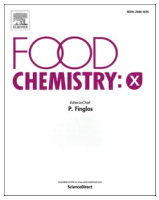
Food Chemistry: X 25 (2025) 102228

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Analysis of fermentation characteristics in fermented grains across seven rounds of sauce-flavored Baijiu: Microbial communities structure, physicochemical parameters, volatile and non-volatile flavor compounds

Xiaomin Liu [a,b], Yingchun Mu [a,*], Xiangxiang Lv [c], Nuo Chen [a], Lei Chen [a], Tangzheng Wen [a], Wei Su [a,b,*]

[a] School of Liquor and Food Engineering, Guizhou University, Guiyang 550025, China
[b] Key Laboratory of Fermentation Engineering and Biological Pharmacy of Guizhou Province, Guiyang 550025, China
[c] Xiaohutuxian Liquor Industry (Group) Co., LTD, Zunyi 563000, China

ARTICLE INFO

Keywords:
Sause-flavor Baijiu
Fermented grains
Microorganisms
Flavor compounds
Correlation analysis

ABSTRACT

Pit fermentation is the critical stage for flavor formation in sauce-flavored Baijiu (SFB). This study aimed to elucidate the fermentation characteristics in fermented grains at the start (EP) and at the end (OP) of pit fermentation across seven rounds of SFB. *Kroppenstedtia* and *Issatchenkia* gradually replaced *Pseudomonas* and *Pichia* in EP, while *Lactobacillus* and *Issatchenkia* maintained dominance in OP. In both groups, volatile flavor compounds (VFCs) and moisture contents initially increased and then declined, non-volatile flavor compounds (N-VFCs) and starch contents overall decreased, and acidity, reducing sugar, and organic acid levels generally increased. Additionally, the VFC and N-VFC contents consistently increased from EP to OP within each round. Correlation analysis revealed *Issatchenkia*, *Kroppenstedtia*, *Monascus*, *Virgibacillus*, and *Pichia* significantly contributed to flavor compound. Finally, a metabolic network was constructed to predict microbial and flavor compound metabolic pathways. This study provided insights into the fermentation characteristics of SFB from a multi-dimensional perspective.

1. Introduction

Sauce-flavor Baijiu (SFB), one of Chinese traditional distilled liquors, is renowned for its intricate brewing process and unique flavor profile. The production involves seven complete fermentation rounds (Chen et al., 2024). The first round begins with two fermentation stages using sorghum as the primary raw material: fresh sorghum is soaked, steamed, cooled to room temperature, and mixed with water and *Daqu*. This mixture undergoes heap fermentation (about 2–7 days) and is then transferred to a "pit" for approximately 30 days of anaerobic fermentation. Subsequently, the fermented grains are distilled to yield based Baijiu. In the second round, the distilled grains from the first round are reused, mixed with water and *Daqu*, and subjected to another cycle of heap fermentation and pit fermentation, followed by distillation to obtain based Baijiu. The process for the third to seventh rounds mirrors that of the second round (Fig. 1). Finally, the based Baijiu is mixed according to different proportions to obtain SFB. Despite the similar brewing processes, the flavor quality of based Baijiu varies across the rounds. Generally, based Baijiu from the first and second rounds is often astringent, the third to fifth rounds is typically of superior flavor and quality, and the sixth and seventh rounds exhibits a roasted aroma.

Currently, microorganisms are recognized as the driving force behind flavor compounds production during SFB fermentation. Xu et al. (Xu et al., 2023) identified *Lactobacillus*, *Virgibacillus*, *Issatchenkia*, and *Byssochlamys* as dominant genera during the first three rounds of SFB fermentation, with *Lactobacillus* playing a significant role in ester synthesis. Chen et al. (2024) revealed that *Lactobacillus*, *Kroppenstedtia*, *Virgibacillus*, and *Issatchenkia* dominated the second and fourth rounds of SFB fermentation, noting a strong correlation between *Lactobacillus* and ester production. Research by Wang, Xu, et al. (2021) on the fifth and sixth rounds of SFB indicated that the reduction in fungal populations contributed to a decrease in ester concentration. Wu et al. (2023) observed microbial succession across all seven rounds of SFB, with *Bacillus*, *Schizosaccharomyces*, and *Torulaspora* showing strong correlations with flavor compounds of based Baijiu.

From a fermentation process perspective, SFB production consists of

* Corresponding authors at: School of Liquor and Food Engineering, Guizhou University, Guiyang 550025, China.
E-mail addresses: myc1886@163.com (Y. Mu), suwei1886@163.com (W. Su).

https://doi.org/10.1016/j.fochx.2025.102228
Received 28 November 2024; Received in revised form 16 January 2025; Accepted 24 January 2025
Available online 28 January 2025

two main stages: heap fermentation and pit fermentation. Heap fermentation occurs in an open environment, where microorganisms accumulate and transform macromolecules into flavor precursors, creating favorable conditions for subsequent pit fermentation (Wang, Wu, et al., 2021). In contrast, pit fermentation takes place under anaerobic conditions, serving as the primary stage for microbial succession and flavor development (Wu et al., 2023). Lu et al. (2022) observed a decline in bacterial diversity and an increase in fungal diversity from day 0 to day 30 of pit fermentation. Furthermore, Wang et al. (2024) reported comparable microbial succession patterns, noting that these changes were associated with increased levels of volatile flavor compounds such as esters, alcohols, and acids. Similarly, Yang, Huang, et al. (2024) identified titratable acidity, starch content, and pH as the main drivers of microbial community assembly during pit fermentation. These findings underscored the direct impact of microbial community succession on flavor formation during SFB fermentation. However, existing studies have predominantly focused on specific fermentation rounds or stages. The fermentation characteristics on fermented grains at the start and end of pit fermentation across all seven fermentation rounds remains inadequately understood.

This study aimed to systematically investigate the dynamic changes and correlations between microorganism and flavor compound in fermented grains at the start and end of pit fermentation across seven fermentation rounds of SFB. High-throughput sequencing, physicochemical test, headspace-solid phase microextraction-gas chromatography–mass spectrometry (HS-SPME-GC–MS), high performance liquid chromatography (HPLC), and liquid chromatography tandem mass spectrometry (LC-MS/MS) were employed to analyze microbial communities, physicochemical indicators, and volatile flavor compounds (VFCs) and non-volatile flavor compounds (N-VFCs). Multivariate statistical analyses were used to explore the relationships between these factors, ultimately elucidating the metabolic pathways of flavor compounds. Particularly, through the comparison between the fermented grains at the start and end of pit fermentation, the influence between them was clarified. These findings provide a theoretical basis for optimizing fermentation processes and improving the quality of SFB.

2. Materials and methods

2.1. Sample collection

The fermented grains were taken from a SFB production enterprise in Xishui County, Guizhou Province, China. The fermentation performance of fermented grains at the start of pit fermentation (EP) influenced the fermented grains at the end of pit fermentation (OP), ultimately affecting the flavor quality of SFB (Chen et al., 2024). However, the fermentation characteristics of EP and OP across all seven fermentation rounds remained insufficiently explored. Therefore, seven rounds of EP were collected and named as EP1, EP2, EP3, EP4, EP5, EP6, EP7, and OP were labeled as OP1, OP2, OP3, OP4, OP5, OP6, OP7. Fermented grains were taken from the upper (A), middle (B), and lower (C) layers of the pit at the center and four corners (Fig. 1). Approximately 50 g of material was collected from each location. The subsamples from all locations point were mixed under sterile conditions to produce a single representative sample. A total of 210 subsamples were collected across seven rounds, yielding 14 composite samples. These composite samples were stored in sterile, sealed bags at −80 °C for subsequent analyses.

2.2. Determination of physicochemical parameters and organic acids

The moisture content of the fermented grains was determined by drying samples at 105 °C to a constant weight. Starch and reducing sugar contents were measured using titration with a glucose standard solution. Titratable acidity was assessed using a 0.1 mol/L NaOH standard solution (Zou et al., 2024). Organic acids, including malic acid, citric acid, tartaric acid, oxalic acid, succinic acid, acetic acid, and lactic acid, were analyzed by HPLC (Agilent1260, Agilent Technologies Co., Ltd., USA). The method was adapted from Chen et al. (2023) with minor modifications. Weighed 1.00 g ground fermented grains and diluted it to 10 mL with distilled water. It was then sonicated for 30 min and centrifuged at 10,000 r/min and 4 °C for 10 min, and the supernatant was filtered through a 0.45 μm membrane. Chromatographic separation was performed on a ZORBAX SB-Aq column (4.6 mm × 250 mm, 5 μm) with a column temperature of 30 °C. The injection volume was 10 μL, and the flow rate was set to 0.8 mL/min. Detection was carried out at a

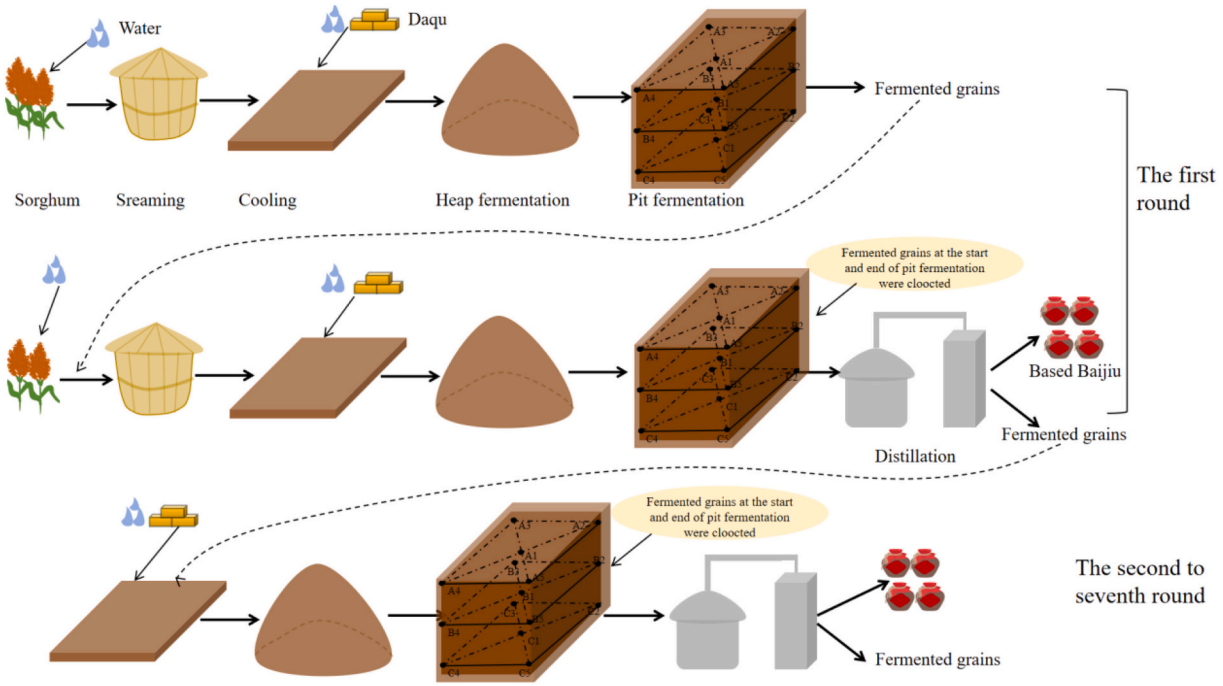


Fig. 1. The production processes of seven rounds of sauce-flavor Baijiu.

wavelength of 210 nm using a mobile phase consisting of methanol and 0.02 mol/L KH_2PO_4. Ultimately, seven organic acids were quantified by peak area external standard method according to the standard curve of organic acids.

2.3. DNA extraction and PCR amplification

A 0.5 g fermented grains was mixed with 1 mL of CATB lysis buffer in a 2.0 mL EP tube. Lysozyme was added, and the mixture was incubated in a water bath at 65 °C, with intermittent inversion to ensure thorough dissolution of the sample. After centrifugation at 12,000 rpm for 10 min at room temperature, the supernatant was transferred to a new tube and mixed with 500 μL of phenol (pH 8.0): chloroform: isoamyl alcohol (25:24:1). The mixture was inverted for 3 min, centrifuged at 12,000 rpm for 10 min, and the supernatant was collected. This step was repeated using 500 μL of chloroform: isoamyl alcohol (24:1). The supernatant was then mixed with isopropanol, gently inverted, and stored at −20 °C to precipitate the DNA. After centrifugation at 12,000 rpm for 10 min, the pellet was washed twice with 75 % ethanol, air-dried in a biosafety cabinet, and dissolved in ddH_2O. To remove RNA, 1 μL of RNase A was added to the solution, and the mixture was incubated at 37 °C for 15 min, yielding total DNA.

For bacterial community analysis, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using primers 338F (5′-ACTCCTACGGGGAGGAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). For fungal analysis, the ITS region was amplified using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′).The PCR reaction (30 μL total) contained 15 μL Phusion Master Mix (2×), 1 μL forward primer (1 μM), 1 μL reverse primer (1 μM), 10 μL genomic DNA (1 ng/μL), and ddH_2O to 30 μL. Amplification was performed using a Bio-Rad T100 thermal cycler under the following conditions: 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 50 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were pooled in equimolar concentrations based on their intensity, purified using 2 % agarose gel electrophoresis, and extracted using a Universal DNA Purification Kit (TianGen, China). Then the library construction was performed using the NEB Next® Ultra DNA Library Prep Kit, and evaluated using the Agilent 5400 (Agilent Technologies Co., Ltd., USA). After confirming the library quality, sequencing was conducted on the NovaSeq 6000 platform. The data processing was performed using the QIIME2 DADA2 plugin, which included quality control, trimming, denoising, merging, and chimera removal, ultimately generating the final feature table. The diversity matrices were calculated using the QIIME2 core-diversity plugin.

2.4. Volatile flavor compound determination

Weighed 2 g of ground sample into a 20 mL headspace vial, followed by the addition of 5 mL of saturated NaCl solution and 30 μL of 2-octanol (internal standard, 30 μg/mL). The headspace vial was tightly sealed using a PEET seal. The sample was incubated at 50 °C for 20 min, and VFCs were extracted for 40 min using a 50 μm/30 μm DVB/CAR/PDMS fiber under shaking conditions. The analysis was held for 3.5 min. The gas chromatography (GC, Thermo Fisher Trace 1300, Agilent Technologies Co., Ltd., USA) conditions were as follows: a DB-WAX capillary column (60 m × 0.25 mm × 0.25 μm, Agilent Technologies Co., Ltd., USA) was used, with an inlet temperature of 250 °C, and splitless injection mode. The temperature program started at 50 °C and was held for 2 min, increased at 4 °C/min to 230 °C, and held for 5 min, with helium as the carrier gas at a flow rate of 1 mL/min. The mass spectrometry (MS, TSQ8000 Evo, Agilent Technologies Co., Ltd., USA) conditions included an electron impact ionization energy of 70 eV, a transfer line temperature of 280 °C, an ion source temperature of 230 °C, a scan range of 50–450 *m/z*, and a scan rate of 1 scan/s. VFCs were identified by comparing their mass spectra with the NIST 5 database, retaining compounds with a match score ≥ 800. The relative contents of each compound were calculated following the method described by Chen et al. (2023).

2.5. Non-volatile flavor compound determination

Weighed 100 mg of ground sample into an EP tube and added 500 μL of 80 % methanol aqueous solution. The mixture was vortexed thoroughly, subjected to an ice bath for 5 min, and then centrifuged at 1500 rpm for 20 min at 4 °C. The supernatant was collected, diluted to a methanol content of 53 %, and centrifuged again under the same conditions. The resulting supernatant was injected into the LC-MS system for analysis. Chromatographic separation was performed using a Hypersil Gold column (100 × 2.1 mm, 1.9 μm, Thermo Fisher, USA) at a temperature of 40 °C and a flow rate of 0.2 mL/min. The mobile phases consisted of 0.1 % formic acid in water (A phase) and methanol (B phase), with the gradient elution program as follows: 98 % A, 0–1.5 min; 98 % - 15 % A, 1.5–3 min; 15 % - 0 % A, 3–10 min; 0 % - 98 % A, 10 - 12 min. MS analysis was performed in both positive and negative ionization modes with a data-dependent MS/MS scanning method. The parameters were set as follows: mass scan range, 100–1500 *m/z*; spray voltage, 3.5 kV; sheath gas flow rate, 35 psi; auxiliary gas flow rate, 10 L/min; ion transfer tube temperature, 320 °C; radio frequency level, 60; and auxiliary gas heater temperature, 350 °C.

2.6. Statistical analysis of data

Each experiment was performed with three biological replicates, and the results were expressed as the mean ± standard deviation. Data analysis was conducted using IBM SPSS Statistics 26.0, with one-way analysis of variance (ANOVA) and independent-samples *t*-test used to determine significance ($P < 0.05$). The bar charts and sline graphs were generated by Origin 2021, while orthogonal partial least squares discriminant analysis (OPLS-DA) was performed by SIMCA 14.1.0. Network node diagrams were visualized with Gephi platform and Cytoscape 3.9.1. Finally, image editing was performed by Adobe Illustrator 2023.

3. Results and discussion

3.1. Microbial diversity analysis

A total of 2,602,413 bacterial and 2,438,428 fungal effective sequences were obtained for EP group, and 2,516,079 bacterial and 2,275,966 fungal for OP group (**Table S1**). These findings indicated that the microbial community in the EP group was more diverse, with bacteria contributing more than fungi. The faith_pd and Shannon curves were employed to assess the adequacy of sequencing depth. In this study, both the faith_pd and Shannon curves reached a plateau (**Fig. S1**), suggesting that the sequencing depth was sufficient to represent the microbial structure and capture the majority of microbial information present in the samples, thus allowing for reliable downstream analysis. The Chao1 index was used to reflect the microbial community richness, while the Shannon and Simpson indices provided insights into microbial community diversity (Wang et al., 2024). For the bacterial communities (Fig. 2a, b, c), Chao1, Simpson, and Shannon indices were significantly higher in the EP group than in the OP group ($P < 0.01$). For fungi (Fig. 2d, e, f), Chao1, Simpson, and Shannon indices were higher in the OP group. These results suggested that the bacterial community in the EP group exhibited greater complexity, whereas the fungal community in the OP group was more intricate.

3.2. Microbial community structure analysis

To investigate the microbial community structure succession, the sequencing and classification were performed at the phylum and genus levels. As shown in Fig. 3a, the dominant bacterial phyla included

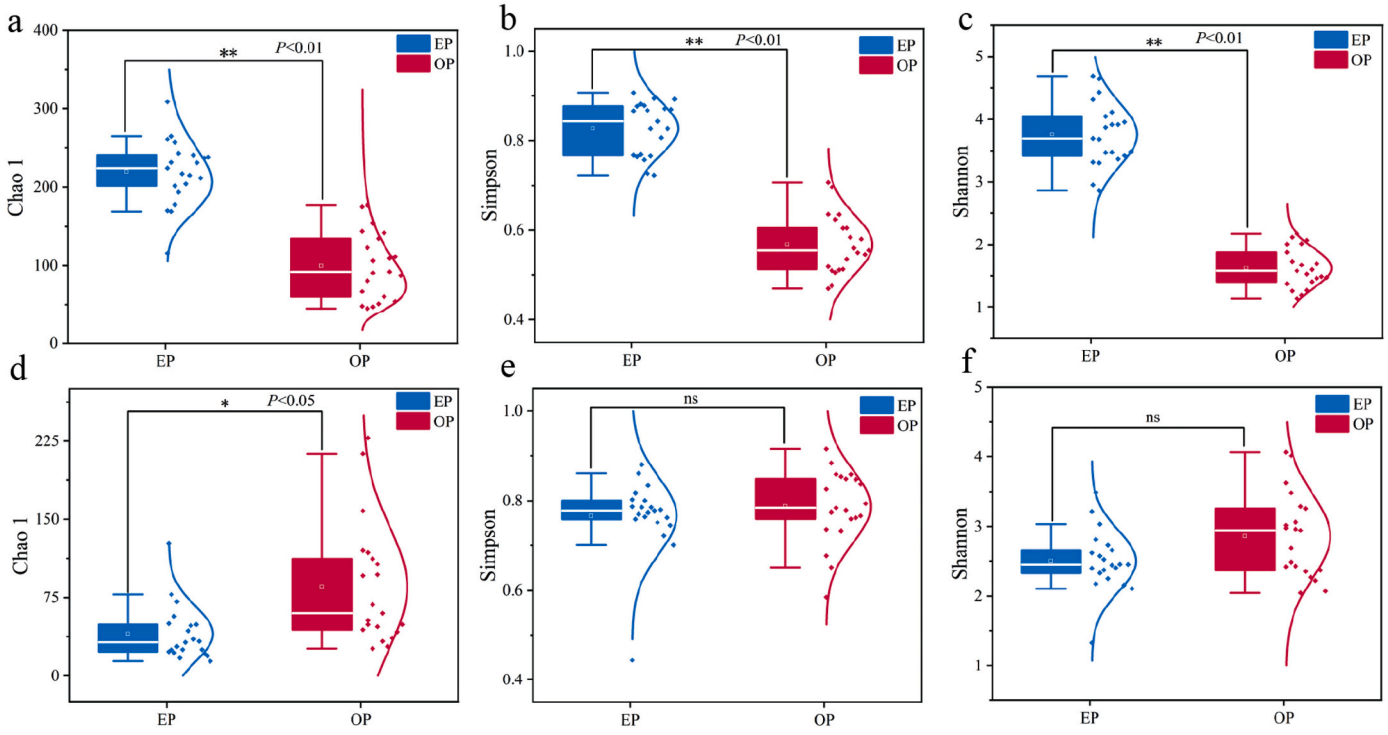


Fig. 2. Analysis of bacterial diversity indices (a: Chao 1 index, b: Simpson index, c: Shannon index) and fungal diversity indices (d: Chao 1 index, e: Simpson index, f: Shannon index) in fermented grains. * indicates significant differences (*: $P < 0.05$, **: $P < 0.01$), while ns indicates no significant difference.

Firmicutes and Proteobacteria (average relative abundance >1.00 %). In the EP group, Proteobacteria had a higher relative abundance from EP1 to EP4. Starting from EP5, Firmicutes began to replace Proteobacteria. In the OP group, Firmicutes predominated, with an average relative abundance of 95.55 %. Additionally, during the fermentation process from EP to OP, a succession phenomenon from Proteobacteria to Firmicutes was observed. This trend reflected the microbial evolution towards a fermentation-favorable environment, with Firmicutes accumulation, which benefit by its ability to form endospores that can withstand the extreme conditions (He et al., 2024). At the genus level (Fig. 3b), 431 bacterial genera were identified in the EP group and 362 in the OP group. A total of 9 dominant (average relative abundance >1.00 %) genera were identified, including *Lactobacillus*, *Kroppenstedtia*, *Pseudomonas*, *Acetobacter*, *Virgibacillus*, *Methyloversatilis*, *Thermoactinomyces*, *Vibrio*, and *Pseudoalteromonas*. Additionally, *Pediococcus* and *Bacillus* were characteristic dominant genera of EP group (relative abundance >1.00 % in EP group). In this study, *Pseudomonas* exhibited the highest relative abundance (27.53 % to 37.48 %) from EP1 to EP4, followed by *Kroppenstedtia*. From EP5 to EP7, the relative abundance of *Pseudomonas* decreased sharply from 27.77 % to 0.22 %, while *Kroppenstedtia* increased from 45.88 % to 67.26 %, establishing dominance. These results indicated a significant bacterial succession, with *Kroppenstedtia* gradually replacing *Pseudomonas* as the dominant genus. *Kroppenstedtia* was a heat-resistant genus commonly found in the high-temperature *Daqu* (Zhang et al., 2022). The later fermentation rounds conducted in the summer, which may be the reason for the significant increase of *Kroppenstedtia*. Additionally, bacterial genera with lower relative abundances also exhibited succession in EP group. For instance, the relative abundance of *Virgibacillus* decreased from EP1 to EP2 (6.31 % to 2.69 %), but increased from EP3 to EP7 (3.02 % to 12.18 %). The relative abundances of *Methyloversatilis*, *Vibrio*, and *Pseudoalteromonas* continuously decreased from EP1 to EP3, followed by an increase starting from EP4, where they maintained relatively high abundances in EP4, EP5, and EP6. In contrast, the relative abundance of *Acetobacter* exceeded 1 % only in EP1, EP2, and EP3 (3.38 %, 18.83 %, and 11.46 %, respectively), after which it continuously declined from EP4 onward, and this genus was typically found in sugar-rich environments, playing a key role in the metabolism of amino acids, terpenes, and polyketides (Yang et al., 2023). *Pediococcus* exhibited a notably high relative abundance in EP1 (10.70 %), significantly higher than other rounds, and demonstrated irregular variations from EP2 to EP6. As a key microorganism in *Xiaoqu*, *Pediococcus* played a critical role in contributing to flavor formation during subsequent fermentation stages (Hao et al., 2022). *Bacillus* showed variation across the seven rounds, with relative abundance greater than 1 % in EP1, EP4, and EP7. This genus can promote the production of pyrazines, acids, and phenolic compounds in Baijiu, as well as enhance the participation of free amino acids in the Maillard reaction, thereby contributing to the unique aroma of Baijiu (Tu et al., 2022). *Lactobacillus* displayed irregular variations with lower relative abundance in the EP group. In contrast, in the OP group, *Lactobacillus* accounted for 85.95 % to 99.00 % of the bacterial community across seven rounds, making it the absolute dominant genus. Furthermore, the bacterial communities gradually transitioned to *Lactobacillus* from EP to OP. This was attributed to the anaerobic conditions of pit fermentation, where the production of acids and ethanol inhibited the growth of other bacteria, while *Lactobacillus*, known for its tolerance to both acid and ethanol, thrived. Overall, the bacterial community in the EP group was more diverse, while the community in the OP group was more homogeneous, indirectly reflecting the different roles of microorganisms in these two groups (Wu et al., 2023). In the EP group, microorganisms primarily contributed to the hydrolysis of carbohydrates and polysaccharides. In the OP group, the microbial function mainly focused on the production of flavor compounds.

Fungi also played a crucial role in fermented grains. At the phylum level, the average relative abundance of Ascomycota in the EP and OP groups was 99.43 % and 96.43 %, respectively, occupying an absolute dominant position in both groups (Fig. 3c). This was consistent with the findings of Wu et al. (2023). At the genus level (Fig. 3d), *Issatchenkia*, *Pichia*, and *Monascus* were identified as the dominant fungal genera common to both the EP and OP groups. *Byssochlamys* and *Candida* were

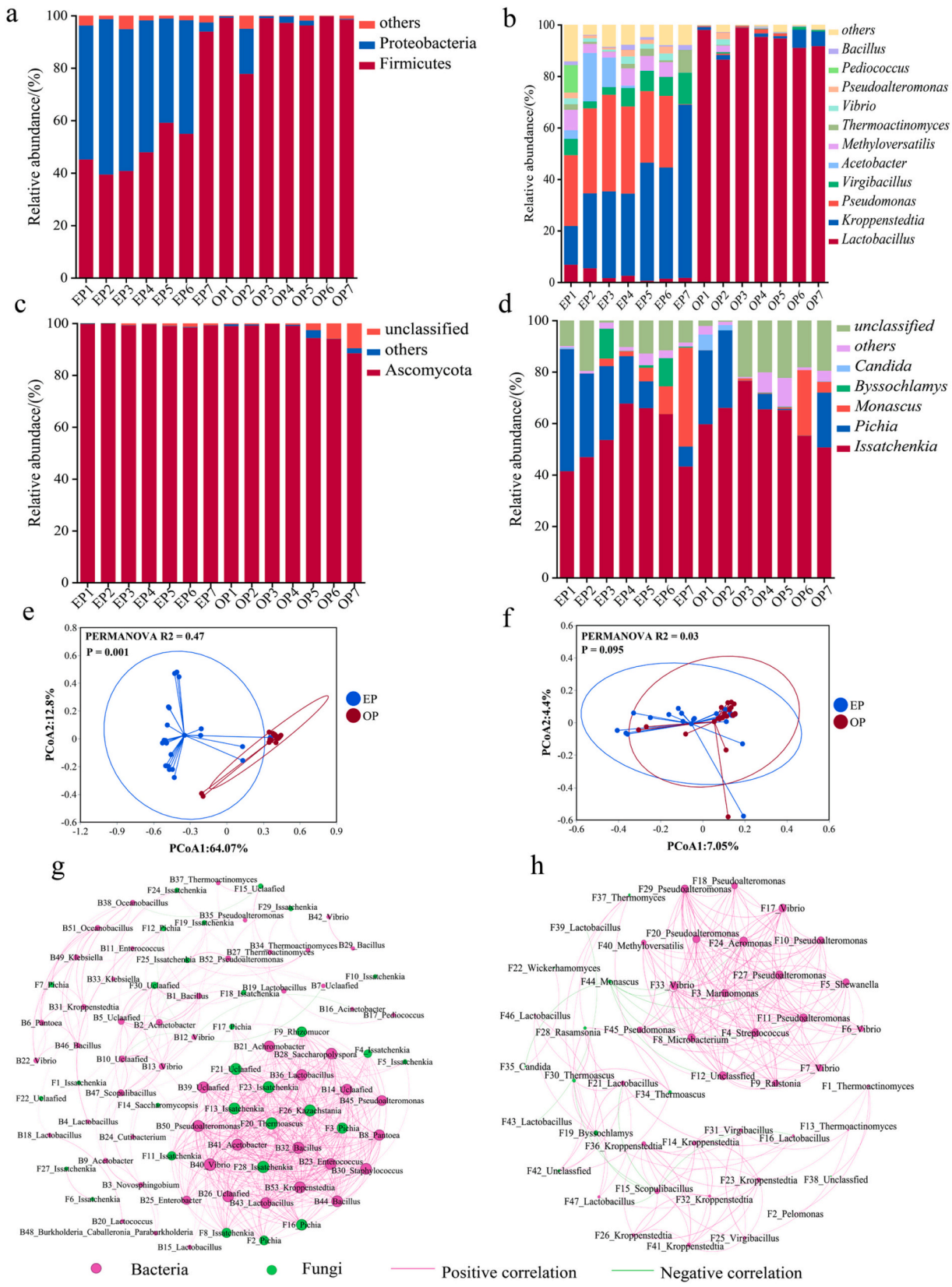


Fig. 3. Relative abundance microbial phylum (a: bacteria, c: fungi) and genus (b: bacteria, d: fungi), PCoA analysis based on OTUs (e: bacteria, f: fungi), as well as microbial interaction networks (g: EP group, h: OP group) in fermented grains.

identified as the dominant genera exclusive to the EP and OP groups, respectively. In the EP group, *Pichia* had a relatively high abundance in EP1 (47.41 %), followed by *Issatchenkia* (41.61 %). However, from EP2 onward, the relative abundance of *Pichia* decreased (32.46 % to 0.01 %) before slightly recovering in EP7 (7.81 %). Meanwhile, *Issatchenkia* consistently increased from EP2 to EP4 (47.06 % to 67.86 %) and subsequently decreased from EP5 to EP7 (66.06 % to 43.36 %), yet it maintained its dominance throughout. These findings indicated significant fungal succession in the EP group, with *Issatchenkia* gradually replacing *Pichia* as the dominant genus. *Pichia* had the ability to produce esterase that enhanced the VFCs, contributing a crisp and mellow taste to Baijiu (Zhang, Meng, et al., 2021). *Issatchenkia* was a prominent functional genus that can produce a variety of alcohols, esters, and aromatic compounds in fermented grains, which enhanced the flavor profile of SFB (Guan et al., 2023). Additionally, the relative abundance of *Monascus* showed a general increasing trend in the EP group (0.01 % to 38.42 %). *Byssochlamys*, as a unique dominant genus in EP group, exhibited irregular changes in relative abundance across EP1 to EP7, peaking at EP3 (11.62 %) and EP6 (10.92 %). In contrast, *Monascus* in the OP group demonstrated a succession pattern similar to that in the EP group, with its relative abundance steadily increasing from OP1 to OP7 (0.05 % to 41.90 %). However, *Issatchenkia* maintained the highest relative abundance among fungal genera in the OP group, increasing from OP1 to OP3 (59.84 % to 76.67 %) before declining from OP4 to OP7 (65.65 % to 50.80 %). Meanwhile, *Pichia* exhibited a rise in relative abundance from OP1 to OP2 (28.68 % to 30.12 %) and irregular fluctuations from OP3 to OP7. Furthermore, *Candida*, the unique dominant genus in the OP group, showed a relative abundance greater than 1 % in OP1 and OP2. *Candida* not only can produce esterases but also generate higher alcohol via the Ehrlich pathway, playing an important role in SFB fermentation (Yang, Yao, et al., 2024). Overall, the fungal composition in both the EP and OP groups was similar, forming a fungal community structure primarily dominated by *Issatchenkia* and *Pichia*.

Principal Coordinate Analysis (PCoA) is commonly used to visualize the differences across multiple samples, and it was performed on Operational Taxonomic Units (OTUs) of the microbial communities in this study. As shown in Fig. 3e, bacterial communities in the EP and OP groups were distinct, indicating significant differences in their bacterial community compositions. Xu et al. (2022) suggested that the fermentation environment greatly influenced bacterial communities and their composition. Bacterial in EP group could source from *Daqu*, air, the ground, tools, and operators (Wang et al., 2018). The OP group was fermentation in a closed environment, and the bacterial community gradually evolved towards a more favorable composition for fermentation. It can be inferred that the environmental differences between the two groups led to the observed differences in their bacterial communities. Additionally, fungi from the *Daqu* made a significant contribution to the fungal community in the fermented grains (Xu et al., 2023), while fewer fungi were introduced from other environments into the co-fermentation system (Wang et al., 2018). This explained the greater similarity in fungal communities between the EP and OP groups (Fig. 3f). To further investigate the interactions between microorganisms, a co-occurrence network of OTUs was constructed based on Spearman correlation coefficient, and the same threshold ($|r| > 0.7$, $P < 0.05$) was applied. As shown in Fig. 3g, h, the EP group network contained 83 nodes and 542 edges (536 positive correlations and 6 negative correlations), while the OP group network contained 47 nodes and 229 edges (216 positive correlations and 13 negative correlations). Compared to the OP group, the EP group exhibited higher centrality in its eigenvector, indicated more microbial connections and a broader ecological niche. Furthermore, several genera with relatively high abundance in the EP group showed strong positive correlations, such as *Lactobacillus*, *Bacillus*, *Issatchenkia*, *Thermoactinomyces*, and *Pichia* ($r = 1$, $P < 0.05$). This was likely due to the more nutrient-rich fermentation environment in the EP group, leading to greater microbial diversity, with competitive and cooperative interactions forming an interaction network. In contrast, the OP group displayed higher modularity and average clustering coefficients, indicating a more modular structure and a significant "small-world" network characteristic (Dan et al., 2024).

3.3. Dynamic changes in physicochemical parameters and organic acids

The physicochemical parameters and microbial communities in FG mutually interacted, which ultimately influenced the flavor quality of SFB. As shown in Fig. 4a, the moisture content in both the EP and OP groups initially increased and then decreased with fermentation rounds, peaking at EP4 (52.21 ± 0.33 %) and OP5 (54.38 ± 0.26 %), respectively. This can be attributed to the closed environment of the pit fermentation, where water produced by microbial metabolism gradually accumulated, leading to an increase in moisture content. As fermentation rounds progress, the depletion of raw materials resulted in slowed microbial metabolic activity and decreased water production, leading to a subsequent decrease in the moisture content of the fermented grains (Ren et al., 2023). Similarly to moisture content, the acidity in the OP group first increased and then decreased, peaking at OP5 (Fig. 4b), with the concentration of 4.64 ± 0.11 mL/g. The fermentation process of SFB included twice of adding ingredients, which led to an accumulation of acid-producing microorganisms in the fermented grains, causing a rapid increase in acidity during the early fermentation rounds. The decrease in acidity during the later rounds (6th and 7th) may be attributed to the depletion of raw materials and the reduced metabolic capacity of the microorganisms. Additionally, acid-producing bacteria such as *Lactobacillus* metabolized acids during pit fermentation, and the partial volatilization of organic acids during distillation further contributed to the higher acidity in the OP group compared to the EP group. As shown in Fig. 4c, the starch content in both groups decreased over the course of fermentation rounds, indicating a temporal continuity of starch content variation across seven fermentation rounds. Furthermore, the addition of *Daqu* in each round provided a certain source of starch to the fermented grains, while also reducing moisture and balancing the acidity. As a result, the starch content in the EP group was higher than in the OP group, while moisture content and acidity were lower in the EP group. The starch hydrolysis and microbial consumption together contributed to the formation and consumption of reducing sugars. As shown in Fig. 4d, the reducing sugar content in both the EP and OP groups generally increased with successive fermentation rounds, suggesting that the rate of reducing sugar production exceeded the rate of consumption. The reducing sugar content in EP group was higher than that in OP group, because starch in EP group was hydrolyzed to reducing sugar after heap fermentation. In contrast, in the OP group, reducing sugars were consumed by yeast strains such as *Issatchenkia*, which produced alcohol during pit fermentation (Chen et al., 2024).

Acidity was considered the primary factor driving the succession of microorganism in the fermentation of SFB (Chen et al., 2024). Organic acids, as important acidic compounds, influenced the synthesis of metabolites and the growth of microorganisms, ultimately affecting the flavor quality of SFB. Fig. 4e-k showed that the total organic acid content increased with fermentation rounds. Further, the content of lactic acid, acetic acid, tartaric acid, and citric acid were higher in OP group, while the levels of oxalic acid, malic acid, and succinic acid higher in EP group. The lactic acid content gradually increased in both groups across fermentation rounds, which can be attributed to the continuous accumulation of lactic acid produced by *Lactobacillus*. Acetic acid imparted a sharp, pungent taste, and its content can be influenced by microorganisms such as *Acetobacter*, *Weissella*, *Lactobacillus*, *Pichia*, and *Candida* (He et al., 2023). Some yeasts also produced acetic acid and tartaric acid along with ethanol in anaerobic environments, leading to an increase in their concentrations. Citric acid imparted a refreshing taste (Zhao et al., 2023), and its content significantly increased after pit fermentation, becoming the highest among the organic acids. Ren et al. (2023) also found that the citric acid content significantly increased after pit fermentation, which was consistent with our findings. Malic acid,

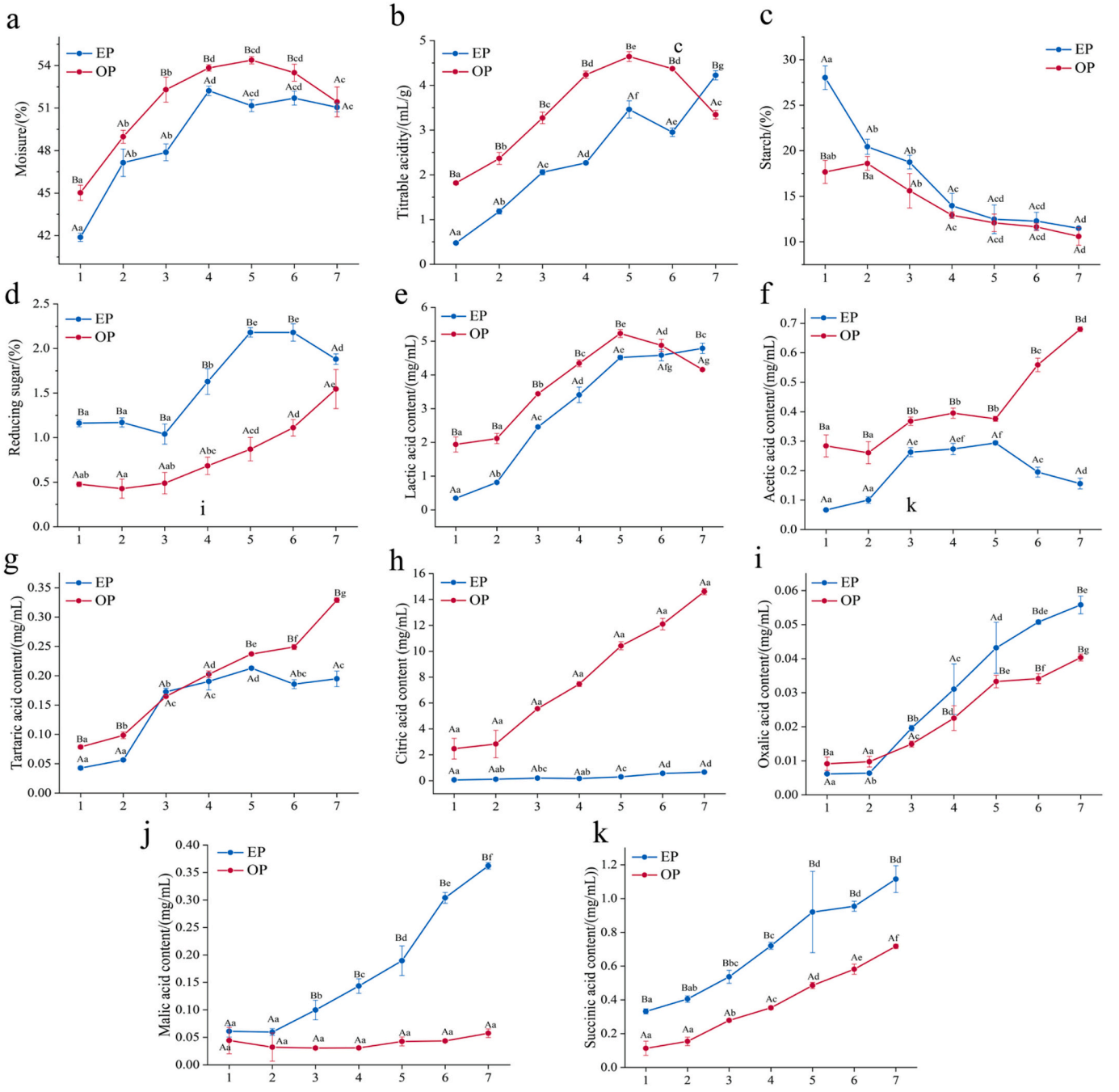


Fig. 4. Changes of the physicochemical properties and organic acids in fermented grains: moisture content (a), titratable acidity (b), starch content (c), reducing sugar content (d), lactic acid content (e), acetic acid content (f), tartaric acid content (g), citric acid content (h), oxalic acid content (i), malic acid content (j), and succinic acid content (k). Different lowercase letters indicate significant differences between fermentation rounds ($P < 0.05$), and different uppercase letters indicate significant differences between EP and OP groups ($P < 0.05$).

succinic acid, and oxalic acid were important metabolites in the tricarboxylic acid (TCA) cycle, primarily derived from the breakdown of amino acids, fatty acids, and carbohydrates. Citric acid, malic acid, and succinic acid can provide a natural sour taste and help inhibit pathogen growth. Oxalic acid, which had a sharp acidic taste, can be utilized by bacteria and yeasts as a carbon source to produce other compounds. This led to higher oxalic acid content in the EP group compared to the OP group (Jiang et al., 2025). Furthermore, interactions between these organic acids can enhance the perceived intensity of the Baijiu's flavor (Zhao et al., 2023).

3.4. Dynamic changes of VFCs in fermented grains

A total of 70 VCFs in fermented grains were detected by HS-SPME-GC–MS, with esters being the most abundant, followed by alcohols (**Table S2**). As illustrated in Fig. 5a, the total VCFs content initially increased and then decreased with successive fermentation rounds, higher in rounds 4–6. In the OP group the total content was significantly higher than in the EP group ($P < 0.05$), indicating that the pit fermentation facilitated VCFs content. As the most abundant and diverse group of compounds in fermented grains, the ester content was relatively higher in rounds 3–5 in both EP and OP groups (Fig. 5b), contributing to its characteristic aroma and mellow taste (Xu et al., 2022). Alcohols and

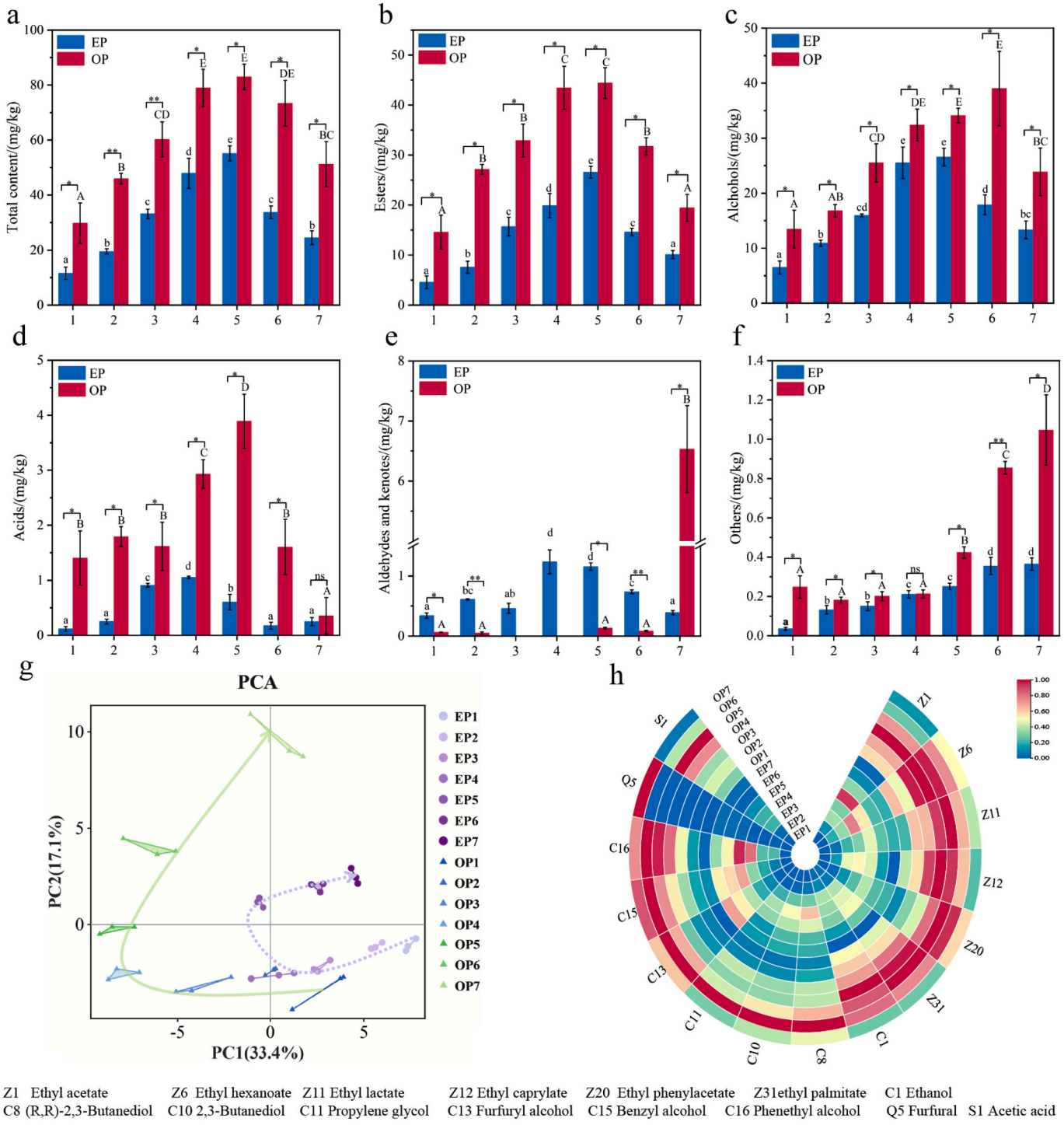


Fig. 5. Changes of VFCs content (a: total compounds, b: esters, c: alcohols, d: acids, e: aldehydes and ketones, f: others) and PCA analysis score plot (g), and changes in 15 key VFCs (h) in fermented grains. Different letters (uppercase: OP group, lowercase: EP group) indicate significant differences between fermentation rounds ($P < 0.05$). * indicates significant differences between EP and OP groups (*: $P < 0.05$, **: $P < 0.01$).

acids were important precursors for ester formation, and their levels were higher in rounds 4–6 and rounds 3–5 of both EP and OP groups, respectively (Fig. 5c, d). *Issatchenkia* can produce alcohols, which served as precursors for the synthesis of esters and other compounds (Chen et al., 2024). In our study, *Issatchenkia* exhibited relatively high abundance in rounds 3–6 of both EP and OP groups, which might explain the elevated levels of esters and alcohols observed during these rounds. In contrast, the aldehyde and ketone content in EP1-EP6 was higher in the OP group (Fig. 5e), which might be attributed to the reduction of aldehydes and ketones to alcohols and acids in the anaerobic fermentation process (Zhang, Wang, et al., 2021). Phenolic compounds, which were beneficial to human health, were found to be more abundant in the early fermentation rounds. This might due to the degradation of tannins from sorghum (Niu et al., 2024). The levels of other VFCs were relatively low (Fig. 5f), but they also contributed to the aroma of the fermented grains. In addition, some VFCs exhibited fluctuating concentrations across fermentation rounds, indicating that these metabolites were both synthesized by microorganisms and degraded during the fermentation

process (Xu et al., 2023).

Principal component analysis (PCA) was explored the changes trend of VFCs in fermented grains across the fermentation rounds. As shown in Fig. 5g, both EP and OP groups exhibited clockwise rotation across fermentation rounds, indicating consistent spatiotemporal fermentation characteristics between the two groups. The EP group displayed a greater degree of dispersion than the OP group, suggesting more significant differences in the composition and concentration of VFCs in the EP group compared to the OP group. In addition, an OPLS-DA model was constructed based on VIP $>$ 1 and $P < 0.05$ to identify key VFCs (**Fig. S2**). A total of 15 key VFCs were identified, including phenethyl alcohol, ethyl lactate, ethanol, furfural, furfuryl alcohol, acetic acid, ethyl palmitate, ethyl acetate, (*R*,*R*)-2,3-Butanediol, propylene glycol, 2,3-butanediol, ethyl caprylate, ethyl phenylacetate, ethyl hexanoate and benzyl alcohol (Fig. 5h). The concentrations of these compounds generally followed an increase-then-decrease trend throughout the seven rounds, with higher concentrations observed in the OP group compared to the EP group. Alcohols, the largest group of key VFCs (6 compounds), which can enhance the penetration of Baijiu aroma (Niu et al., 2024). In the EP group, the contents of phenylethyl alcohol, 2,3-butanediol, propylene glycol, and benzyl alcohol initially increased and then decreased across fermentation rounds. Phenylethyl alcohol, 2,3-butanediol, and propylene glycol peaked in EP4, with concentrations of 13.11 ± 1.22 mg/kg, 1.29 ± 0.48 mg/kg, and 1.32 ± 0.59 mg/kg, respectively, while benzyl alcohol reached its maximum in EP5 (0.48 ± 0.03 mg/kg). Conversely, furfuryl alcohol accumulated steadily over rounds, achieving its highest concentration in EP7 (1.53 ± 0.19 mg/kg). In the OP group, phenylethyl alcohol, 2,3-butanediol, propylene glycol, benzyl alcohol, and furfuryl alcohol also exhibited an initial increase followed by a decline, with all compounds peaking in OP6 (13.67 ± 0.60 mg/kg, 2.43 ± 0.94 mg/kg, 3.31 ± 1.24 mg/kg, 0.68 ± 0.09 mg/kg, and 2.18 ± 0.23 mg/kg, respectively). Among them, phenyl alcohol imparted rose scents, 2,3-butanediol contributed buttery flavors, and furfuryl alcohol provided a sugar burnt flavor (Harada et al., 2017; Wang, Sun, et al., 2023), all of which could enhance the smoothness and richness of the Baijiu. Ethanol, a precursor for many flavor compounds, had the highest content in OP4 (13.26 ± 1.77 mg/kg). It can interact with acids to form ethyl esters, thus influencing the Baijiu aroma. Furthermore, the ester compounds found among the differential VFCs were all ethyl esters. Ethyl lactate (fruit aroma), ethyl acetate (pineapple aroma), and ethyl hexanoate (pineapple, pit aroma) were considered to contribute significantly to the Baijiu flavor, generally with relatively high concentrations in Baijiu (Xu et al., 2022). In this study, ethyl acetate exhibited the highest average content among all compounds. Its concentration fluctuated irregularly in the EP group, peaking in EP5 (7.47 ± 1.22 mg/kg), while in the OP group, it initially increased and then decreased, with the highest level observed in OP4 (7.94 ± 1.93 mg/kg). Besides, both EP and OP groups displayed a similar trend for ethyl lactate and ethyl hexanoate, where concentrations increased initially and then decreased. Ethyl lactate reached its maximum in the fifth round (EP5: 7.72 ± 0.11 mg/kg; OP5: 16.42 ± 0.16 mg/kg), while ethyl hexanoate peaked in the fourth round (EP4: 2.18 ± 0.14 mg/kg; OP4: 2.78 ± 0.21 mg/kg). Higher fatty acid esters, such as ethyl caprylate and ethyl palmitate, can contribute fruity aromas, enhance the richness of the Baijiu, and reduce astringency (Wang et al., 2022). Their concentrations in both EP and OP groups initially increased and then declined. Ethyl caprylate peaked in the fourth round (EP4: 1.27 ± 0.16 mg/kg; OP4: 2.21 ± 0.11 mg/kg), while ethyl palmitate reached its highest level in the fifth round (EP5: 2.67 ± 0.14 mg/kg; OP5: 4.67 ± 0.58 mg/kg). Ethyl phenylacetate (rose and osmanthus aroma), formed through esterification of phenyl alcohol with acetic acids (Wang et al., 2022), also had the highest concentration in the fifth round (EP5: 13.11 ± 1.22 mg/kg; OP5: 2.19 ± 0.17 mg/kg). Acetic acid, which can combine with alcohols to form ethyl acetate and enhance Baijiu flavor characteristics, was detected across all seven rounds. Its content varied irregularly, peaking in EP3 (0.91 ± 0.03 mg/kg) and OP5 (3.89 ± 0.49 mg/kg), respectively. Furfural alcohol, recognized as a characteristic compound of SFB, imparted a sweet taste (Wang et al., 2022), and its highest concentration was observed in OP7 (5.85 ± 0.68 mg/kg). On the whole, the content of key volatile flavor compounds was generally higher in the intermediate rounds 4–6, consistent with findings by Wu et al. (2023), suggesting that the VFCs profile of SFB exhibits spatiotemporal variation.

3.5. Analysis of non-volatile flavor compound content changes and differences

N-VFCs played a crucial role in forming the complex flavor profile of SFB, and LC-MS/MS was used to measure them in the fermentation grains from seven rounds. A total 181 effective N-VFCs were identified (**Table S2**). Amino acids and derivatives content were the most abundant N-VFCs, followed by fatty acids and derivatives (Fig. 6a). Amino acids contributed various flavors, including umami, sweetness, bitterness, and astringency, and served as precursors for many other metabolites. Fatty acids can be reduced to fatty alcohols to form ethyl esters during the fermentation, which were important components of the flavor profile. A moderate amount of organic acids can smooth the Baijiu and balance the taste. In addition to the seven organic acids quantified, we also detected betaine, DL-salicylic acid, watercress alkaloid, α-ketoglutaric acid, folic acid, and other organic acids and derivatives. Among the detected N-VFCs, L-glutamic acid, betaine, *L*-phenylalanine, oleamide, 2-hydroxyphenylalanine, choline, proline, oleic acid, 3-phenyl lactic acid, and hypoxanthine were the most abundant across all seven rounds. They might originate from the raw materials or *Daqu*, and be progressively decomposed or consumed as flavor precursors during the fermentation.

The PCA result showed that EP1-EP3 exhibited a higher degree of dispersion (Fig. 6b), which was likely due to the abundant raw materials and the availability of oxygen in the early rounds, which activated the microorganisms, accelerating the changes in N-VFCs (Chen et al., 2023). Further, an OPLS-DA model was constructed (**Fig. S3**), and a total of 32 significantly key N-VFCs were identified based VIP $>$ 1, $P < 0.05$ (Fig. 6c, d). The concentrations of amino acids, including proline, L-pyroglutamic acid, valine, 2-hydroxyphenylalanine, leucylproline, and valylproline, exhibited dynamic changes across the seven fermentation rounds in both EP and OP groups, with more pronounced fluctuations observed during the initial four rounds. In detail, the proline content in both groups gradually decreased throughout fermentation rounds, whereas 2-hydroxyphenylalanine content steadily increased. In contrast, the levels of L-pyroglutamic acid, valine, leucylproline, and valylproline displayed irregular variations. Proline could contribute a sweet taste to Baijiu, while valine was closely related to the synthesis of higher alcohols and esters (Sun et al., 2022). For fatty acids and derivatives, 3-hydroxy-3-methylglutaric acid and 1-palmitoylglycerol accumulated across fermentation rounds, whereas oleamide and 3-isopropylmalic acid decreased in both EP and OP groups. 1-palmitoylglycerol could contribute an oily aroma and was an important precursor for the synthesis of ethyl palmitate. Oleamide, known for its sleep-inducing and anti-anxiety properties, can prevent and improve obesity (Kobayashi et al., 2022). Flavonoids, considered health-promoting factors in Baijiu, exhibited antimicrobial and antioxidant properties. The content of flavonoids, including luteolin, naringenin, apigenin, genistein, and quercetin, decreased over the fermentation rounds in both EP and OP groups. Zou et al. (2024) also noted that flavonoids in SFB were present in trace amounts and decreased as fermentation progresses. For nucleosides, nucleotides, and analogues, the content of cytosine increased across rounds, while 4-hydroxybenzaldehyde and adenosine generally decreased, and hypoxanthine exhibited irregular changes. 4-hydroxybenzaldehyde and D-(+)-arabinitol increased with fermentation rounds. 4-hydroxybenzaldehyde had anticoagulant effects and protects the blood-brain barrier (Zhou et al., 2022). *Lactobacillus* and *Saccharomyces* can generate ethanol, acetic acid, and other flavor

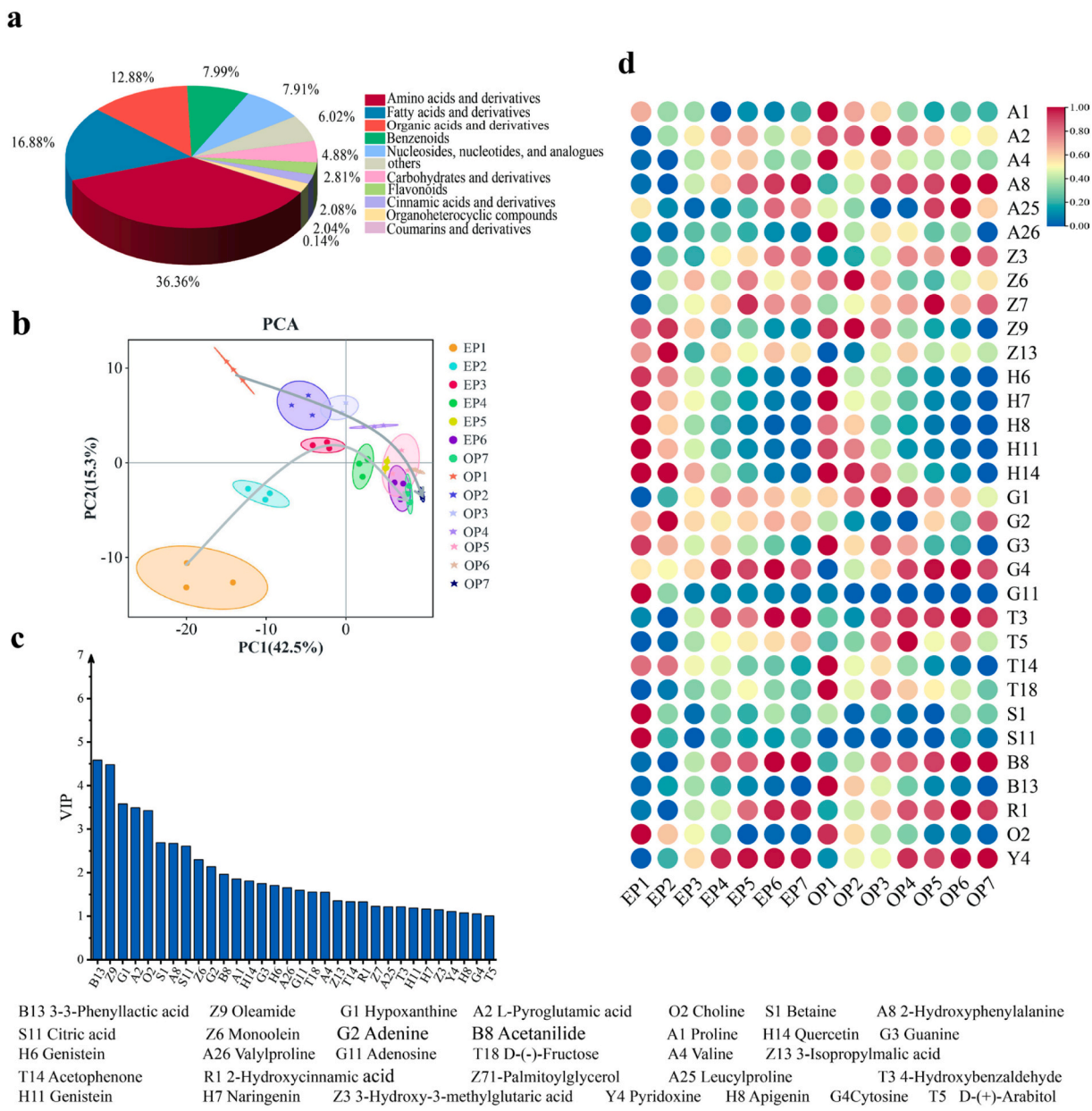


Fig. 6. Proportion (a) and PCA analysis (b) of N-VFCs content in fermented grains. VIP scores (c) and content changes (d) of 34 key N-VFCs in fermented grains.

components from arabinose (Wang, Huang, & Huang, 2021). Additionally, N-VFCs such as acetanilide, 2-hydroxycinnamic acid, and pyridoxine accumulated during fermentation rounds, although at lower concentrations. Overall, N-VFCs were continuously generated and consumed through multiple metabolic pathways during the fermentation of SFB. The total content of N-VFCs compounds in the OP group was higher than in the EP group, suggesting that the generation of N-VFCs in the pit fermentation process outweighed their consumption. However, as the raw materials were progressively decomposed and consumed, the content of N-VFCs tends to decrease over successive rounds.

3.6. Microbial and metabolite correlation analysis

To elucidate the interactions between microorganisms and metabolites, we performed a correlation analysis based on the Spearman correlation coefficient ($|r| > 0.8$, $P < 0.05$) between dominant microorganisms and key VFCs and N-VFCs, as well as seven organic acids. The results showed that in the EP group, a total of 133 significant correlations were identified, with 71 positive and 62 negative correlations (Fig. 7a). In the OP group, 59 significant correlations were observed, with 22 positive and 27 negative correlations (Fig. 7b). In the EP group, *Issatchenkia* exhibited the most correlations with VFCs (8 pairs), showing positive correlations with ethyl hexanoate, ethyl lactate, ethyl palmitate, phenylethyl acetate, 2,3-butanediol, phenylethanol, propylene glycol, and acetate. It has been found that *Issatchenkia* could produce alcohols and acids, also generate esterases that combine them to generate ester compounds (Guan et al., 2023). In our study, the total content of the aforementioned eight VFCs and the relative abundance of *Issatchenkia* were notably higher in EP4-EP6. Therefore, it was hypothesized that *Issatchenkia* might be a key contributor to the VFCs of fermented grains. Additionally, *Monascus*, *Kroppenstedtia*, and *Virgibacillus* showed positive correlations with furfuryl alcohol, which was typically associated with the Maillard reaction (Harada et al., 2017). *Lactobacillus* and *Pichia* were positively correlated with benzyl alcohol, while *Kroppenstedtia* and *Monascus* exhibited negative correlations. In terms of organic acids, *Kroppenstedtia*, *Monascus*, and *Virgibacillus* showed

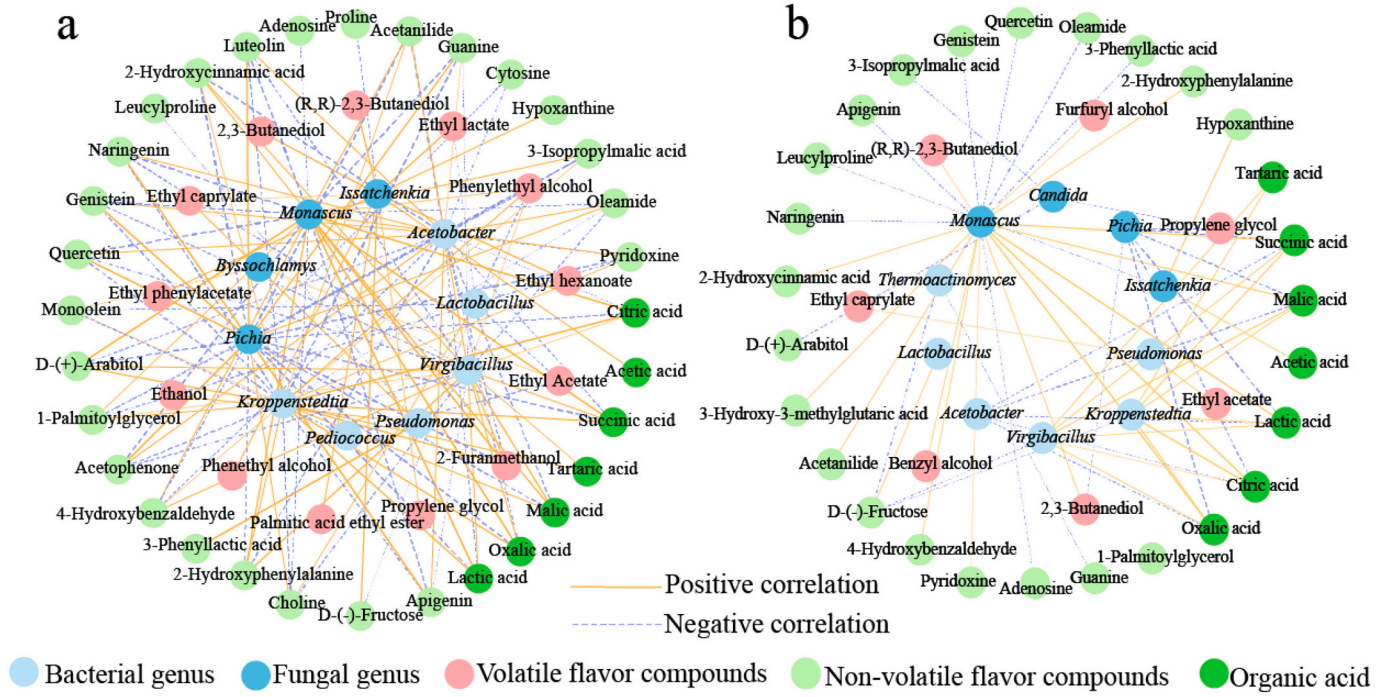


Fig. 7. Correlation networks between microorganisms and VFCs, N-VFCs, and organic acids in the EP group (a) and OP group (b) ($|r| > 0.8$, $P < 0.05$; the thickness of the lines represents the strength of the correlation).

positive correlations with oxalic acid, lactic acid, succinic acid, and malic acid. *Kroppenstedtia* can produce amylase, which hydrolyzes starch into sugars, indirectly promoting the TCA cycle and the generation of organic acids (Zhu et al., 2022). Regarding N-VFCs, *Pichia*, *Monascus*, and *Kroppenstedtia* exhibited strong correlations ($|r| > 0.9$, $P < 0.05$) with more than 8 N-VFCs. *Pichia* showed strong positive correlations with compounds such as apigenin, luteolin, naringenin, genistein quercetin, acetophenone, and guanine. Interestingly, both *Monascus* and *Kroppenstedtia* showed strong negative correlations with these compounds. Furthermore, *Monascus* and *Kroppenstedtia* demonstrated strong positive correlations with D-(+)-arabitol and 2-hydroxyphenylalanine. *Acetobacter* exhibited positive correlations with the five flavonoids mentioned above and acetophenone, but a negative correlation with D-(+)-arabitol.

In the OP group, there were fewer significant correlations than in the EP group, which could be attributed to the reduced microbial diversity during the pit fermentation process. For VFCs, *Monascus* showed positive correlations with alcohols like 2,3-butanediol, phenylethanol, and furfural alcohol, while *Pseudomonas* was positively correlated with ethyl acetate and ethyl caprylate. *Pichia* showed a negative correlation with propylene glycol and 2,3-butanediol. During Baijiu fermentation, *Monascus* played a crucial role in promoting ester production (Cheng et al., 2024), which could facilitate the formation of alcohol precursors. As for organic acids, *Kroppenstedtia*, *Virgibacillus*, and *Monascus* exhibited positive correlations with oxalic acid, lactic acid, succinic acid, and malic acid, while *Acetobacter* and *Pichia* showed negative correlations. Moreover, *Monascus* displayed the highest number of correlations with N-VFCs (13 pairs). It showed positive correlations with 2-hydroxyphenylalanine, 3-hydroxy-3-methylglutaric acid, 4-hydroxybenzaldehyde, acetanilide, 2-hydroxycinnamic acid, and pyridoxine, while showing negative correlations with oleamide, naringenin, apigenin, genistein, quercetin, and adenosine, which was similar to that observed in the EP group. Overall, from EP to OP, the fermentation system tended to stabilize, with microbial succession becoming more uniform, leading to a reduction in the number of significant correlations between microorganisms and metabolites.

3.7. Metabolic network construction

To explore the metabolic pathways in the fermented grains, we conducted an enrichment analysis of all detected flavor compounds. Using a threshold of $P < 0.05$ and impact >0.2, five key pathways closely related to the flavor compounds were identified: phenylalanine, tyrosine, and tryptophan biosynthesis, phenylalanine metabolism, alanine, aspartate, and glutamate metabolism, Citrate cycle (TCA cycle), and histidine metabolism (Fig. 8a). These findings suggested that amino acid metabolism was one of the crucial pathways.

Besides, based on microbial annotations of related enzymes, and integrating the KEGG database and relevant literature, we constructed a metabolic network for flavor compounds (Fig. 8 **b, c**). Proteins were degraded into peptides, amino acids, and their derivatives, such as valine, threonine, L-pyroglutamic acid, 2-hydroxyphenylalanine, and leucylproline, with the participation of *Bacillus*, *Monascus*, and *Thermoascus*. Some of these amino acids could serve as precursors for higher alcohols, aldehydes, and ketones (Jiang et al., 2023). Notably, leucine and phenylalanine can be derived from carbohydrate degradation and subsequently converted into higher alcohols during the fermentation of SFB. For instance, leucine can be transformed into 3-methyl-1-butanol through EC.1.1.1.1 (Wang, Sun, et al., 2023). Phenylalanine, on the other hand, was decarboxylated by EC:4.1.1.28 to form phenylacetaldehyde, which was then catalyzed by EC:1.1.1.90 to finally produce phenyl alcohol. During this process, *Lactobacillus* and *Bacillus* secreted EC.4.1.1.28, while *Aspergillus* and *Saccharomyces* produced EC.1.1.1.1 and EC.1.1.1.90. Currently, enzymes related to the synthesis of phenyl alcohol included EC 4.1.1.28, EC 1.1.1.1, EC 1.1.1.90, EC 2.6.1.57, EC 4.1.1.43, and EC 1.2.4.4 (Jiang et al., 2023). Our results annotated all these enzymes except for EC 4.1.1.43, and their abundance was higher in the OP group, which explained the higher phenyl alcohol content in the OP group.

Carbohydrates, a rich energy source for microorganisms in the fermented grains, were broken down into glucose by amylases secreted by *Bacillus* and *Aspergillus* (Huang et al., 2020). On one hand, glucose served as precursors for certain amino acids like L-histidine and leucine. Leucine can be converted into 3-methyl-1-butanol through EC:1.1.1.1

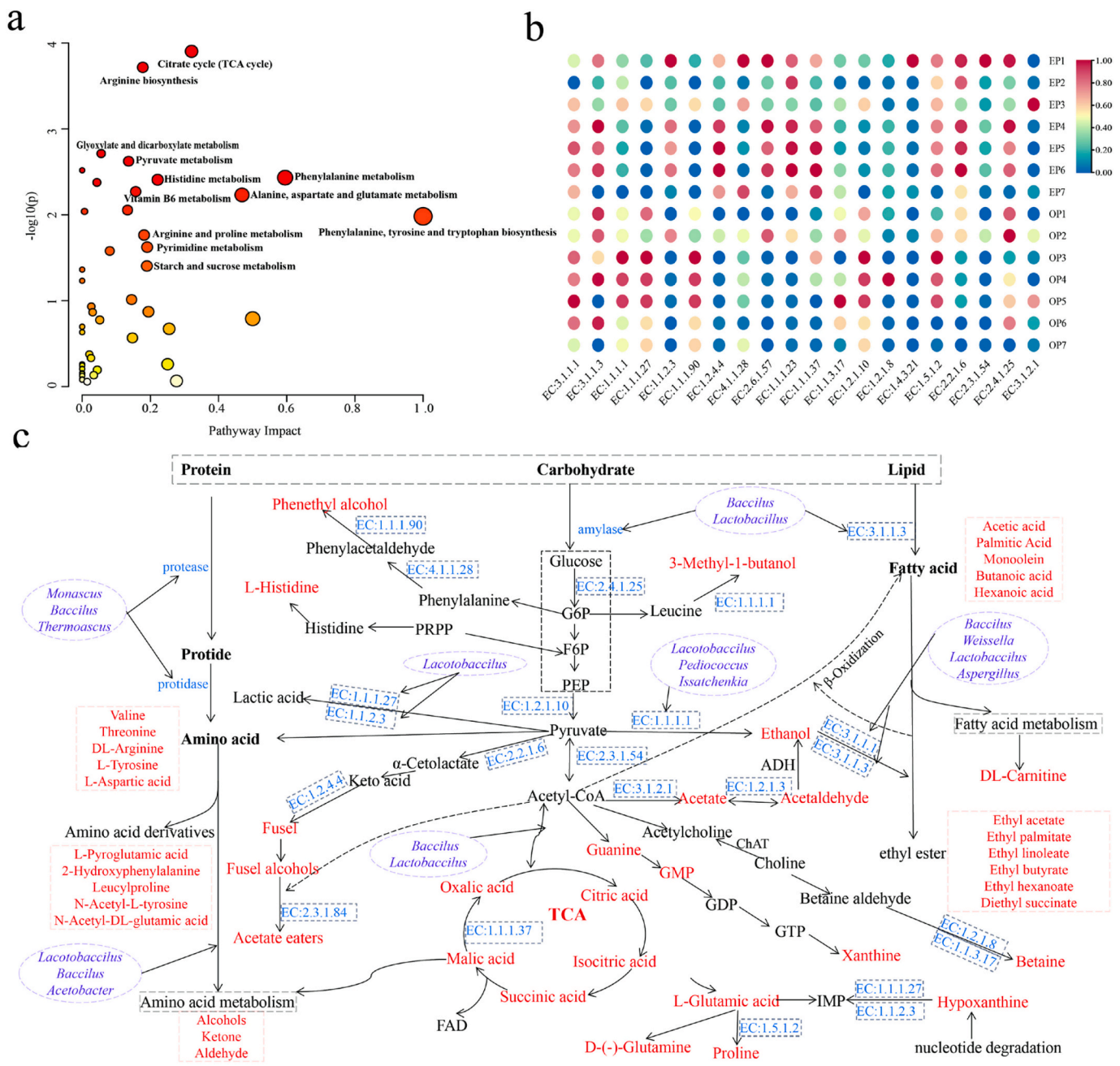


Fig. 8. Enrichment bubble chart of flavor compound pathways (a), changes in the abundance of related functional enzymes (b), and predicted microorganism and flavor metabolic pathway networks (c) in fermented grains.

(Wang, Zhang, et al., 2023). On the other hand, glucose underwent glycolysis pathway (EMP) to produce pyruvate, a key intermediate in fermented grains metabolism. Pyruvate can be catalyzed by EC:1.1.1.27 and EC:1.1.2.3 to form lactic acid, it also can be converted into ethanol through EC:1.1.1.1. *Lactobacillus*, *Pediococcus*, and *Issatchenkia* played crucial roles in these processes (Zou et al., 2024). The higher abundance of *Lactobacillus*, *Issatchenkia*, EC:1.1.1.1, EC:1.1.1.27, and EC:1.1.2.3 in the OP group could explain the higher content of alcohol in this group. Furthermore, pyruvate also can be converted into acetate eaters and acetyl-CoA. Acetyl-CoA was involved in several metabolic pathways, including the production of acetic acid and betaine. These compounds were present at higher levels in the OP group, indicating a more active acetyl-CoA metabolism. Acetyl-CoA can also generate guanine, GMP, and others through the guanylic acid metabolic pathway, ultimately leading to the production of xanthine. Additionally, acetyl-CoA participated in the TCA cycle, an important metabolic pathway in Baijiu fermentation. The TCA cycle not only generated organic acids such as oxalic acid, citric acid, isocitric acid, succinic acid, malic acid, and oxalic acid, contributing to the characteristic sour and umami flavors, but also produced L-glutamic acid, which can be further converted into D-(−)-glutamine, proline, and hypoxanthine, contributing to the development of bitterness (Zhang et al., 2023).

Esters can be categorized into acetic acid esters and fatty acid esters. In the fatty acid ester metabolic pathway, lipids were first broken down into fatty acids such as palmitic acid, butyric acid, and hexanoic acid. Then, enzymes such as EC 3.1.1.3 and EC 3.1.1.1, secreted by *Bacillus*, *Lactobacillus*, *Weissella*, and *Aspergillus*, catalyzed the reversible condensation of acid and alcohols to form ester compounds such as ethyl palmitate, ethyl butyrate, and hexyl acetate (Zou et al., 2024). These enzymes were annotated in our study, and their expressions were higher

in rounds 3–6 of both EP and OP groups, which explained the relatively higher content of these compounds in these rounds. In addition, certain fatty acids, such as palmitic acid, can be transformed to DL-carnitine via fatty acid metabolism. These findings provided some insight into the metabolic pathways of microorganisms and flavor compounds in SFB. However, these results should be interpreted cautiously, as they do not necessarily imply that these microorganisms directly produce these metabolites.

4. Conclusion

This study investigated the microbial communities, physicochemical indicators, VFCs and N-VFCs in fermented grains across seven fermentation rounds of SFB. The result showed that from EP group to OP group, bacterial diversity decreased and fugal diversity increased, as well as *Lactobacillus*, *Issatchenkia* occupied the dominant position. In terms of metabolites, a total of 72 VFCs and 181 N-VFCs were detected. The total content of VFCs and N-VFCs in the OP group was higher than that in the EP group, with the VFCs concentration increased initially and then decreased and N-VFCs concentration decreased as rounds progressed. Furthermore, the organic acid content and physicochemical parameters in the OP group showed significant differences compared to the EP group ($P < 0.05$). Correlation analysis indicated that *Issatchenkia* played a key role in the production of VFCs, while *Kroppenstedtia*, *Monascus*, *Virgibacillus*, and *Pichia* contributed significantly to N-VFCs. These findings provided a preliminary analysis of the characteristics of the fermented grains, offering data support for future research on SFB flavor quality improvement.

CRediT authorship contribution statement

Xiaomin Liu: Writing – original draft, Validation, Investigation. **Yingchun Mu:** Writing – review & editing, Supervision. **Xiangxiang Lv:** Resources, Investigation. **Nuo Chen:** Investigation. **Lei Chen:** Investigation. **Tangzheng Wen:** Investigation. **Wei Su:** Supervision, Funding acquisition.

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.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (32160554) and the industry-university-research cooperation project of Xiaohutuxian Liquor Industry (Group) Co., LTD (701332212213).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102228.

Data availability

Data will be made available on request.

References

Chen, T., Wang, H., Su, W., Mu, Y., & Tian, Y. (2023). Analysis of the formation mechanism of volatile and non-volatile flavor substances in corn wine fermentation based on high-throughput sequencing and metabolomics. *Food Research International, 165*, Article 112350, 112350.10.1016/j.foodres.2022.112350.

Chen, X., Du, B., Liu, J., Zhang, C., Zhu, H., Wang, K., et al. (2024). Exploring the significance of the 2nd and 4th round fermentations in the brewing process of sauce-flavor baijiu. *Food Bioscience, 59*, Article 104114. https://doi.org/10.1016/j.fbio.2024.104114

Cheng, W., Chen, X., Xue, X., Lan, W., Zeng, H., Li, R., et al. (2024). Comparison of the correlations of microbial community and volatile compounds between pit-mud and fermented grains of compound-flavor baijiu. *Foods, 13*(2), 203. https://doi.org/10.3390/foods13020203

Dan, H., Song, X., Xiang, G., Song, C., Dai, H., Shao, Y., et al. (2024). The response pattern of the microbial community structure and metabolic profile of *jiupei* to *Bacillus subtilis* JP1 addition during *baijiu* fermentation. *Journal of the Science of Food and Agriculture, 104*(9), 5021–5030, 5021-5030.10.1002/jsfa.13345.

Guan, T., Wu, X., Hou, R., Tian, L., Huang, Q., Zhao, F., et al. (2023). Application of *clostridium butyricum*, *Rummeliibacillus suwonensis*, and *Issatchenkia orientalis* for *Nongxiangxing baijiu* fermentation: Improves the microbial communities and flavor of upper fermented grain. *Food Research International, 169*, Article 112885. https://doi.org/10.1016/j.foodres.2023.112885

Hao, H., Yan, R., Miao, Z., Wang, B., Sun, J., & Sun, B. (2022). Volatile organic compounds mediated endogenous microbial interactions in Chinese baijiu fermentation. *International Journal of Food Microbiology, 383*, Article 109955, 109955.10.1016/j.ijfoodmicro.2022.109955.

Harada, R., Yuzuki, M., Ito, K., Shiga, K., Bamba, T., & Fukusaki, E. (2017). Influence of yeast and lactic acid bacterium on the constituent profile of soy sauce during fermentation. *Journal of Bioscience and Bioengineering, 123*(2), 203–208. https://doi.org/10.1016/j.jbiosc.2016.08.010

He, G., Gao, L., Deng, Y., Jiao, W., Wang, J., Wei, Y., et al. (2024). Microbial succession in different years of pit mud from a distillery in Sichuan for nong-xiang baijiu fermentation. *Food Science and Biotechnology, 33*(13), 3083–3092. https://doi.org/10.1007/s10068-024-01558-4

He, G., Xie, F., Ren, X., Yin, B., Du, L., Wei, Y., et al. (2023). Revealing the functional microbiota for acetic acid formation in Daqu starter for Chinese *nong-Xiang baijiu* fermentation. *Food Bioscience, 53*, Article 102782. https://doi.org/10.1016/j.fbio.2023.102782

Huang, X., Fan, Y., Lu, T., Kang, J., Pang, X., Han, B., et al. (2020). Composition and metabolic functions of the microbiome in fermented grain during light-flavor *baijiu* fermentation. *Microorganisms, 8*(9), 1281. https://doi.org/10.3390/microorganisms8091281

Jiang, L., Mu, Y., Su, W., Tian, H., Zhao, M., Su, G., et al. (2023). Effects of *Pediococcus acidilactici* and *Rhizopus Oryzae* on microbiota and metabolomic profiling in fermented dry-cure mutton sausages. *Food Chemistry, 403*, Article 134431. https://doi.org/10.1016/j.foodchem.2022.134431

Jiang, L., Shen, S., Zuo, A., Chi, Y., Lu, Y., & He, Q. (2025). Characterizing flavor development in low-salt Chinese horse bean-chili paste through integrated metabolomics and metagenomics. *Food Chemistry, 463*, Article 141076. https://doi.org/10.1016/j.foodchem.2024.141076

Kobayashi, Y., Kubota, M., Sugimoto, K., Kitakaze, T., Harada, N., & Yamaji, R. (2022). Dietary oleamide attenuates obesity induced by housing mice in small cages. *Bioscience, Biotechnology, and Biochemistry, 86*(8), 1095–1105. https://doi.org/10.1093/bbb/zbac082

Lu, Y., Zhang, C., Zhao, H., Min, W., Zhu, H., Wang, H., et al. (2022). Effect of environmental microorganisms on fermentation microbial Community of Sauce-Flavor *baijiu*. *Foods, 12*(1), 10. https://doi.org/10.3390/foods12010010

Niu, Y., Yang, Y., Mao, C., & Xiao, Z. (2024). Effects of gallic acid on the release of aroma compounds in Moutai baijiu. *Food Research International, 176*, Article 113655. https://doi.org/10.1016/j.foodres.2023.113655

Ren, T., Su, W., Mu, Y., Qi, Q., & Zhang, D. (2023). Study on the correlation between microbial communities with physicochemical properties and flavor substances in the Xiasha round of cave-brewed sauce-flavor baijiu. *Frontiers in Microbiology, 14*. https://doi.org/10.3389/fmicb.2023.1124817

Sun, H., Ni, B., Yang, J., & Qin, Y. (2022). Nitrogenous compounds and Chinese baijiu: A review. *Journal of the Institute of Brewing, 128*(1), 5–14. https://doi.org/10.1002/jib.686

Tu, W., Cao, X., Cheng, J., Li, L., Zhang, T., Wu, Q., et al. (2022). Chinese baijiu: The perfect works of microorganisms. *Frontiers in Microbiology, 13*, 919044.10.3389/fmicb.2022.919044.

Wang, B., Wu, Q., Xu, Y., & Sun, B. (2021). Multiple sugars promote microbial interactions in Chinese baijiu fermentation. *LWT, 138*, Article 110631. https://doi.org/10.1016/j.lwt.2020.110631

Wang, G., Jing, S., Song, X., Zhu, L., Zheng, F., & Sun, B. (2022). Reconstitution of the flavor signature of *Laobaigan*-type baijiu based on the natural concentrations of its odor-active compounds and nonvolatile organic acids. *Journal of Agricultural and Food Chemistry, 70*(3), 837–846. https://doi.org/10.1021/acs.jafc.1c06791

Wang, H., Huang, Y., & Huang, Y. (2021). Microbiome diversity and evolution in stacking fermentation during different rounds of Jiang-flavoured baijiu brewing. *LWT, 143*, Article 111119. https://doi.org/10.1016/j.lwt.2021.111119

Wang, H., Sun, C., Yang, S., Ruan, Y., Lyu, L., Guo, X., et al. (2023). Exploring the impact of initial moisture content on microbial community and flavor generation in Xiaoqu baijiu fermentation. *Food Chemistry: X, 20*, Article 100981. https://doi.org/10.1016/j.fochx.2023.100981

Wang, H., Zhang, M., Qu, C., Fei, Y., Liang, J., Bai, W., et al. (2023). Characterization and correlation of microbiota and higher alcohols based on metagenomic and metabolite profiling during Rice-flavor baijiu fermentation. *Foods, 12*(14), 2720. https://doi.org/10.3390/foods12142720

Wang, L., Tang, P., Zhao, Q., Shan, Q., Qin, L., Xiao, D., et al. (2024). Difference between traditional brewing technology and mechanized production technology of jiangxiangxing baijiu: Micro ecology of zaopei, physicochemical factors and volatile composition. *Food Research International, 192*, Article 114748. https://doi.org/10.1016/j.foodres.2024.114748

Wang, W., Xu, Y., Huang, H., Pang, Z., Fu, Z., Niu, J., et al. (2021). Correlation between microbial communities and flavor compounds during the fifth and sixth rounds of sauce-flavor baijiu fermentation. *Food Research International, 150*(Pt A), Article 110741. https://doi.org/10.1016/j.foodres.2021.110741

Wang, X., Du, H., Zhang, Y., & Xu, Y. (2018). Environmental microbiota drives microbial succession and metabolic profiles during Chinese liquor fermentation. *Applied and Environmental Microbiology, 84*(4). https://doi.org/10.1128/AEM.02369-17

Wu, Y., Duan, Z., Niu, J., Zhu, H., Zhang, C., Li, W., et al. (2023). Spatial heterogeneity of microbiota and flavor across different rounds of sauce-flavor baijiu in northern China. *Food Chemistry: X, 20*, Article 100970. https://doi.org/10.1016/j.fochx.2023.100970

Xu, Y., Wu, M., Niu, J., Lin, M., Zhu, H., Wang, K., et al. (2023). Characteristics and correlation of the microbial communities and flavor compounds during the first three rounds of fermentation in Chinese sauce-flavor baijiu. *Foods, 12*(1), 207. https://doi.org/10.3390/foods12010207

Xu, Y., Zhao, J., Liu, X., Zhang, C., Zhao, Z., Li, X., et al. (2022). Flavor mystery of Chinese traditional fermented baijiu: The great contribution of ester compounds. *Food Chemistry, 369*, Article 130920. https://doi.org/10.1016/j.foodchem.2021.130920

Yang, L., Huang, X., Hu, J., Deng, H., He, J., & Zhang, C. (2024). The spatiotemporal heterogeneity of microbial community assembly during pit fermentation of soy sauce flavor baijiu. *Food Bioscience, 61*, Article 104438. https://doi.org/10.1016/j.fbio.2024.104438

Yang, L., Xian, C., Li, P., Wang, X., Song, D., Zhao, L., et al. (2023). The spatio-temporal diversity and succession of microbial community and its environment driving factors during stacking fermentation of Maotai-flavor baijiu. *Food Research International, 169*, Article 112892. https://doi.org/10.1016/j.foodres.2023.112892

Yang, X., Yao, J., Hu, Y., Qin, Z., & Li, J. (2024). Fungal community succession and volatile compound changes during fermentation of Laobaigan *baijiu* from Chinese Hengshui region. *Foods, 13*(4), 569. https://doi.org/10.3390/foods13040569

Zhang, H., Wang, L., Tan, Y., Wang, H., Yang, F., Chen, L., et al. (2021). Effect of *Pichia* on shaping the fermentation microbial community of sauce-flavor *baijiu*. *International Journal of Food Microbiology, 336*, Article 108898. https://doi.org/10.1016/j.ijfoodmicro.2020.108898

Zhang, K., Zhang, T., Guo, R., Ye, Q., Zhao, H., & Huang, X. (2023). The regulation of key flavor of traditional fermented food by microbial metabolism: A review. *Food Chemistry: X, 19*, Article 100871. https://doi.org/10.1016/j.fochx.2023.100871

Zhang, X., Meng, L., Lu, Z., Chai, L., Wang, S., Shi, J., et al. (2021). Identification of age-markers based on profiling of baijiu volatiles over a two-year maturation period: Case study of Lu-flavor baijiu. *LWT, 141*, Article 110913. https://doi.org/10.1016/j.lwt.2021.110913

Zhang, Y., Xu, J., Jiang, Y., Niu, J., Chen, X., & Han, B. Z. (2022). Microbial characteristics and metabolite profiles of high-temperature *Daqu* in different maturation stages. *World Journal of Microbiology and Biotechnology, 38*(12), 234. https://doi.org/10.1007/s11274-022-03428-9

Zhao, P., Liu, C., Qiu, S., Chen, K., Wang, Y., Hou, C., et al. (2023). Flavor profile evaluation of soaked greengage wine with Different Base liquor treatments using principal component analysis and Heatmap analysis. *Foods, 12*(10), 2016. https://doi.org/10.3390/foods12102016

Zhou, J., Hou, D., Zou, W., Wang, J., Luo, R., Wang, M., et al. (2022). Comparison of widely targeted metabolomics and untargeted metabolomics of wild *Ophiocordyceps sinensis*. *Molecules, 27*(11), 3645. https://doi.org/10.3390/molecules27113645

Zhu, Q., Chen, L., Peng, Z., Zhang, Q., Huang, W., Yang, F., et al. (2022). Analysis of environmental driving factors on Core functional community during *Daqu* fermentation. *Food Research International, 157*, Article 111286. https://doi.org/10.1016/j.foodres.2022.111286

Zou, T., Mu, Y., Qi, Q., Ren, T., & Su, W. (2024). Analysis of microbial community succession and its metabolic characteristics in the first and second rounds of cave-brewed sauce-flavor baijiu. *Food Bioscience, 60*, Article 104485. https://doi.org/10.1016/j.fbio.2024.104485