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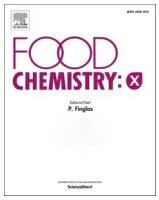

Microbial community succession and their relationship with the flavor formation during the natural fermentation of Mouding sufu

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ABSTRACT

Mouding sufu, a traditional fermented soybean product in China, has been recognized by the public in the southwestern regions of China. To reveal the microbial community succession and their relationship with the flavor formation during the natural fermentation of Mouding sufu, microbial community, non-volatile flavor compounds and volatile flavor compounds were analyzed by high-throughput sequencing, high-performance liquid chromatography, gas chromatography ion migration spectroscopy, respectively. The results showed that *Lactobacillus* and *Klebsiella* were the most abundant bacterial genus, whereas the main fungal genera were *unclassified-f-Dipodascaceae* and *Issatchenkia*. In addition, Glutamic acid, Aspartic acid, Alanine, Valine, Lysine, Histidine, lactic acid, succinic acid, and acetic acid were the main non-volatile flavor substances. Furthermore, the taste activity values of glutamic acid, aspartic acid and lactic acid reached 132, 68.9, 18.18 at H60, respectively, meaning that umami and sour were the key taste compounds. Simultaneously, ethyl 3-methylbutanoate-M, ethyl propanoate, methyl 2-methylbutanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate-D, ethyl isobutyrate, linalool-M, linalool-D, cis-4-heptenal, 2-methylpropanal were the characteristic volatile flavor of Mouding sufu. Finally, correlation analysis showed that *g_Erwinia* and *g_Acremonium* correlated with most of the key aroma compounds. 20 bacteria and 21 fungi were identified as core functional microbe for Mouding sufu production.

Introduction

Mouding sufu, a naturally fermented sufu, is produced in Mouding prefecture, Yunnan province (Wei, et al., 2018). It is considered a "natural" product with characteristic flavor and taste, which has been widely accepted and enjoyed by the local people. Since 2014, it has been China's National Geographic indication product. Its "natural" attributes mainly reflect in unique producing technology in the tofu-making process in which acidic whey replaced traditional calcium salt or acidulant coagulants, such as glucono-δ-lactone, as the coagulant (Henao Ossa, Wagner, & Palazolo, 2022; Wei, et al., 2018). Acidic whey is usually made from tofu whey naturally fermented, a liquid by-product of the tofu-making process, which has been used as a coagulant in Mouding sufu with a long history (Dai, et al., 2023).

It is generally recognized that the flavor formation of fermented products highly relies on the active microbes involved in the fermentation environment (Guan, et al., 2022; Yao, et al., 2021). For instance, K. Li, et al. (2022) revealed the dominant bacterial and fungal genera in Chaling red Sufu and discovered that *Debaryomyces*, *Leuconostoc*, *Lactobacillus*, *Pichia Weissella* and *Tausonia* were the main microorganisms responsible for the flavor formation of Sufu. Zhao, Hu, and Chen (2022) found that *Ralstonia*, *Staphylococcus* and *Cobetia* were the dominant bacterial genera for flavor formation in dry-cured fish. Huang, et al. (2018) explored the main microbiota for producing the volatile flavor of Wuyi Hong Qu glutinous rice wine, indicating that *Gluconacetobacter*, *Lactobacillus*, *Lactococcus*, *Pichia*, *Wickerhamomyces*, and *Saccharomyces* were the core functional microbiota to enhance the production of key volatile flavor. Thus, it is meaningful to explore the succession of the microbes responsible for the formation of characteristic flavor compounds.

Food flavor is considered an essential attribute that largely determines its acceptance by consumers (Xie, Zeng, Wang, Xu, & Qin,

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2018). Volatile flavor compounds of sufu have been investigated in previous studies. Huang, Yu, Han, and Chen (2018) employed headspace solid-phase microextraction and gas chromatography and mass spectrometry (HS-SPME-GC–MS) to analyze volatile compounds in sufu. 72 volatile compounds were identified, including 15 alcohols, 7 phenols, 6 acids, 17 esters, 17 aldo-ketones, and 10 heterocyclic compounds. Wang, et al. (2019) reported that 106 volatile compounds were identified in Wangzhihe red sufu samples using gas chromatography-mass spectrometry (GC–MS) analysis. Several previous research on the flavor of sufu mainly focuses on GC–MS techniques. However, it requires tedious pre-processing and a long detection time, which limits the efficacy of GC–MS. Gas chromatography ion migration spectroscopy (GC-IMS) is a novel nondestructive gas separation and detection technology. Gas chromatography is used to separate complex mixtures in a short time, making the mixture into a single component. Then characterizes the compounds based on the mobility of gas phase ions (Zhu, et al., 2022). Because its samples do not need to be pretreated such as enrichment and concentration, they can be directly injected into headspace, thereby maintaining the stability of the flavor substance (Li, et al., 2023). However, the application of GC-IMS in flavor components of sufu was rarely reported.

For Mouding sufu, current researches mainly focus on the effect of reducing salt on the microflora, enzymatic activity, textural and sensory properties, characterizing aroma profiles of sufu with aging solutions during fermentation (Wei, et al., 2018; Wei, Yang, Regenstein, Liu, & Liu, 2020). However, the composition of the microbial community in Mouding sufu, the correlation between microbiota community succession and flavor compounds during the natural fermentation of Mouding sufu are poorly understood. Therefore, the aim of the work was to study the dynamic changes in microbial communities of Mouding sufu using MiSeq sequencing and analyze flavor compounds, such as organic acids, amino acids, volatile flavor compounds using amino acid analyzer, high performance liquid chromatography, GC-IMS during sufu fermentation. In addition, a two-way orthogonal partial least squares method (O2PLS) was used to analyze the correlation between the microbiota community and flavor compounds. These results will provide useful information for an in-depth understanding of the volatile compound formation mechanism and provide references for the quality control of food fermentation.

Materials and methods

Preparation of sufu and sample collection

Sufu samples were provided by Yunnan Yangquan Biotechnology Co., Ltd, Mouding City, Yunnan Province, China, which holds the largest production scale in Yunnan Province. The production technology of Mouding sufu is shown in the flow chart (Fig. 1). Simply, selected soybeans went through a series of washing, soaking, boiling, filtering, coagulating, and squeezing to make tofu according to the local methods. During the coagulation period, 1/45–1/50 (v:v) already fermented acidic whey was added into fresh soybean milk, similar to the making-tofu using calcium chloride as the coagulant. Subsequently, the tofu pieces were put on a grill at intervals and sent to an incubation room with controlled temperature (16–23 °C) and relative humidity (60–75%) for 3 days in natural fermentation. After the tofu surface was covered with mycelium, the pehtzes were added 15% − 18% salt remaining for 13–15 h so that the dense mycelium was erased. Subsequently, the salt-pehtze was transferred outdoors to be dried. After these procedures, the salt-pehtze was washed with sterile water and put into a glass bottle containing a dressing mixture lasting 60 days. The dressing mixture contained chili 3%-6%, flour 5%-8%, pepper 2%-5%, alcohol 5%-9%, ginger 4%-6%, sugar 4%-8% and some spices. Sufu samples were randomly collected at the fermentation of days 0 (DF), 1 (F1), 3 (F3), 5 (LS), 30 (H30), 60 (H60). DF was the tofu samples. F1, F3 were sampled on day 1 and day 3 of fermentation, respectively. LS was the samples after air drying. H30, H60 samples were collected at the fermentation of days 30 and 60, respectively. Three parallel samples from different locations or bottles were analyzed for each stage. 18 samples were transported into the lab on ice and stored at −80 °C until further use.

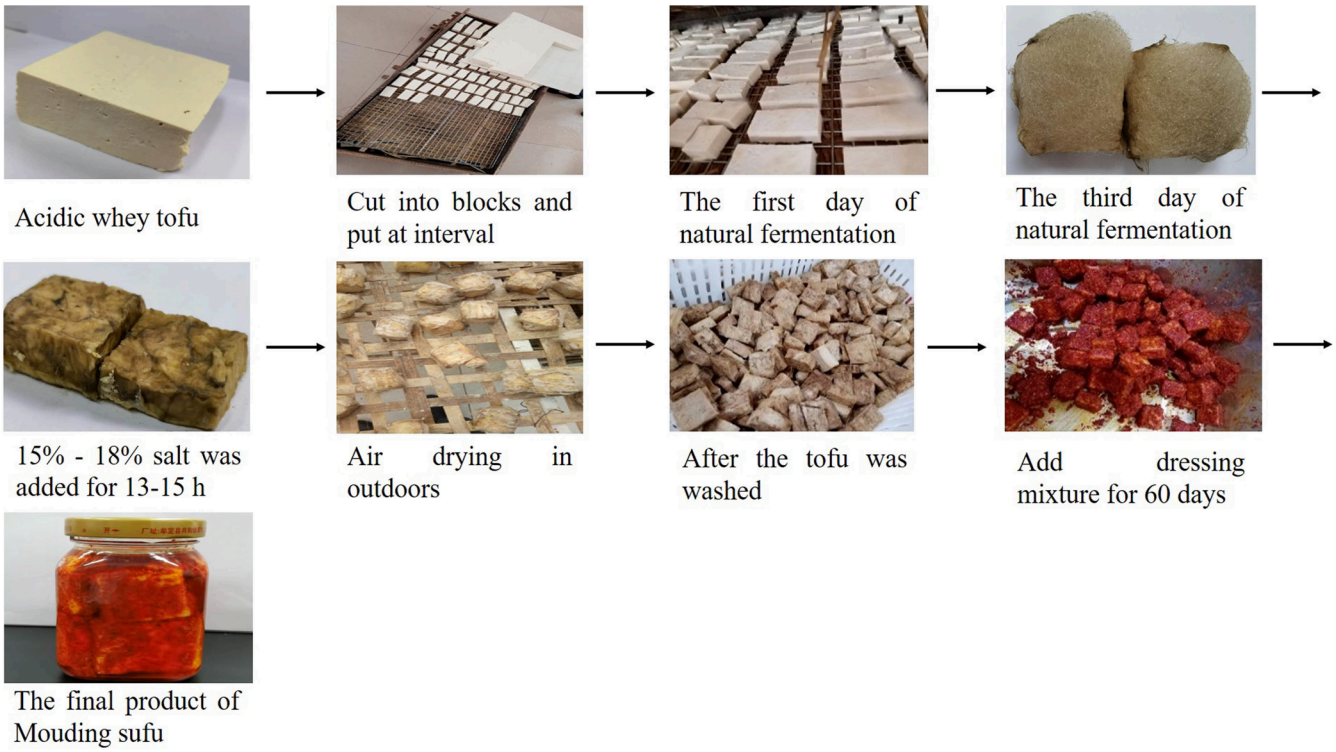

Fig. 1. The production diagram of Mouding sufu.

DNA extraction and Illumina Miseq sequencing

The total genomic DNA was extracted from 2.0 g of each sufu sample using the E.Z.N.A Soil DNA kit (Omega Bio-tek, Norcross, GA, USA), according to the method described in our previous reports (Chen et al., 2022b). The total DNA extracted was detected by agarose gel electrophoresis and polymerase chain reaction (PCR). Primer F341 5′-ACTCCTACGGGRSGCAGCAG -3′and R806 5′- GGACTACVVGGGTATCTAATC -3 were used to amplification for V3-V4 regions of 16S rRNA. Primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) were applied to amplification for fungal ITS region of 18S rDNA. PCR procedure was implemented as follows: predegeneration at 95 °C for 3 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and extension at 72 °C for 10 min. The gene amplicons were sequenced by Illumina Miseq PE300 (Majorbio Biopharm Technology Co., Ltd, Shanghai, China). After being merged by FLASH and quality filtered, the sequences were clustered into Operational Taxonomic Units (OTU) at 97% similarity threshold by UPARSE. The UCHIME was employed to identify chimaeras.

The determination of amino acids

Free amino acids (FAA) were detected according to the methods described in our previous reports (Chen, et al., 2022a). Ultra- HPLC-tandem MS method (UHPLC-MS/MS; model 1290/6460; Agilent Ltd.) was used to detect the concentrations of FAA. In brief, Minced sufu samples (2 g) were dissolved in 100 mL extraction solution (volume ratio of acetonitrile: methanol: water = 2:2:1). Then it was shaken well and conducted with ultrasound wave for 5 min. The prepared sample was centrifuged at 8000 g for 10 min for UHPLC-MS/MS analysis. An ACQUITY UPLC BEH HILIC (2.1 × 100 mm, 1.7 μm; Waters Corp.) liquid chromatographic column was used to separate FAA at a temperature of 35 °C with liquid chromatography phase A (1% formic acid aqueous solution) as well as phase B (1% formic acid acetonitrile). The injection volume was 1 μL.

The determination of organic acids

Organic acids were measured according to a previous report (Zhang, et al., 2020) with some modifications. LC-20 HPLC (Shimadzu, Japan) was equipped with a C_{18} column (4.6 mm × 150 mm, 5 μm; Agilent, Palo Alto, CA, USA) to detect organic acids. First of all, 2.5 g sufu samples were dissolved in ultra-pure water, bathing at 60 °C for 15 min. Subsequently, the mixture was diluted to 50 mL, and centrifuged at 6000 r /min for 20 min. Then, the supernatants were collected and filtered through 0.22 μm filter membrane. Lactic acid, citric acid, acetic acid, and succinic acid were considered external standards. 1% methanol and 0.06 mol/L KH_2PO_4 (pH 2.50) were applied to the mobile phases. 210 nm was selected for the detection absorbance. The injection volume and column temperatures were 15 μL and 28 °C, respectively.

Analysis of volatile flavor compounds by GC-IMS

The volatile fingerprints of Mouding sufu samples were analyzed on GC-IMS system (the G.A.S.Department of Shandong Hai Neng Science Instrument Co., Ltd., Shandong, China). 1.0 g sample was incubated in a 20 mL headspace vial at 60 °C for 20 min. The centrifuge speed was 500 r /min, and the temperature of injecting needle was 85 °C. Ultimately, 500 μL samples were injected. N_2 was used as carrier gas (purity ≥ 99.99 %), and the nitrogen flow rate was maintained as follows: 2 mL/min for 0–2 min, 10 mL/min for 2–10 min, and 100 mL/min for 10–20 min; after 20 min, the analysis was stopped. The temperature of the IMS was set at 45 °C and the analysis time was 30 min. Retention indexes (RI) of volatile compounds were calculated with *n*-alkanes (C4-C9) (chromatographic reagent) as external references for the identification of compounds. The RI and the reference of the NIST library and IMS database were used to determine the volatile compounds. The relative contents of the volatile compounds were expressed by the signal intensity.

The Relative odor activity value (ROAV) was used to evaluate the key volatile flavor compounds and weigh their contributions to the whole flavor. The calculation method is based on the following formula: $ROAV_i = 100\,(C_i/C_{max})(T_{max}/T_i)$, C_i and T_i are the relative concentration and threshold in the water of an arbitrary flavor component; Threshold value referenced to (Gernert, 2003). Tmax and Cmax are the thresholds and relative concentrations of the volatile component with the highest odor activity values (OAV), respectively (K. Li, et al., 2022).

Statistical analysis

SPSS 19 software (SPSS Inc., Chicago, IL, USA) and Origin 2021 pro software (OrginLab, Northampton, MA, USA) were used for statistical analysis and mapping, respectively. To elucidate the relationships between the microbial and flavor compounds, the O2PLS model was performed using SIMCA-14.1 software (Umetrics AB, Umea, Vasterbotten, Sweden). Cytoscape (v.3.7.1) was used to build the network figures between microbes and volatile compounds. Each experiment was implemented in triplicate.

Results and discussion

Changes of alpha diversity during Mouding sufu fermentation

The bacterial Sobs, Shannon, Simpson, Ace, Chao indexes are shown in Supplement Fig. 1. In general, the Sobs index represents the observed richness, while the Ace and Chao indexes are usually applied to evaluate the number of OTU in the samples (Wang, et al., 2022). The Shannon and Simpson indexes are used to characterize community diversity. The Shannon index is positively related to community diversity and the Simpson index is negatively associated with community diversity. As shown in Supplement Fig. 1a, 1d, 1e, the Sobs, Ace and Chao indexes showed a similar tendency, namely, first rising and then falling, then increasing during the fermentation of Mouding sufu. This result meant that the bacterial community richness in fresh tofu was lower, but as the fermentation proceeded, it showed a gradually increasing trend until the LS stage. However, bacterial community richness from the LS stage to H60 first decreased and then increased. This phenomenon illuminated that the exogenous bacteria's growth might be inhibited because of the addition of dressing solution. But after a period of time fermentation, the richness of the bacterial community recovered, which meant that the number of bacteria in the end products of Mouding sufu was the most abundant. The results of Shannon and Simpson indexes (Supplement Fig. 1b, 1c) revealed that bacterial community diversity of samples from tofu samples to the F3 sample remained at a stable level, while in the LS stage, bacterial community diversity took great changes. This result meant that the bacterial diversity was improved after the drying technology, which may relate to the open natural fermentation environment.

The alpha diversity of the fungal community in the production of Mouding sufu is shown in Supplement Fig. 1f, 1g, 1h, 1i, 1j. The results of the Sobs, Ace and Chao indexes showed a high level in tofu samples. After that, they kept at a low level and did not change significantly throughout the Mouding sufu production. This suggested that fungal richness in Mouding sufu was less rich than bacterium. The Shannon index value peaked at DF samples, indicating that the fungal community diversity decreased as time prolonged.

Microbial succession during Mouding sufu fermentation

The microbial composition at the phylum and genus levels in Mouding sufu at different fermentation stages is shown in Fig. 2a, 2b. Firmicutes and Proteobacteria were the main phyla. Especially for DF,

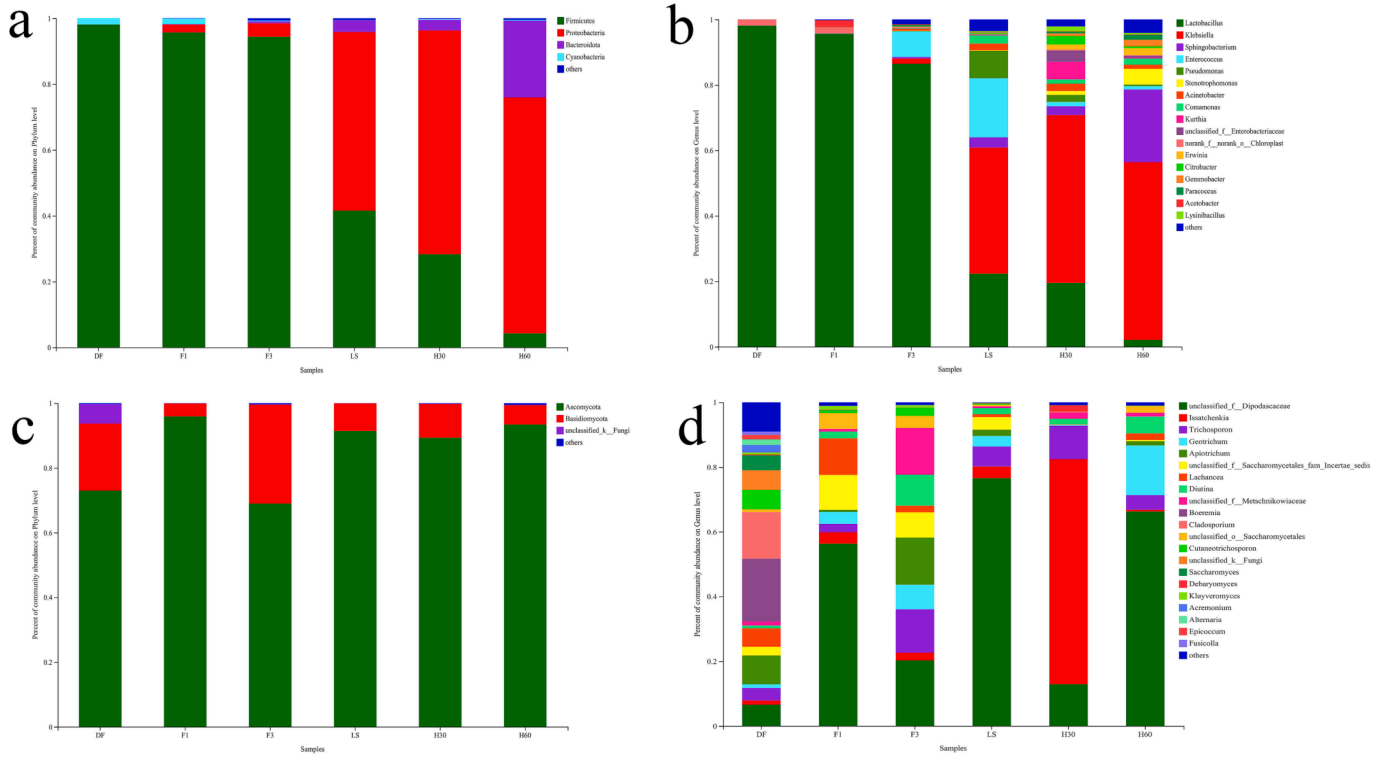

Fig. 2. Relative abundance of bacterial and fungal composition in sufu samples during the fermentation process. Bacterium: at phylum level (a) and genus level (b). Fungus: at phylum level (c) and genus level (d).

F1, F3 samples, Firmicutes were their predominant phyla, while Proteobacteria was the dominant phyla for samples of LS, H30, H60. Zhang, Wang, Xiang, Hou, and Guo (2021) reported that Proteobacteria, Firmicutes, and Bacteroides were the dominant bacterial phyla in Huase sufu, whose results were in agreement with ours. Xu, et al. (2020) reported that Proteobacteria, Firmicutes, and Bacteroidetes were the dominant phyla throughout the entire stages of red sufu fermentation. In contrast, Proteobacteria were the most abundant phylum at every stage in red sufu, which differed from our result. At the genus level, *Lactobacillus* was the most abundant classified genus from DF samples to F3 samples, which accounted for above 86%. From LS to H60, the relative abundance of *Lactobacillus* gradually declined to 2.10%. At the same time, the relative abundance of *Klebsiella* exhibited a dramatically increasing trend and became the principal genus, reaching a peak of 54.27% by the end. Notably, the relative abundance of *Enterococcus* from F3 to LS increased by 1.25 times. *Enterococcus* is pathogenic bacteria. However, their abundance increased as the fermentation process progressed, probably as the fermentation of Mouding sufu was under no-sterile environment and the bacteria composition of Mouding sufu was greatly affected by the environmental conditions. Previous studies reported that *Enterococcus* could secrete various proteases, playing a vital role in developing umami peptides (Yang, et al., 2022). Similarly, *Pseudomonas* appeared during the LS period and they have also been considered as the spoilage bacteria detected in many fermented foods, such as pickled chili pepper (Ye, et al., 2022), Chouguiyu (Yang, et al., 2022), chickpeas sufu (Yao, et al., 2021). Although *Enterococcus* and *Pseudomonas* are undesirable, the quality guarantee of the final products of Mouding sufu would not be an issue because they were found to be abundant only in the LS sample but declined sharply in the H30 sample and disappeared from the fermentation environment ultimately. Besides, *Sphingobacterium* increased by 6.33 times from LS to H60. It was found by (K. Li, et al., 2022) that *Sphingobacterium* exhibited an upward trend in Chaling red sufu. They discovered that several sweet amino acids, such as alanine, glycine, proline, and most free fatty acids positively correlated with *Sphingobacterium*.

As shown in Fig. 2c, *Ascomycota* and *Basidiomycota* were the predominant fungus phyla, especially *Ascomycota*. It occupied absolute predominance during the entire fermentation process. As shown in Fig. 2d, compared with bacteria, the overall changing trend of the fungus of Mouding sufu was more complex. In the DF samples, no species was particularly predominant, with the highest community diversity. But in the F1 sample, relative abundance of *unclassified-f-Dipodascaeae* increased considerably and became absolutely dominant at 56.35%, and the relative abundance of *Lachancea* was 11.33%. In contrast, earlier article reports about the function of *unclassified-f-Dipodascaeae* were limited. On the fermentation of day 3 (F3), the relative abundance of *Trichosporon*, *Geotrichum*, *Apiotrichum*, and *unclassified-f-fMetchnikowiaceae* increased. *Trichosporon* was the main genera in pre-fermentation in red sufu and various carbohydrates and carbon sources could be utilized by it to degrade urea (Xu, et al., 2020). *Geotrichum* was the predominated genus in Baixi sufu after the pehtze salting and correlated significantly and positively with amino nitrogen (Wan, et al., 2020). *Apiotrichum* was an important fungi genus in cabernet sauvignon (*Vitis vinifera* L.) wine (Wei, et al., 2022). Whereas *unclassified-f-Dipodascaeae* became the dominant fungi again in the LS samples, this indicated that drying might lead to those heat-labile microorganisms disappearing and the drying stage significantly influenced the change of microbial communities. In H30 samples, the relative abundance of *Issatchenkia* closed to 70%. Previous studies found that *Issatchenkia* was successfully detected in the traditional fermentation process of Pixian Doubanjiang, Wuyi hong qu glutinous rice wine, and paocai (Huang, et al., 2018; Xiao, et al., 2018; Yang, et al., 2021). Therefore, we could speculate that the emergence of *Issatchenkia* may be related to the fact that dressing solution containing chili paste and liquor was added to Mouding sufu during manufacturing. But at H60 samples, *unclassified-f-Dipodascaeae* and *Geotrichum* were the main fungi with the relative abundance of 66.28%, 15.35%, respectively, suggesting that at the end of the fermentation, several fungi from tofu, dressing solution, and tools can't fit the fermentation circumstances, thereby resulting to a certain fungus extinction. In contrast, the dominant fungi grew faster and gradually

tended to be stable in the airtight fermentation environment.

Comparison of microbial communities during Mouding sufu fermentation

The PCoA were used to evaluate the variation and similarity of microbial diversity among the samples (Fig. 3a). The PCoA score plot showed 77.37% and 10.60% variance by PC1 and PC2, respectively. The 18 samples of bacteria were clustered into three groups (Fig. 3a). DF, F1, F3 samples showed clear clustering trends despite some overlap in spatial arrangement, which were mainly distributed on the negative axis of the PCoA plots, indicating that sufu samples from different fermentation times had similar microbial diversity. The more similar microbial communities in samples are, the closer the distance reflected in the PCoA diagram is. Besides, the distance among F3 samples is farther, indicating a greater difference between sample groups. LS samples on the fourth quadrant showed that there were significant differences in the structure of bacterial communities contrasted with other samples, which may demonstrate that drying process alters the microbiota structure in sufu. H30, H60 samples clustered a group, indicating that although sampled time was at an interval of 30 d, the bacterial communities were similar during the post-fermentation. Besides, the two samples of H30, H60 were away from the other groups, which illustrated that bacterial communities of post-fermentation were different from DF to F3 stage and LS stage. Therefore, according to the succession of bacterial communities, the fermentation of Mouding sufu could divide into three stages: Tofu, F1, F3 (initial stage, group 1); LS (middle stage, group 2); H30, H60 (late stage, group 3). Cai, et al. (2021) used the PCoA to distinguish the similarities and differences of microbial compositions in sufu from different regions in China and they found that samples from eastern China could be generally clustered into one group, which indicated that communities of sufu samples could be influenced by the geographical factor.

The 3 distinct clusters were used for the discrepancy analysis using the *t*-test between groups. As shown in Fig. 3b, the Y-axis indicates the species name at a given taxonomic level, and the X-axis indicates the mean relative abundance in different groupings of species, with different colored bars indicating different groupings; the rightmost column shows P values, *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Because significant differences ($p < 0.05$) in fifteen indicators of the bacterial community were observed among the three fermentation stages of samples, it can be concluded that great differences existed in the relative abundance of the bacterial community. In the initial stage, the relative abundance of *Lactobacillus* was biologically more significant ($P < 0.01$); in the middle stage, the relative abundance of *Enterococcus* and *Pseudomonas* were biologically more significant ($P < 0.05$); in the late stage, the relative abundance of *Klebsiella* and *Sphingobacterium* were biologically more significant ($P < 0.01$). These results indicated that the differential microbes of three fermentation stages were revealed, which will help us to understand microbial succession.

LEfSe analysis was further used to evaluate species that differ

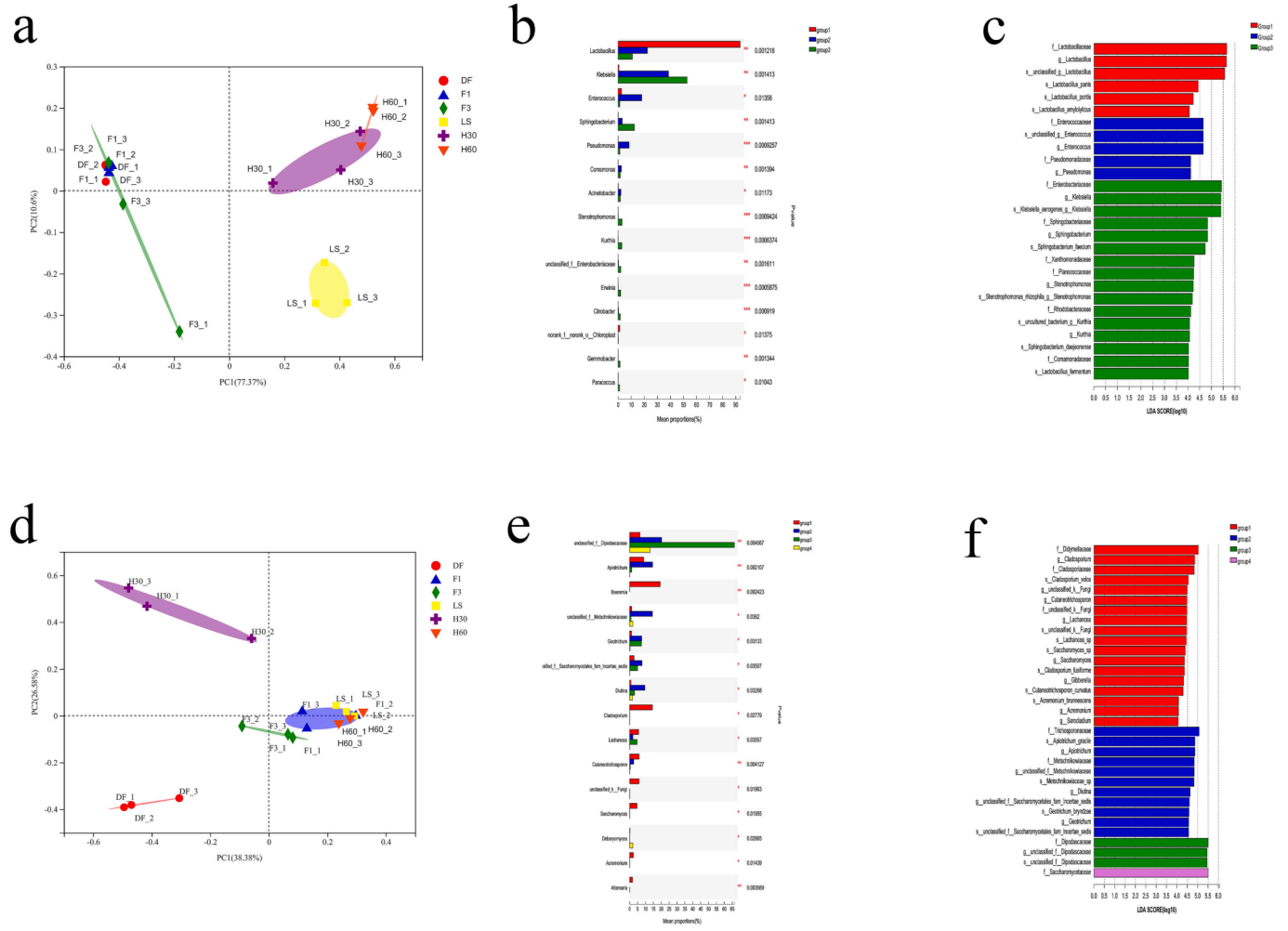

Fig. 3. PCoA score plot of all the samples of Mouding sufu during fermentation with unweighted unifrac distance, LEfSe and biomarker analysis. PCoA score plot of Mouding sufu (a: bacteria, d: fungi); LEfSe analysis of different fermentation stage samples in Mouding sufu (b: bacteria, e: fungi); Biomarker analysis (c: bacteria, f: fungi).

significantly in abundance among sample groups and the length of the bar graph represents the effect size of the significantly different species. From LEfSe analysis in Fig. 3c, at the genera level, *Lactobacillus* was significantly enriched (LDA 5.5), and it contributed greatly to the differences of species for the initial stage. This result confirmed that *Lactobacillus* played a dominant role in early stage of fermentation; *Enterococcus*, *Pseudomonas* were predominant and LDA score were higher than 4.5, which were identified as the biomarkers in the middle stage, indicating that there might be a small number of harmful microorganisms in sufu. Therefore, it is especially necessary to optimize the processing environment of sufu. Compared with other fermentation stages, the species diversity in group 3 was significantly higher; however, the main enriched species were from *Klebsiella*, *Sphingobacterium*, *Stenotrophomonas*, *Kurthia* and LDA score were higher than 4. The final quality of sufu might decide by these different species.

From PCoA analysis in Fig. 3d, we observed that the fungal community of all samples formed 4 clusters. DF clustered group 1; F3 sample clustered group 2; F1, LS, H60 clustered group 3; H30 clustered group 4. Each group had good repeatability within the group, and obvious differences among groups were obtained, indicating that these microflorae showed dynamic changes during fermentation. The cluster results could be interpreted in terms of the results of Fig. 2d. Owing to DF samples containing the kinds of fungi were abundant, without especially predominant microbes. Analogously, the dominant fungi flora of the F3 sample was not outstanding and community structure had discrepancies with DF. So, DF and F3 were grouped into one category, respectively. However, F1, LS, H60 samples were grouped based on containing higher proportion *unclassified_f_Dipodascaeae*. H30 sample characterized by *Issatchenkia* at a ratio of 69.54%. So, F1, LS, H60 samples and H30 samples were divided into two distinct clusters. The results of PCoA analysis showed fungal succession was more complex than that of bacteria. Days 3 and 30 of fermentation likely were the critical time points for fungal changes. In addition, drying process had little influence on the changes in the structure of the fungal community.

The 4 clusters were used for the discrepancy analysis using the *t*-test between groups. As shown in Fig. 3e, the relative abundance of *Boeremia*, *Cladosporium*, *Lachancea*, *Cutaneotrichosporon*, *unclassified_k_Fungi*, *Saccharomyces*, *Acremonium*, *Alternaria* in group 1 was biologically significant ($P < 0.05$). *Apiotrichum*, *Unclassified_f_Metschnikowiaceae*, *Geotrichum, Diutina* in group 2 were biologically significant ($P < 0.05$). But only *Unclassified_f_Dipodascaceae and Debaryomyces* in group 3 and 4 were biologically significant ($P < 0.05$), respectively. This result suggested that the with the fermentation processing, several fungal growths not gradually adapted to the post-fermentation surrounding, so the numbers of biologically significant species decreased. In group 4, namely after 30 d fermentation, the relative abundance of *Debaryomyces* was biologically significant, which had previously been detected in sourdoughs, vinegar, or soysauce and may contribute to the formation of the unique flavors of fermented foods.

As shown in Fig. 3f, *Sarocladium*, *Acremonium*, *Gibberella*, *Saccharomyces*, *Lachancea*, *Cutaneotrichosporon*, *Cladosporium* (LDA > 4) were significantly enriched in group 1 compared with other fermentation stages, with *Cladosporium* contributing greatly to the differences in the samples (LDA 5.3). *Geotrichum, Diutina, Unclassified_f_Metschnikowiaceae*, *Apiotrichum*, *Unclassified_f_Metschnikowiaceae* were significantly enriched in group 2 with LDA score were higher than 4.5. In group 3, fewer microbes were significantly enriched; enriched species were from *Unclassified_f_Dipodascaceae* (LDA > 5).

Dynamic changes of amino acids, organic acids during Mouding sufu fermentation

As shown in Fig. 4a, the amino acids content of DF samples was the lowest. But with the fermentation time prolonged, amino acids content peaked at 2.33 g/100 g in the LS stage. These phenomena may be related to the fact that via 4 days of growth, amount of microbe had adapted to the environment and had higher growth vitality, thereby secreting highly abundant protease, lipase and amylase, which can hydrolyze protein in soybeans into amino acids through a series of biochemical processes (Hu, et al., 2021). In addition, moisture content evaporated during the LS period, and the texture of tofu changed, which was

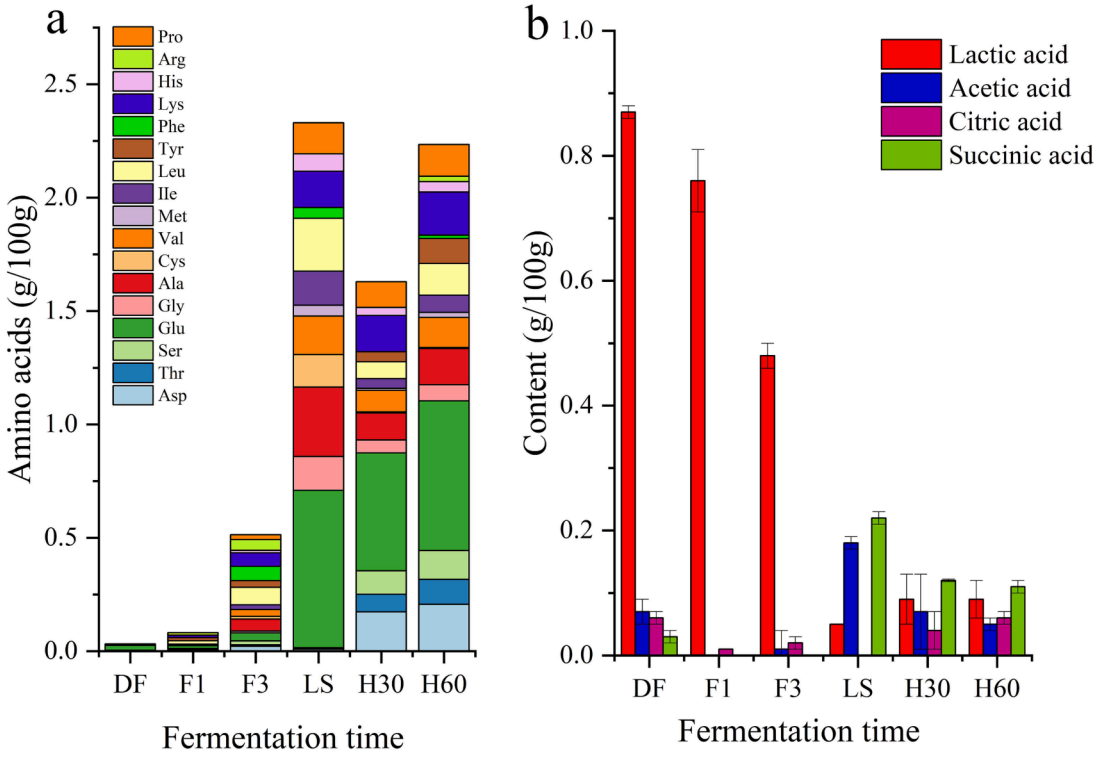

Fig. 4. The composition of organic acids, amino acids (a: organic acids, b: amino acids).

different from the initial status. It might be good for the release of amino acids. In contrast, it showed a significant decline in H30 samples, especially Valine (Val), Alanine (Ala), Glutamic acid (Glu). These might relate to the fact that free amino acids which served as flavor precursors were further metabolized to a variety of volatile compounds, such as acids, ketones, esters (Wang, et al., 2021). And then reached a comparatively high level of 2.23 g/100 g in H60 samples, close to the peak value. These results were consistent with the earlier report about low-salt oil sufu (Wei, et al., 2018). However, our results had a litter discrepancy with other results that significant accumulation of total amino acids occurred during the post-fermentation process (Yao, et al., 2021). At H60 samples, sufu fermentation gradually tended to mature. The microorganisms in the sufu were inhibited due to limited oxygen and osmotic pressure, and their number and vitality were gradually reduced, and the activity of enzymes were affected, making the growth rate of free amino acid significantly slower.

Taste activity values (TAV) were used to evaluate the contribution of each volatile compound to the overall odor. TAV for each odorant was calculated according to the method used in a previous study (He, Wan, Yi, Liu, & Chen, 2020). TAV is calculated as the ratio between the measured concentration of taste compounds and their predetermined taste threshold values. The higher the TAV is, the greater contribution of the volatile flavor substance to the overall flavor of sufu. As shown in Table 1, the TAV of Glu exceeded 1 during the entire fermentation period. At the fermentation of LS period, TAV of Aspartic acid (Asp), Glu, Glycine (Gly), Ala, Val, Methionine, Isoleucine, Leucine, Lysine (Lys), Histidine (His) were higher than 1, implying that taste compounds were the richest and most complicated after the dry technology. It was particularly necessary for the production of unique taste of Mouding sufu. In addition, TAV of most bitterness-related amino acids showed the highest TAV in the LS stage, whereas, at the H60 stage, they had lower TAV that ranged from 0.163 to 3.333, reflecting that they contributed a bitter taste that was difficult to notice. The high NaCl content and umami of the sufu could inhibit the bitter taste and provide a palatable taste.

However, during the post-fermentation (H30 and H60), only TAV of Asp, Glu, Ala, Val, Lys, His were greater than 1, demonstrating that the six amino acids were the characteristic tastes substances of Mouding sufu as the fermentation time longed. Furthermore, the TAV of Glu and Asp peaked at 132, 68.9 at H60, respectively. It is well known that Asp and Glu are responsible for producing umami. Therefore, it was assumed that umami was the key taste of Mouding sufu. Our results agreed with the previous report (Yao, et al., 2021).

As shown in Fig. 4b, the total content of organic acids decreased gradually from DF to F3. At the beginning stage, lactic acid content accounted for above 85% of all organic acids. This result might be

Table 1
The content and TAV of amino acids and organic acids.

Amino acids		threshold (g/100 g)	DF (g/100 g)	TAV	F1 (g/100 g)	TAV	F3 (g/100 g)	TAV	LS (g/100 g)	TAV	H30 (g/100 g)	TAV	H60 (g/100 g)	TAV
Asp	Umami	0.003	0.0014 ± 0.0006	0.467	0.0049 ± 0.0021	1.633	0.0217 ± 0.004	7.233	0.0085 ± 0.0004	2.833	0.1733 ± 0.015	57.767	0.2067 ± 0.015	68.9
Thr	Sweet	0.26	0.0007 ± 0.0001	0.003	0.0043 ± 0.002	0.017	0.0057 ± 0.001	0.022	0.0049 ± 0.001	0.019	0.0777 ± 0.002	0.299	0.11 ± 0.01	0.423
Ser	Sweet	0.15	0.0018 ± 0.0003	0.012	0.0027 ± 0.0003	0.018	0.018 ± 0.003	0.12	0.0018 ± 0.0007	0.012	0.1033 ± 0.025	0.689	0.1267 ± 0.025	0.845
Glu	Umami	0.005	0.0213 ± 0.004	4.26	0.0123 ± 0.003	2.46	0.0353 ± 0.004	7.06	0.6933 ± 0.025	138.66	0.52 ± 0.021	104	0.66 ± 0.06	132
Gly	Sweet	0.13	0.0008 ± 0.0021	0.006	0.0006 ± 0.0002	0.005	0.008 ± 0.002	0.062	0.150 ± 0.001	1.154	0.0563 ± 0.001	0.433	0.072 ± 0.01	0.554
Ala	Sweet	0.06	-	-	-	-	0.0527 ± 0.003	0.878	0.3067 ± 0.025	5.112	0.12 ± 0.02	2	0.16 ± 0.01	2.667
Cys	-	-	-	-	0.0009 ± 0.0004	-	0.012 ± 0.002	-	0.1433 ± 0.015	-	0.0036 ± 0.0005	-	0.0035 ± 0.0002	-
Val	Bitter	0.04	0.0006 ± 0.0007	0.015	0.0039 ± 0.0005	0.098	0.03 ± 0.004	0.75	0.17 ± 0.01	4.25	0.0967 ± 0.003	2.418	0.1333 ± 0.015	3.333
Met	Bitter	0.03	-	-	-	-	-	-	0.0473 ± 0.002	1.577	0.0083 ± 0.0005	0.277	0.022 ± 0.002	0.733
Ile	Bitter	0.09	-	-	0.0016 ± 0.0002	0.018	0.0203 ± 0.003	0.226	0.15 ± 0.017	1.667	0.0433 ± 0.002	0.481	0.0757 ± 0.003	0.841
Leu	Bitter	0.19	-	-	0.0147 ± 0.003	0.077	0.0777 ± 0.004	0.409	0.2333 ± 0.006	1.228	0.0737 ± 0.002	0.388	0.14 ± 0.01	0.737
Tyr	-	-	-	-	0.0133 ± 0.003	-	0.0293 ± 0.003	-	-	-	0.0443 ± 0.002	-	0.11 ± 0.01	-
Phe	Bitter	0.09	-	-	-	-	0.0637 ± 0.007	0.708	0.0477 ± 0.004	0.53	-	-	0.0147 ± 0.002	0.163
Lys	-	0.05	0.0023 ± 0.0009	0.046	0.0113 ± 0.003	0.226	0.0587 ± 0.003	1.174	0.16 ± 0.02	3.2	0.16 ± 0.01	3.2	0.19 ± 0.02	3.8
His	Bitter	0.02	0.0024 ± 0.001	0.12	-	-	0.011 ± 0.003	0.55	0.0767 ± 0.005	3.835	0.0357 ± 0.001	1.785	0.046 ± 0.004	2.3
Arg	Bitter	0.05	-	-	0.011 ± 0.003	0.22	0.048 ± 0.005	0.96	-	-	-	-	0.0237 ± 0.003	0.474
Pro	-	0.3	-	-	-	-	0.0163 ± 0.009	0.181	0.1433 ± 0.11	0.478	0.14 ± 0.14	0.467	0.14 ± 0.01	0.467
lactic acid	Acid	0.005	0.87 ± 0.01	173.34	0.76 ± 0.05	151.07	0.48 ± 0.02	95.58	0.05 ± 0	9.66	0.09 ± 0.04	17.59	0.09 ± 0.03	18.18
acetic acid	Acid	0.035	0.07 ± 0.02	1.88	-	-	0.01 ± 0.03	0.31	0.18 ± 0.01	5.26	0.07 ± 0.06	1.87	0.05 ± 0.01	1.36
citric acid	Acid	0.07	0.06 ± 0.01	0.88	0.01 ± 0.0002	0.16	0.02 ± 0.01	0.25	-	-	0.04 ± 0.03	0.58	0.06 ± 0.02	0.79
Succinic acid	Acid	0.055	0.03 ± 0.01	0.52	-	-	-	-	0.22 ± 0.01	3.91	0.12 ± 0.002	2.19	0.11 ± 0.01	2.01

- not detected. Thresholds reference Gernert, L. J. V. (2003). Compilations of flavor threshold values in air, water and other media. 665 Netherlands, Oliemans Punter & Partners.

because *Lactobacillus* were significantly enriched for the initial stage. On the other hand, this phenomenon was likely related to the unique technology of the tofu-making in which acidic whey containing higher lactic acid was used as tofu coagulant (Dai, et al., 2023). At the LS stage, acetic acid and succinic acid accounted for a higher ratio. At the end of fermentation, succinic acid and lactic acid were the most abundant organic acids. Lactic acid is a good flavoring agent with a milder acidity, giving sufu a unique flavor and appetite enhancing effect. Succinic acid has a strong sour taste, it also has a slightly astringent taste. In contrast, lactic acid, acetic acid and citric acid have previously been reported as the predominant organic acids in red and white sufu (Tan, et al., 2020). The phenomenon may be attributed to the fact that the formation of organic acids in sufu was influenced by raw materials, environmental conditions, production technology, and microorganisms involved in

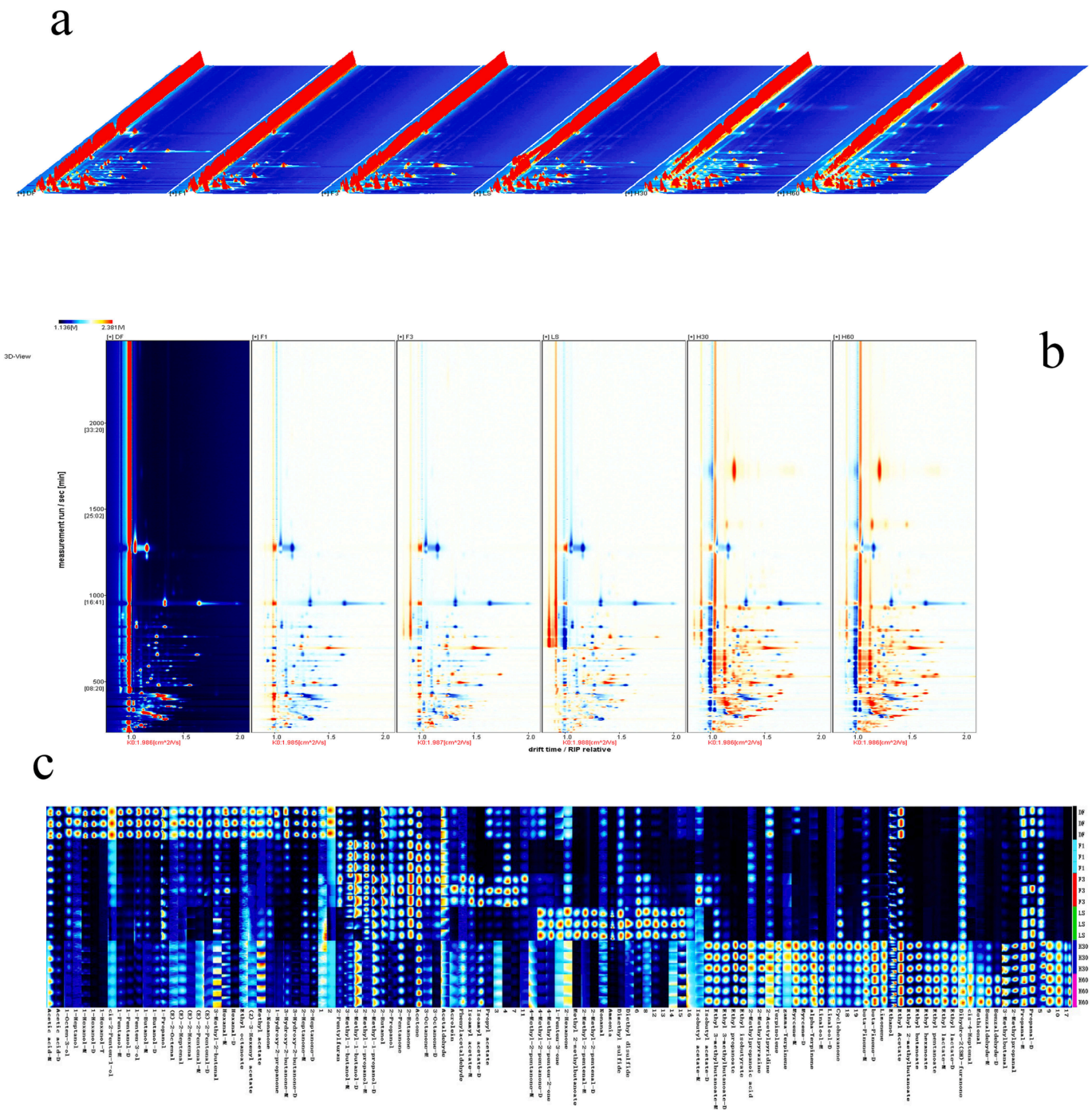

Fig. 5. (a) 3D-topographic imaging of volatile compounds were presented based on the data obtained by HS-GC-IMS in different fermentation stages of sufu samples. (b) Comparison of volatile compounds in different fermentation stages of sufu samples via GC-IMS (If the concentrations of volatile compounds were the same, the background after deduction was white, while red indicated that the concentration of the substance was higher than the reference, and blue indicated that the concentration of the substance was lower than that the reference). (c) Gallery plot of the selected signal peak areas obtained from sufu samples of different fermentation periods via GC-IMS. Each row represents the signal peaks selected in sufu sample, and each column represents the signal peaks of the same volatile compound in different sufu samples. Individual dot represents a volatile substance, and the color intensity represents the content levels of the volatile substance, as the brighter the color, the higher the content. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fermentation. As shown in Table 1, at the end of fermentation (H60), the TAV of lactic acid, succinic acid and acetic acid were 18.18, 2.01, 1.36, respectively, which indicated that the three organic acids were the key tastes substances of Mouding sufu.

Volatile flavor compound analysis of Mouding sufu by GC-IMS

GC-IMS was utilized to analyze the volatile flavor compounds. Fig. 5a shows a three-dimensional spectrum obtained by HS-GC-IMS. From Fig. 5a, we can conclude that the volatile flavor compounds in different sufu samples were similar during fermentation. Nevertheless, some differences were found between the red points and peak signal intensity. In order to have a convenient observation, the top view was taken for comparisons in detail (Fig. 5b). Comparing the DF sample with F3 and LS, it can be found that the red points in LS were richer than those of F3 and DF. Liang, et al. (2019) reported that when tofu was processed to pehtze, the number of ester compounds increased to 9 kinds. The reasons may be because at the beginning of fermentation, organic acids in tofu and acids secreted by several microorganisms have an activating effect on endogenous enzymes in sufu of the initial fermentation stage. Furthermore, endogenous enzymes with strong protein hydrolysis ability can degrade protein in sufu, leading to the liberation of free amino acids, which are considered important flavor-enhancing compounds in many fermented foods (Wei, Chitrakar, Regenstein, Sang, & Zhou, 2023). However, comparing the samples of H30 and H60 with other four fermentation stages, there were more red points in samples of H30 and H60. But there were small differences between them, which means that as the fermentation time prolonged, volatile flavor substances showed an increasing trend and the flavor tended to a stable level after the post-fermentation. An increasing trend of volatile flavor substances of H30 and H60 was likely related to a wide array of enzymes released by microbe in sufu, such as protease, glutaminase, peptidase, lipase, cellulase, hemicellulose, α-amylase, β-glucosidase, etc. These enzymes break down amino acids, proteins, lipids and carbohydrates in raw materials into short peptides, free amino acids, fatty acids and sugars to form the special flavor or flavor precursor compounds of Sufu. (Liu, et al., 2022).

Volatile compound fingerprints of sufu in different fermentation stages were revealed by the gallery plot (Fig. 5c). 98 volatile compounds were detected by GC-IMS, including 22 aldehydes, 21 alcohols, 20 esters, 18 ketones, 8 alkenes, 3 acids, 6 others. The main volatile compounds were aldehydes, alcohols and esters, which was in accordance with previous research (Chen et al., 2022b; He & Chung, 2020). As shown in Fig. 5c, different fermentation phases had various volatile substances. The volatile substances of the DF sample were mainly alcohols and aldehydes, F1 sample (alcohols and ketone), F3 sample (aldehydes and esters), LS sample (ketone and aldehydes), H30 and H60 sample (esters and aldehydes). Among them, the compounds of isoamyl acetate, isobutyl acetate, ethyl lactate, acetic acid, 1-hexanol, 1-pentanol, 3-methyl-1-butanol, 1-butanol, 2-methyl-1-propanol, linalool, (E)-2-pentenal, hexanal, propanal, 2-methyl-2-pentenal, 3-hydroxy-2-butanone, 2-heptanone, 4-methyl-2-pentanone, 3-octanone, beta-pinene, myrcene had the forms of monomers and dimers.

Aldehydes are produced by Strecker degradation of amino acids and peroxidation and degradation of esters (Yu, et al., 2022), because it had low odor thresholds and intense odor properties, giving the sufu sweet, fruity, nutty and caramel flavor. The aldehydes identified in Mouding sufu are mainly cis-4-Heptenal, Hexanal-M, Hexanal-D, Propanal-M, Propanal-D, 2-Methylpropanal, Methional, Phenylacetaldehyde, Nonanal. Methional has been described as a meaty smell in white tofu. It is derived from methionine and is produced through the Maillard reaction or enzymatic degradation (Chen & Chung, 2018). Phenylacetaldehyde and Nonanal were also the key odorants of Guilin Huaqiao white sufu. Phenylacetaldehyde can improve the umami aftertaste and the palatability of the umami solution. Nonanal originated from the autoxidation of ω-9 fatty acids such as oleate (He, Wan, Yi, Liu, & Chen, 2020).

The type and content of alcohol are usually related to the external addition. Ethanol is often added as a preservative during the processing of Mouding sufu. Due to the metabolism of fungi and bacteria, alcohols increased in DF and F1 stages. After fermentation, alcohol content began to decrease, because esterification or oxidation reaction occurred to produce esters substances, giving sufu with wine, rose and vanilla flavor.

Esters, produced by the esterification of organic acids and alcohols, are recognized as the most important flavor components in sufu (He, Wan, Yi, Liu, & Chen, 2020). The relative content of esters in tofu was low, and it increased in H30 stage. In the post-fermentation stage, the degradation of macromolecular substances such as proteins in the salt-pehtzes and the synergistic action between the generated products and the chemicals in the ingredients continuously enriched ester substances, mainly including (Z)-3-Hexenyl acetate, Ethyl 3-methylbutanoate-M, Ethyl propanoate, Methyl 2-methylbutanoate, Ethyl 2-methylbutanoate, Ethyl 3-methylbutanoate-D, Ethyl isobutyrate, which provide the sufu with fruity flavor and creamy flavor. Esterification and alcoholysis are the two main catalytic actions for the biosynthesis of ester flavors. Esterification is the formation of esters from alcohols and carboxylic acids. Simultaneously, alcoholysis is the formation of esters from alcohols and acylglycerols or from alcohols and fatty acids, amino acids, and lipidyl CoA produced by carbohydrate metabolism (Zang, et al., 2022).

ROAV analysis of Mouding sufu by GC-IMS

ROAV $>$ 1 of all volatile flavor substances detected by GC-IMS were shown in Table 2. From Table 2, we concluded that the total numbers of ROAV $>$ 1 flavor compounds of LS stage were the highest, up to 23 kinds which were the richest. Particularly, the ROAV of methyl 2-methylbutanoate increased by 99 times from the DF stage to LS stage. However, in H30 and H60 samples, the number of ROAV $>$ 1 flavor compounds reduced to 10 kinds. Ethyl 3-methylbutanoate-M, ethyl propanoate, methyl 2-methylbutanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate-D, ethyl isobutyrate, linalool-M, linalool-D, cis-4-heptenal, 2-methylpropanal were the characteristic volatile flavor of Mouding sufu analyzed by GC-IMS. Furthermore, ethyl isobutyrate, ethyl 2-methylbutanoate had the highest ROAV. Xie, Zeng, Wang, Xu, and Qin (2018) reported that ethyl isobutyrate and ethyl 2-methylbutanoate were the key characteristic flavor compound of sufu. Ethyl 3-methylbutanoate-M might bestow fresh fruity aromas (H. Zhao, et al., 2022). Ethyl propanoate is described as sweet, pineapple-like, floral, and green-leafy flavor (Wang, et al., 2022). Methyl 2-methylbutanoate imparts sweet, fruity, blueberry, floral, caramel and methyl 3-methylbutanoate may produce fruity, sweet, rancid-cheesy, pungent, sharp, blueberry (Forney, Qiu, Jordan, McCarthy, & Fillmore, 2022). 2-Methylpropanal can give malt, fresh, floral, pungent, green (Sharan, et al., 2022). Linalool produces hot pepper, pure, sweet aromas (Lee, et al., 2020). These flavor compounds jointly contribute sweet, floral, fruity, pungent, caramel and malt to the unique flavor profiles of Mouding sufu. For the fermentation of LS, free amino acids and ROAV $>$ 1 flavor compound measured by GC-IMS were remarkably rich, which meant the drying stage was an important process for the release of flavors. S. Zhao et al. (2022) indicated that the drying stage might be involved in the synthesis of esters during fermentation, or stimulate the metabolic activity of microorganisms, or act as an external catalyst to accelerate enzymatic reactions. This matter needs further research in the future.

Correlation between microorganisms and flavor components

O2PLS model was constructed to illustrate the correlation between bacterial community and volatile flavor compounds during the production of sufu. For bacteria, the R^2 and Q^2 values of the model were 0.735 and 0.567, respectively, indicating that the O2PLS model was a good fit for analysis and prediction. The variable importance for predictive components (VIP) of the top 50 genera varied in the range of 0.61–1.17, among which 29 genera had a VIP $>$ 1 (Fig. 6a). *g_Erwinia,*

Table 2
The relative content and ROAV of sufu detected by GC-IMS.

Flavor compound	CAS	threshold (mg/Kg)	DF (%)	ROAV	F1 (%)	ROAV	F3 (%)	ROAV	LS (%)	ROAV	H30 (%)	ROAV	H60 (%)	ROAV
Ester (7)														
(Z)-3-Hexenyl acetate	3681-71-8	0.0121	0.15 ±0.006	0.76	0.05 ±0.013	0.39	0.06 ±0.021	0.44	0.08 ±0.014	2.11	0.04 ±0.003	0.03	0.04 ±0.002	0.04
Ethyl 3-methylbutanoate-M	108-64-5	0.0002	0.03 ±0.003	8.29	0.09 ±0.004	44.36	0.22 ±0.054	98.04	0.33 ±0.011	536.77	0.48 ±0.037	21.66	0.43 ±0.006	21.74
Ethyl propanoate	105-37-3	0.004	0.05 ±0.002	0.81	0.03 ±0.012	0.82	0.06 ±0.035	1.29	0.08 ±0.022	6.92	2.14 ±0.022	4.83	1.55 ±0.026	3.88
Methyl 2-methylbutanoate	868-57-5	0.0002	0.03 ±0.002	9.11	0.02 ±0.003	7.82	0.03±0	12.69	0.55 ±0.14	911.56	0.03 ±0.001	1.41	0.03 ±0.003	1.68
Ethyl 2-methylbutanoate	7452-79-1	0.00015	0.03 ±0.004	13.95	0.03 ±0.009	22.79	0.09 ±0.02	51.05	0.14 ±0.01	301.11	1.34 ±0.023	80.77	1.31 ±0.024	87.04
Ethyl 3-methylbutanoate-D	108-64-5	0.0002	0.01 ±0.001	2.52	0.01 ±0.004	5.96	0.02 ±0.003	8.15	0.05 ±0.01	82	0.27 ±0.01	12.21	0.2 ±0.003	9.78
Ethyl isobutyrate	97-62-1	0.0001	0.03 ±0.003	18.12	0.03 ±0.006	30.88	0.02 ±0.004	22.03	0.03 ±0.003	96.64	1.11 ±0.008	99.81	0.94 ±0.025	93.97
Alcohol(4)														
3-Methyl-1-butanol-M	123-51-3	0.3	0.59 ±0.005	0.12	3.15 ±0.102	1.05	1.92 ±0.116	0.58	0.97 ±0.231	1.06	0.64 ±0.019	0.02	0.62 ±0.005	0.02
3-Methyl-1-butanol-D	123-51-3	0.3	0.17 ±0.003	0.03	5.22 ±0.145	1.74	5.37 ±0.299	1.61	1.86 ±1.87	2.05	3.21 ±0.254	0.1	3.59 ±0.053	0.12
Linalool-M	78-70-6	0.0038	0.23 ±0.046	3.62	0.31 ±0.133	8.29	0.31 ±0.071	7.31	0.22 ±0.029	19.51	7.98 ±0.205	18.97	7.58 ±0.042	19.96
Linalool-D	78-70-6	0.0038	0.75 ±0.008	11.84	1.26 ±0.263	33.21	1.12 ±0.118	26.47	0.89 ±0.178	76.93	1.85 ±0.076	4.4	1.63 ±0.026	4.28
Aldehyde(9)														
cis-4-Heptenal	6728-31-0	0.00006	0.1 ±0.009	102.62	0.06 ±0.005	99.46	0.05 ±0.011	81.04	0.02 ±0.006	129.65	0.25 ±0.058	37.37	0.38 ±0.024	63.85
Hexanal-M	66-25-1	0.02	1.28 ±0.027	3.85	0.3 ±0.070	1.48	0.63 ±0.313	2.82	0.28 ±0.036	4.67	0.54 ±0.004	0.24	0.55 ±0.008	0.28
Hexanal-D	66-25-1	0.02	1.33 ±0.189	3.99	0.03 ±0.014	0.16	0.14 ±0.124	0.64	0.04 ±0.009	0.62	0.19 ±0.019	0.08	0.2 ±0.0110	0.1
Propanal-M	123-38-6	0.06	0.83 ±0.009	0.83	0.62 ±0.076	1.03	0.82 ±0.232	1.24	0.84 ±0.030	4.64	0.68 ±0.046	0.1	0.67 ±0.010	0.11
Propanal-D	123-38-6	0.06	1.15 ±0.066	1.14	0.2 ±0.041	0.34	0.89 ±0.029	1.34	1.42 ±0.128	7.81	1.13 ±0.080	0.17	1.25 ±0.044	0.21
2-Methylpropanal	78-84-2	0.001	0.05 ±0.002	3.21	0.04 ±0.007	3.75	0.03 ±0.007	2.85	0.02 ±0.001	7.23	0.31 ±0.008	2.81	0.31 ±0.008	3.09
Methional	3268-49-3	0.016	0.06 ±0.004	0.21	0.08 ±0.009	0.5	0.08 ±0.011	0.42	0.06 ±0.005	1.25	0.11 ±0.035	0.06	0.26 ±0.059	0.16
Phenylacetaldehyde	122-78-1	0.009	0.24 ±0.038	1.58	0.43 ±0.107	4.79	0.61 ±0.14	6.11	0.24 ±0.019	8.82	0.34 ±0.03	0.34	0.34 ±0.045	0.38
Nonanal	124-19-6	0.04	0.1 ±0.009	0.15	0.11 ±0.035	0.27	0.1 ±0.015	0.23	0.18 ±0.014	1.47	0.11 ±0.015	0.03	0.11 ±0.002	0.03
Ketone(2)														
2-Pentanone	107-87-9	0.3	1.84 ±0.047	0.37	2.6 ±0.166	0.87	3.12 ±0.753	0.94	2.37 ±0.091	2.61	1.56 ±0.007	0.05	1.34 ±0.029	0.04
1-Penten-3-one	1629-58-9	0.0012	0.04 ±0.004	2.18	0.01 ±0.002	0.92	0.02 ±0.004	1.33	0.09 ±0.022	25.23	0.01 ±0.001	0.09	0.02 ±0.001	0.19
Other														
2-Pentylfuran	3777-69-3	0.0048	0.79 ±0.077	9.93	1.43 ±0.05	29.89	1.6 ±0.461	29.84	0.13 ±0.035	9.02	0.3 ±0.056	0.56	0.3 ±0.008	0.63
Dimethyl sulfide	75-18-3	0.005	0.52 ±0.035	6.28	0.81 ±0.036	16.09	0.61 ±0.139	11.04	0.88 ±0.06	58.37	0.13 ±0.006	0.23	0.11 ±0.003	0.22

Thresholds reference Gernert, L. J. V. (2003). Compilations of flavor threshold values in air, water and other media. 665 Netherlands, Oliemans Punter & Partners.

g_Klebsiella, g_Empedobacter, g_Lactobacillus were correlated with 21, 17, 14, 13 flavor components, respectively (Fig. 6c), suggesting that they were the important contributors to the production of flavors during sufu fermentation. *g_Erwinia* was positively linked to 4 aldehydes (benzaldehyde-M, cis-4-heptenal, 3-methylbutanal, 2-methylpropanal), 7 esters (ethyl 2-methylbutanoate, ethyl isobutyrate, ethyl butanoate, ethyl hexanoate, ethyl lactate-M, ethyl lactate-D, ethyl pentanoate), 4 alkenes (beta-pinene-D, myrcene-D, limonene, alpha-terpinene), 4 amino acids (Asp, Threonine, Serine), 1 organic acid. *g_Erwinia* was detected from dajiang and vacuum-packaged peeled potatoes (Z. Li, et al., 2022; Liu, et al., 2022) and it was positively correlated with ethanol, p-mentha-1,8-dien-7-ol, (E)-2-octen-1-ol, (E)-2-hexenal, heptanal and (E)-2-heptenal. 4-Methylpentyl-2-methylbutanoate was positively correlated with *g_Erwinia* (Xu, et al., 2021). *g_Lactobacillus* was positively correlated with (E)-2-octenal, cis-2-penten-1-ol, acetaldehyde, lactic acid, while negatively related to beta-pinene-M, ethyl 3-methylbutanoate-M, cyclohexanone, Glu, Val, Lys, His, Proline, succinic acid. *Lactobacillus* has been reported to have positive correlation with the production of volatile flavor compounds and organic acids in many fermented foods. For instance, (Zang, et al., 2020) reported that *Lactobacillus* showed the largest number of positive correlations with flavors and contributed to the production of ethyl lactate, ethyl octanoate. (Jia, et al., 2021) reported that *Lactobacillus* was indispensable for producing amino acid nitrogen, titratable acid and amino acids in broad bean paste fermentation.

To identify the core functional bacteria in the Mouding sufu, the key

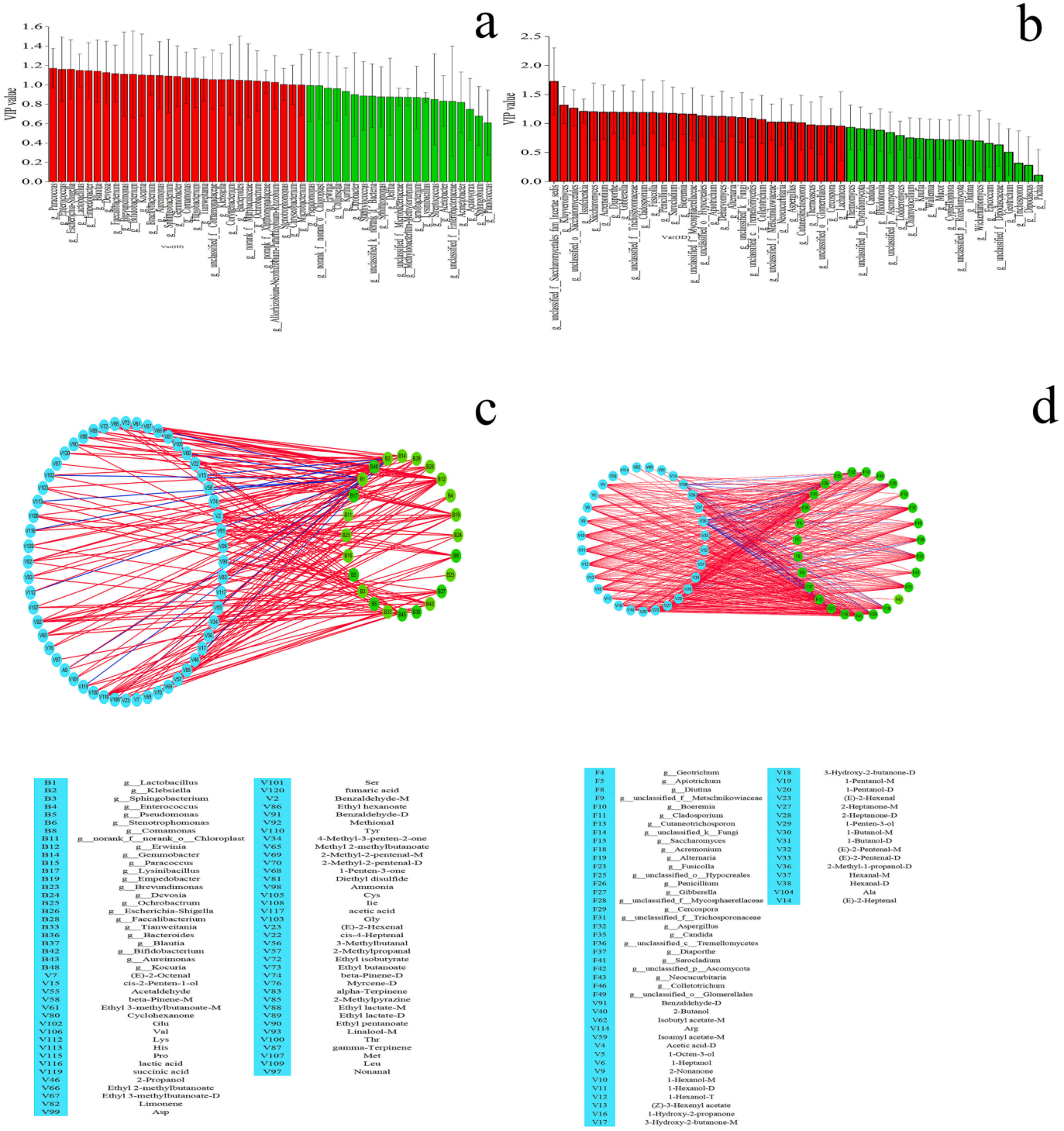

Fig. 6. Correlation analysis between microbial communities and flavor compounds. (a) A VIP (variable importance for predictive components) plot of bacterial genera. (b) A VIP plot of fungal genera. (c) The correlating network between the bacteria and flavor compounds at the genus level and the left-hand circles represent flavor compounds (blue), while the right-hand circles (green) represent the bacterial genera ($|\rho| > 0.8$). (d)The correlating network between the fungus and flavor compounds at the genus level and the left-hand circles represent flavor compounds the (blue), while the right-hand circles (green) represent fungal genera ($|\rho| > 0.8$). Positive correlation represented by the red solid line; negative correlation represented by blue soild line. A high-resolution version of the image is available as eSlide: VM06864. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

functional strains must meet several conditions, such as VIP value ≥ 1, correlation coefficient $|r| \geq 0.8$, and the number of microbes highly correlated ($|r| \geq 0.8$) with chemical compounds ≥ 1. Based on these criteria, 20 genera, including *g_Lactobacillus, g_Klebsiella, g_Sphingobacterium, g_Enterococcus, g_Stenotrophomonas, g_Comamonas, g_Gemmobacter, g_Paracoccus, g_Empedobacter, g_Brevundimonas, g_Devosia, g_Ochrobactrum, g_Escherichia-Shigella, g_Faecalibacterium, g_Tianweitania, g_Bacteroides, g_Blautia, g_Bifidobacterium, g_Aureimonas, g_Kocuriawere,* identified as core functional bacteria for Mouding sufu production. *g_Lactobacillus, g_Sphingobacterium, g_Stenotrophomonas* were also identified as core functional microbiotas with significant impact on the production of flavor compounds in naturally fermented

plain sufu (He & Chung, 2020). Characteristic volatile flavor compounds ethyl isobutyrate was positively correlated with *g_Kocuria* of core functional bacteria. Characteristic volatile flavor compound ethyl 3-methylbutanoate-M was negatively correlated with *g_Lactobacillus* of core functional bacteria, but positively correlated with *g_Klebsiella*. Characteristic volatile flavor compounds methyl 2-methylbutanoate positively correlated with core functional bacteria, including *g_Enterococcus*, *g_Faecalibacterium*, *g_Bacteroides*, *g_Blautia*. In our study, there was strong agreement between the results of ROAV analysis and the correlation analysis of bacteria.

For fungus, based on the O2PLS model, the R^2 and Q^2 value was 0.825 and 0.467, indicating that the O2PLS model was eligible for analysis and prediction. The VIP of top 50 genera varied in the range of 0.11–1.72, among which 26 genera had a VIP > 1 (Fig. 6b). *g_Boeremia*, *g_Saccharomyces*, *g_Acremonium*, *g_Fusicolla*, *g_Penicillium* were correlated with 25, 25, 26, 25 flavor components (Fig. 6d), respectively, suggesting that they were the important contributors to the production of flavors during sufu fermentation.

For fungus, based on the above criteria for screening core functional fungus, 21 genera, including *g_Apiotrichum, g_unclassified_f_Metschnikowiaceae, g_Boeremia, g_Cladosporium, g_Cutaneotrichosporon, g_unclassified_k_Fungi, g_Saccharomyces, g_Acremonium, g_Alternaria, g_Fusicolla, g_unclassified_o_Hypocreales, g_Penicillium, g_Gibberella, g_unclassified_f_Mycosphaerellaceae, g_unclassified_f_Trichosporonaceae, g_Aspergillus, g_unclassified_c_Tremellomycetes, g_Diaporthe, g_Saroccladium, g_Neocucurbitaria, g_Colletotrichum* were identified as core functional fungus for Mouding sufu production. *g_Alternaria* was also identified as core functional microbiotas in naturally fermented plain sufu (He & Chung, 2020). Notably, although *unclassified_f_Dipodascaceae, Issatchenkia* and *Trichosporon* were the top three dominant fungus genera, they didn't show correlation with any flavor compounds, while *g_Boeremia* and *g_Cladosporium* were positive with 11 alcohols (1-Octen-3-ol, 1-Heptanol, 1-Hexanol-M, 1-Hexanol-D, 1-Hexanol-T, 1-Pentanol-M, 1-Pentanol-D, 1-Penten-3-ol, 1-Butanol-M, 1-Butanol-D, 2-Methyl-1-propanol-D), 6 ketones (2-Nonanone, 1-Hydroxy-2-propanone, 3-Hydroxy-2-butanone-M, 3-Hydroxy-2-butanone-D, 2-Heptanone-M, 2-Heptanone-D). 1-Octen-3-ol and 1-Hexanol also were identified as the aroma-active compounds in Guilin Huaqiao white sufu and 1-Hexanol is derived from the autoxidation of ω-6 fatty acids, such as linoleic acid and arachidonic acid (He, Wan, Yi, Liu, & Chen, 2020). *Boeremia* and *Cladosporium* were marker fungi contributing to the fermentation of paocai (Xiao, et al., 2018). Although core microbiota in fungus correlated with several flavors, it did not correlate with characteristic flavor compounds, implying fungus had less impact on the overall flavors.

Conclusions

In this work, the microbial diversity, amino acids, organic acids and volatile flavor compounds were investigated during the natural fermentation of Mouding sufu and the correlations between microbial community and flavor compounds were further explored. *Lactobacillus* and *Klebsiella* were the most abundant bacterial genus, while for the fungal genus, the *unclassified-f-Dipodascaeae* and *Issatchenkia* were the most dominant. A total of 19 compounds, including 6 amino acids, 3 organic acids and 10 volatile compounds were identified as the characteristic flavor compounds of Mouding sufu. In addition, the drying stage was an important process for the release of flavors. The analysis of the correlation between odorants and microorganisms demonstrated that *g_Erwinia* and *g_Klebsiella* were correlated with 21, 17 flavor components, respectively, while *g_Boeremia*, *g_Saccharomyces*, *g_Acremonium* were correlated with 25, 25, 26 flavor components, suggesting that they were the important contributors to the production of flavors during sufu fermentation. These findings are expected to enhance our understanding of the flavor formation mechanism in fermented soybean foods.

CRediT authorship contribution statement

Zhongai Chen: Writing – original draft, Software. **Lijing Liu:** Methodology, Visualization, Investigation. **Huan Du:** Investigation, Data curation. **Kaixiang Lu:** Data curation, Formal analysis. **Cong Chen:** Software, Formal analysis, Conceptualization, Data curation. **Qiaoli Xue:** Supervision, Writing – review & editing. **Yongjin Hu:** Resources, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100686.

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