be attributed to its presence. Throughout the entire aging process, we only detected 4-ethylguaiaicol in Y3 and Y4, especially its content reaching 167.11 $\mu\text{g}/\text{kg}$ in Y4. Hexylalcohol, characterized by a fruity aroma, was also found in two samples (Y3 and Y9). A common sulphur-containing substance called methional in Huangjiu was regarded as a maker for the classification of young and aged rice wines (Chen, Wang, Qian, Li, & Xu, 2019; Wang et al., 2022). However, we detected it in only Y4 and Y9 samples. This discrepancy in results may be attributed to regional variations within the Huangjiu production. The category of eaters, crucial for bestowing fruity and floral attributes upon Huangjiu, is typically generated through microbial metabolism and esterification reactions. Ethylacetate, which is characterized by a fruity and sweet odor, reached its peak concentration in Y6 at 464.2 $\mu\text{g}/\text{kg}$, while Y3 exhibited the highest levels of ethylcaproate and ethylcaprylate. Throughout the entire aging process, the concentration of 16 key aroma compounds exhibited dynamic fluctuations, ultimately contributing to the development of distinctive aroma profiles in Huangjiu with different aging durations.

Microbial diversity throughout the aging of Huangjiu

To evaluate the variations of diversity and composition within microbial communities in the Huangjiu aged for different years, high-throughput sequencing was conducted on all samples. The sequences

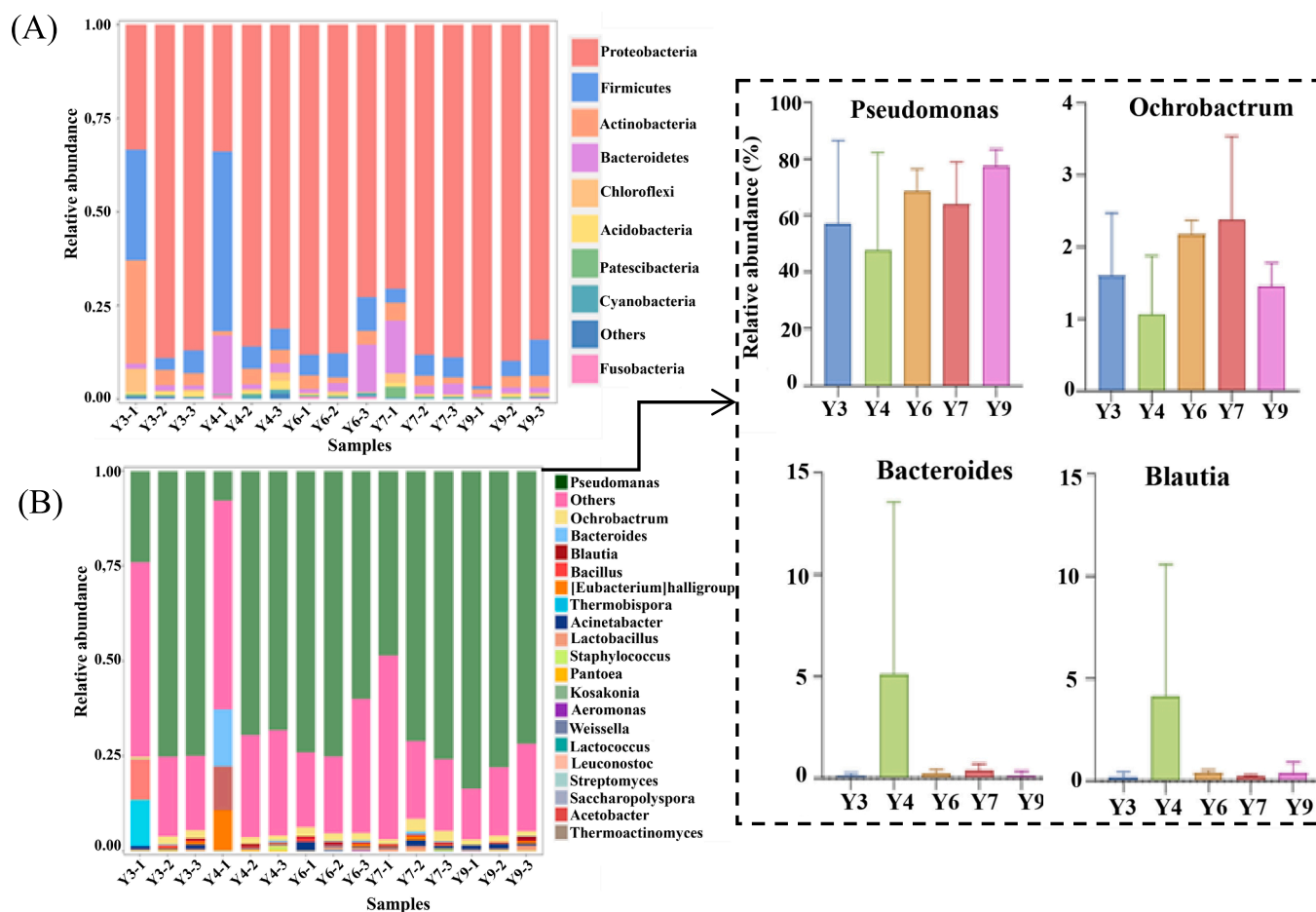


Fig. 3. Distribution of bacteria at phylum (A) and genus (B) level during XCHJ aging.

with 97 % similarity threshold were clustered as one Operational Taxonomic Unit (OTU). As shown in Table 2, 101-369 OTUs of bacteria were obtained while 239-476 OTUs of fungus were generated. Moreover, the Alpha diversity analysis was used to evaluate the richness and diversity of microbial community. The Chao1 index and Goods_coverage represented the richness and the sequencing depth, respectively; Shannon and Simpson could be as the indices of diversity. All Goods_coverage indexes were 1, indicating that the sequencing depth was sufficient, allowing for further analysis to proceed (Zhang et al., 2023).

Microbial communities underwent significant changes throughout the aging process. Regarding bacteria, Y3 displayed the highest Chao1 and Shannon indices, whereas Y9 exhibited the lowest values for both Chao1 and Shannon. The Simpson index peaked in Y4 but hit its lowest point in Y9. The fluctuations in these indices among bacteria across different aging periods could reflect dynamic changes in the richness and diversity of the microbial communities. These could potentially be attributed to alterations in environmental factors during the aging process. In terms of fungi, Shannon and Simpson indices initially increased and then gradually decreased, which indicated that fungal diversity showed an upward trend during the early stages of aging until it reached its peak at year 4, and then followed by an opposite trend. Y9 had the highest richness of fungi and its Chao1 index reached 262.13. In comparison, the fungal diversity in aged XCHJ exceeded that of bacteria, although the lower richness of fungi was found than bacteria on the whole. In summary, the richness and diversity of microbial community fluctuated as XCHJ aging advanced.

Composition of microbial communities in the samples

A deep insight into the succession of microbial community in aged XCHJ is invaluable for comprehending the mechanisms underlying Huangjiu quality formation and for identifying effective strategies to enhance the quality of XCHJ. Fig. 3 depicted the alterations in bacterial composition at both the phylum and genus levels. Consistent with a previous research (Yu et al., 2023), we also observed that *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* dominated at the phylum level in all samples (Fig. 3A). Of these, *Proteobacteria*, with a relative abundance ranging from 66.90 % to 90.09 %, consistently represented the most abundant phyla throughout the entire aging period, while *Firmicutes* accounted for 4.86 %–20.01 %. Both of these phyla encompass numerous beneficial species involved in Huangjiu fermentation (Xie et al., 2013). Overall, phyla *Proteobacteria* exhibited the ascending trend as the aging progressed, while *Firmicutes* was on the decline. At the genus level, 20 bacteria communities with higher relative abundance were detected in aged XCHJ (Fig. 3B), with *Pseudomonas* having the most abundance, followed by *Ochrobactrum*, *Bacteroides* and *Blautia*. It was noteworthy that Y9 had the highest abundance (77.38 %) of *Pseudomonas*. *Pseudomonas*, as one of main contributors to production of amino acid decarboxylase (Luo et al., 2020), played a vital role in the biosynthesis of aroma compounds in Huangjiu (Tian et al., 2022). Y7 showed an important presence of *Ochrobactrum* in comparison with other samples, which was possibly concerned with the lowest alcohol content due to its growth inhibition as ethanol increased (Liang et al., 2023). The abundances of *Bacteroides* and *Blautia* in Y4 were far higher than in others. *Bacteroides* is known for its capacity to produce acids such as succinic acid, while *Blautia* has the potential to produce short-chain

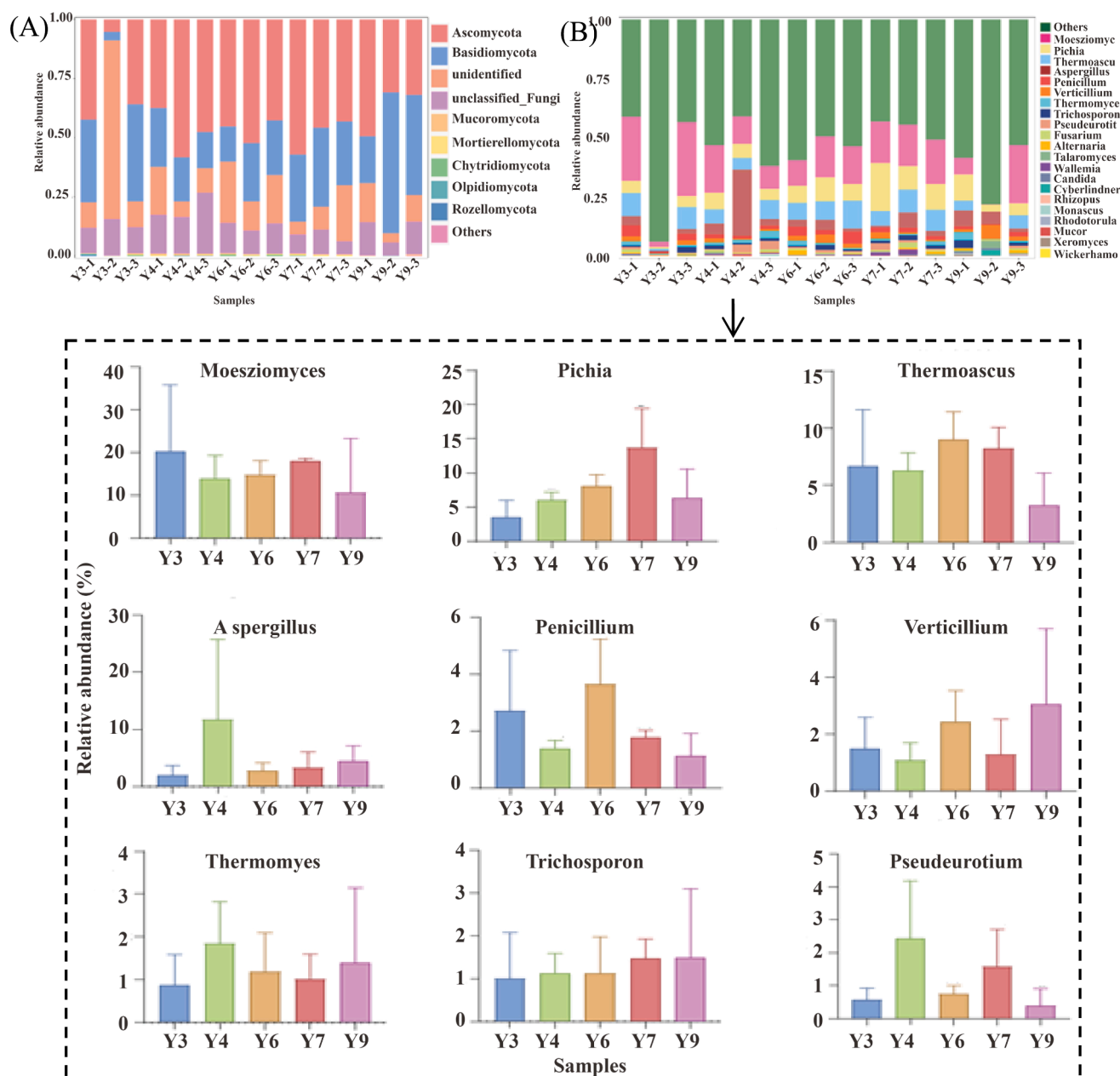


Fig. 4. Distribution of fungi at phylum (A) and genus (B) level during XCHJ aging.

fatty acids (Isar, Agarwal, Saran, Kaushik, & Saxena, 2007; Ren et al., 2020). Therefore, both of these genera could play pivotal roles in shaping the aroma quality of Huangjiu.

As illustrated in Fig. 4A, a total of seven identified and classified fungi phyla were detected, with notable populations of *Ascomycota* (41.65 %) and *Basidiomycota* (27.38 %). More specifically, 21 genera were identified, among which nine fungal genera, namely *Moesziomyces*, *Pichia*, *Thermoascus*, *Aspergillus*, *Penicillium*, *Verticillium*, *Thermomyces*, *Trichosporon* and *Pseudeurotium*, exhibited a relative abundance of ≥ 1.00 % (Fig. 4B). It's worth noting that these nine genera displayed variations across samples with different aging durations. The XCHJ aged four years (Y4) had highest frequencies of *Aspergillus*, *Thermomyces* and *Pseudeurotium* when compared to other samples, while the abundance of *Thermoascus* and *Penicillium* were highest in Y6. Interestingly, the *Pichia* genus displayed a clear pattern throughout the entire aging period, with its abundance gradually increasing from Y3 to Y7 and then sharply decreasing. This yeast is a significant contributor to the generation of aroma in food as it facilitates the transformation of alcohols into esters

(Mingorance-Cazorla, Clemente-Jiménez, Martínez-Rodríguez, Las Heras-Vázquez, & Rodríguez-Vico, 2003). Consequently, its fluctuations might contribute to variations in aroma quality during the aging of XCHJ.

Correlations between microorganism and key aroma compounds/physicochemical properties/amino acids in XCHJ

To reveal the intrinsic relationship between the microbial community and key aroma compounds in aged XCHJ, a heatmap was plotted based on Pearson correlation coefficients of the 16 volatiles and the core microorganisms (16 bacteria and 21 fungi). Fig. 5A illustrated a significant positive correlation between *Ochrobactrum* and three compounds possessing fruity notes, namely isoamylalcohol, benzaldehyde and 2-octanol ($p < 0.05$). Conversely, *Ochrobactrum* exhibited a notably negative correlation with phenylacetaldehyde. *Staphylococcus* played a crucial role in the generation of methional and 4-ethylguaiacol flavour substances in aged XCHJ. Notably, this bacterium is sensitive to acidic

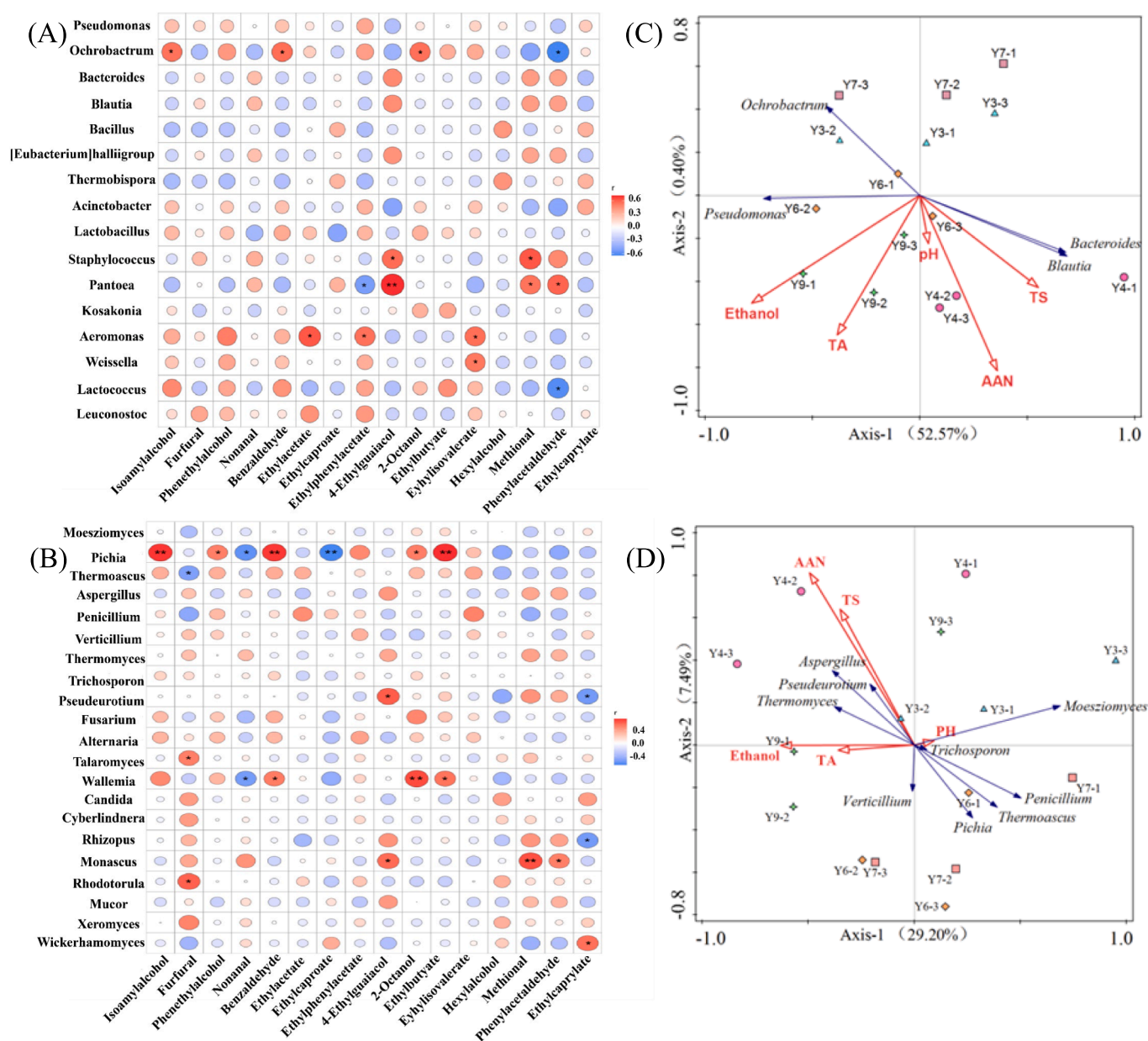


Fig. 5. The relationship of microorganisms and key aroma compounds in aged samples (A,B); RDA analysis of physicochemical indexes, bacteria (C) and fungi (D) in Xiecen Huangjiu. Ethanol: Alcohol (v/v,%); AAN: amino acid nitrogen; TS: Total sugar; TA: Total acid.

environments (Wang et al., 2021), which implies the potential to control the production of methional and 4-ethylguaiacol by regulating acidity levels within the system. *Aeromonas* played a key role on generation of some ethyl esters including ethylacetate, ethylphenylacetate, ethylisovalerate. In addition, *Weissella* also had significant effect on ethylisovalerate. Overall, the aforementioned four bacteria genus demonstrated a superior capacity for flavor production in aged XCHJ.

Besides bacteria, the fungi group also plays an essential role in the formation of distinctive aroma in Huangjiu. Eight of twenty-one genus, namely *Pichia*, *Pseudeurotium*, *Wallemia*, *Monascus*, *Rhodotorula*, *Talaromyces*, *Wickerhamomyces*, and *Rhizopus*, were main aroma-promoting contributors during the aging process of Huangjiu due to their strong correlations with many key aroma compounds (Fig. 5B). Among them, *Pichia* was significantly positively associated with isoamylalcohol, benzaldehyde and ethyl butyrate ($p < 0.01$) and positively correlated with phenylethanol and 2-octanol ($p < 0.05$), while negatively correlated with nonanal and ethylcaproate. As a non-saccharomyces

cerevisiae, this genus contributes to the production of significant metabolites, such as esters and alcohols, during the brewing process of Hungjiu, thereby augmenting the intricacy of flavor profile. Positive correlations between *Pseudeurotium* and 4-ethylguaiacol were observed, whereas *Pseudeurotium* was negatively related to ethylcaprylate. Moreover, ethylcaprylate formation might be influenced by *Wickerhamomyces* and *Rhizopus* ($|p| < 0.05$) as well. The presence of *Rhodotorula* and *Talaromyces* was strongly correlated with the occurrence of furfural, which imparts a caramel-like aroma. Being readily detectable in specific plants and the surrounding air (Yang et al., 2023), we observed a close association between *Wallemia* and the generation of four aroma compounds in this study, namely benzaldehyde, 2-octanol, ethylbutyrate (positively correlated), and nonanal (negatively correlated). Additionally, the presence of *Monascus* exhibited a positive correlation with 4-ethylguaiacol, methional, and phenylacetaldehyde. Based on the aforementioned findings, it can be inferred that a specific microorganism plays a pivotal role in the synthesis of multiple compounds,

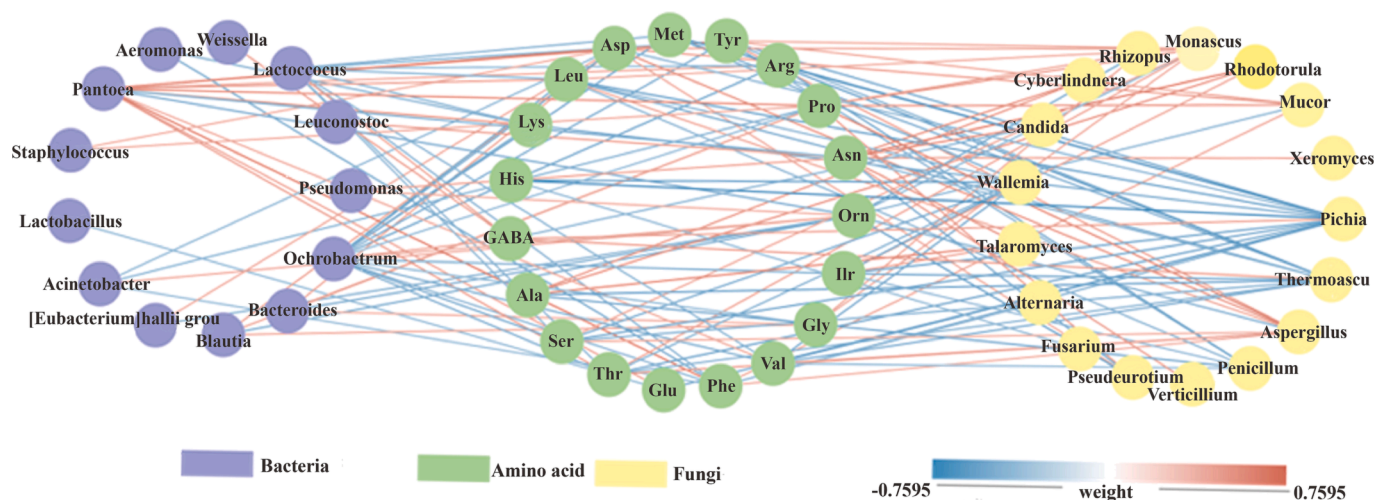


Fig. 6. The correlation between amino acids and microorganisms in XCHJ.

whereas the production of specific aroma components is likely attributed to the combined influence of multiple microorganisms.

The microbial growth of aged XCHJ is significantly influenced by various factors, including pH, total acid, and total sugar levels within the system. Hence, it is imperative to elucidate the intricate interactions between microorganisms and these physicochemical factors to identify potential target parameters for controlling specific microbial growth in aged Huangjiu. The redundancy analysis (RDA), an extension of PCA that provides the advantage of capturing the regression between diverse microorganisms and multiple environmental variables (Zhao et al., 2023), was employed to elucidate the aforementioned relationship (Fig. 5C and Fig. 5D). As shown, the length of an arrow serves as a proxy for the strength of the relationship. We found that *Pseudomonas* with the most abundance in bacteria showed a significant correlation with alcohol content. The *Bacteroides* and *Blautia*, in conjunction with pH, total sugar, and amino acid nitrogen levels, was located in the fourth quadrant, indicating a strong correlation between these two bacteria and the three physicochemical indices (Fig. 5C). For fungi, *Aspergillus*, *Pseudeurotium* and *Thermomyces* displayed strong correlations with total sugar, alcohol content and amino acid nitrogen levels (Fig. 5D).

Amino acids in Huangjiu serve not only as nutrients but also as flavor precursors, influencing aroma and taste through microbial conversion or decomposition (Wang et al., 2022). The connection between microorganisms and amino acids was elucidated through a network analysis based on their correlation coefficient (Fig. 6), wherein the red line represents a positive correlation, while the blue line indicates an inverse relationship. A total of 27 co-occurrence relationships (represented by red lines) of bacteria and 36 co-occurrence relationships of fungi were identified, while a combined count of 79 co-exclusion relationships was observed (34 blue lines in bacteria and 45 blue lines in fungi) (Jin et al., 2019). By comparison, *Pantoea* exhibited a greater number of positive correlations with various amino acids among bacteria, while *Lactobacillus* demonstrated no significant associations with amino acids except for threonine. The study revealed a higher frequency of interactions between fungi and amino acids compared to bacteria. Specifically, *Rhodotorula* and *Monascus* exhibited significant positive correlations, while *Penicillium* and *Pichia* demonstrated notable negative correlations.

Conclusions

In conclusion, our investigation found that 4 (phenethylalcohol, isoamylalcohol, benzaldehyde, and ethylbenzoate) of 16 key aroma compounds exhibited an initial increase followed by a subsequent decrease throughout the aging period of XCHJ. The pH was consistently maintained within the range of 3.95 to 4.25. The levels of total sugar,

total acidity, amino acids, and alcohol content exhibited fluctuating variations. We detected nineteen amino acids in aged XCHJ, with lysine consistently dominating the profile throughout the aging process. Moreover, the richness and diversity of microbial community also exhibited variations as XCHJ aging progressed. *Pseudomonas Ochrobactrum*, *Bacteroides*, *Blautia*, *Moesziomyces*, *Pichia*, *Thermoascus*, *Aspergillus*, *Penicillium*, *Verticillium*, *Thermomyces*, *Trichosporon* and *Pseudeurotium* consistently displayed higher relative abundance in aged XCHJ. The correlation analysis highlighted that four bacteria and eight fungi were key microorganisms involved in the formation of key aroma compounds. Additionally, the physicochemical indices displayed notable correlations with *Pseudomonas*, *Bacteroides*, *Blautia*, *Aspergillus*, *Pseudeurotium* and *Thermomyces*, while *Pantoea*, *Rhodotorula* and *Monascus* exhibited significant associations with changes in amino acid profiles. These findings offer fundamental insights into the mechanisms underlying aroma formation and provide a robust foundation for quality control of XCHJ. Future research could explore the impact of specific microorganisms on the formation of key aroma compounds and develop strategies to modulate their activity for desired flavor profiles.

Chemical compounds

Phenethylalcohol (PubChem CID: 6054); 2-Octanol (PubChem CID: 20083); Furfural (PubChem CID: 7362); Benzaldehyde (PubChem CID: 240); Ethylcaprylate (PubChem CID: 7799); Ethylphenylacetate (PubChem CID: 7590); Ethylbutyrate (PubChem CID: 7762); Ethylacetate (PubChem CID: 8857); Isoamylalcohol (PubChem CID: 31260); Phenylacetaldehyde (PubChem CID: 998); 4-Ethylguaiacol (PubChem CID: 62465); Ethylisovalerate (PubChem CID: 7945); Hexylalcohol (PubChem CID: 8103); Nonanal (PubChem CID: 31289); Ethylcaproate (PubChem CID: 31265).

CRediT authorship contribution statement

Yu Zhao: Writing – original draft, Validation. **Min Li:** Investigation. **Ping Zhan:** . **Peng Wang:** Supervision, Formal analysis. **Wanying He:** Project administration, Writing – review & editing. **Honglei Tian:** Funding acquisition, Project administration.

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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