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Deciphering the role of traditional flipping crafts in medium-temperature Daqu fermentation: Microbial succession and metabolic phenotypes

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ABSTRACT

Medium-temperature Daqu (MTD) serves as the saccharification and fermentation starter for Nongxiangxing Baijiu. Flipping Daqu (FD) during fermentation is a key craft in traditional MTD preparation. However, the mechanism underlying this flipping craft remains unclear. To address this, we systematically compared FD with non-flipping Daqu (NFD) to elucidate microbial succession dynamics, metabolic phenotypes, and environmental drivers. Our results demonstrated divergent microbial community succession patterns between FD and NFD during the stable fermentation phase (days 9–25). FD exhibited significantly higher enzyme activities and volatile ketone content, along with lower core temperatures compared to NFD. Metabolite production in FD was influenced by both bacteria and fungi, whereas fungi predominantly controlled metabolite production in NFD. Co-occurrence network analysis revealed that the microbial community in FD was simpler yet more stable compared to that in NFD. Microbial succession in MTD was primarily driven by interspecies interactions and environmental factors. Furthermore, deterministic processes and stochastic processes jointly governed microbial assembly both FD and NFD, with temperature, moisture, and acidity as the key driving factors. These findings highlight the pivotal role of the flipping crafts in enhancing microbial functionality and metabolic diversity, offering a theoretical basis for optimizing MTD production and advancing intelligent fermentation systems.

1. Introduction

Daqu is a spontaneously produced microbial fermentation starter for Baijiu, playing multiple roles in saccharification, fermentation, and aromatization. It serves as an essential raw material and core component in Baijiu brewing during multi-species fermentation by the rich microorganisms, diverse enzymes, and flavor compounds. (Huang et al., 2023; Shi et al., 2024; Wu et al., 2023). According to the maximum fermentation temperature of the Qu-core, Daqu is classified into low-temperature Daqu (LTD), medium-temperature Daqu (MTD), and high-temperature Daqu (HTD) (Huang et al., 2024; Liu et al., 2018).

MTD is the main starter for brewing Nongxiangxing Baijiu (N-Baijiu), which primarily utilizes wheat as the raw material, cultivating diverse microorganisms through natural inoculation and generating abundant metabolites under long-cycle (lasting approximately 30-day) solid-state fermentation conditions (Liu et al., 2023; Zou et al., 2018). Multiple flipping operations, which are performed by experienced artisans to turn and rearrange Daqu brick positions based on the fermentation status, are essentially required to ensure uniform fermentation and maturation (Zhu et al., 2022). The flipping processes, at day 6 and 12 during fermentation, are considered crucial for regulating and maintaining consistent fermentation conditions within the fermentation chamber, including temperature, humidity, oxygen, and carbon dioxide levels. Traditional practices suggest that premature or delayed flipping can adversely affect Daqu quality. Operators must perform labor-intensive flipping in high-temperature and high-humidity fermentation rooms to

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ensure proper Daqu fermentation, exposing themselves to significant health risks. This process seriously restricts the scalability of Daqu production. With advancements in science and technology, the traditional manual production of Daqu has transitioned toward mechanized manufacturing to reduce labor intensity, improved efficiency, and increased production capacity (Liu et al., 2023; Shi et al., 2024). Hence, it is imperative to investigate the impact of traditional flipping crafts on the fermentation mechanism of Daqu when shift to automated technologies under semi-controlled conditions.

The microbial community and its metabolites in MTD play a pivotal role in shaping Baijiu's flavor and quality during fermentation. High-throughput sequencing and metabolomics provide insights into microbial community and the processes underlaying metabolite formation during Daqu fermentation. Comparative analyses revealed the specialized community structures among three types of Daqu, which are responsible to various enzyme activities (e.g., saccharifying, liquefying, fermenting, and esterifying abilities) (Huang et al., 2024; Kang et al., 2022a). Different microbial communities between the surface and core of MTD demonstrated a spatial heterogeneity for microbial community assembly during fermentation (Tang et al., 2023). The microbial metabolic network responsible for flavor development during MTD fermentation, showing that Lactobacillales, Mucorales, and Eurotiales sequentially dominate, producing lytic enzymes and flavor precursors/compounds (Yang et al., 2021). In addition, Environmental factors (such as acidity, moisture, and temperature) and microbial interactions drove microbial community succession during fermentation of MTD (Ma et al., 2022). Variables such as moisture, pH, acidity, and temperature significantly affect microbial composition during MTD fermentation (Li et al., 2016), ultimately leading to functional and metabolic differences (Kang et al., 2022a).

While previous studies have characterized microbial diversity in Daqu, the mechanistic relationship between traditional flipping crafts and microbial ecological principles remains poorly understood. Current research predominantly relies on either taxonomic profiling or single-omics approaches, which have failed to capture the spatiotemporal dynamics of microbial community succession and assembly under craft-specific environmental conditions. To address this gap, we systematically compared microbial taxa and metabolites in MTD under flipping and non-flipping regimes. Through integrated multi-omics and ecological network analyses, we deciphered the microbial interactions governing community succession and established correlations between environmental parameters and core functional microorganisms. Furthermore, we identified the key drivers for microbial community assembly. These findings not only advance our mechanistic understanding of traditional Daqu fermentation but also provide actionable insights for optimizing MTD production protocols to ensure batch-to-batch consistency and quality control. Importantly, this work establishes a theoretical framework for transitioning from empirical craftsmanship to data-driven intelligent manufacturing in traditional fermentation industries.

2. Materials and methods

2.1. Sample collection

Medium-temperature Daqu (MTD) samples were obtained from a Nongxiangxing Baijiu (N-Baijiu) distillery in Yibin, Sichuan, China. The flipping Daqu (FD, a traditional craft) refers to the practice in Daqu fermentation where experienced craftsmen adjust the fermentation environment by flipping the Daqu based on its fermentation status, typically on the 6th and 12th days of fermentation. In contrast, the non-flipping Daqu (NFD) involves no flipping operations throughout the entire fermentation period, while all other procedures remain consistent with the flipping process. Representative FD and NFD samples were collected at ten different fermentation time points: day 1, 2, 4, 6, 9, 12, 15, 18, 21, and 25. Daqu bricks were randomly selected from door, center, and window locations at each sampling time to ensure reliable samples (Fig. S1). A total of 78 Daqu bricks were collected. Daqu bricks from the same fermentation room and sampling time were thoroughly mixed and transferred to sterile bags, then stored at −80 °C until further analysis.

2.2. Determination of physicochemical properties and enzymatic activities, and evaluation of sensory characteristics

To investigate the dynamics of MTD fermentation, we assigned six fermentation parameters, including temperature (Qu-core), moisture, starch, reducing sugar, pH, and total acidity. In addition, according to the national professional standard techniques (QB/T 4257–2011), four enzyme activities were measured, including saccharifying activity (amylase), liquefying activity (glucoamylase), acidic protease activity, and esterifying activity. Each sample were measured in triplicate. A panel of nine seasoned evaluators (comprising four women and five men), specialized in the sensory evaluation of matured MTD (day 25), conducted the sensory analysis of the Daqu.

2.3. Quantitative analysis of sugars, alcohols, organic acids, and amino acids

We analyzed the main components of Daqu sample, including sugar, alcohol, organic acid, and amino acid, using a high-performance liquid chromatography (HPLC) system. Briefly, 5.0 g of the sample was mixed with 45.0 mL of ultrapure water, and placed in a 4 °C ultrasound bath for 30 min. Then, the mixture was centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was filtered through a 0.22 μm nylon membrane and injected into the HPLC system. Quantification of sugars, alcohols, organic acids was performed using an HPLC system (LA-20A, Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm, Bio-Rad, Contra Costa, USA), based on a previous study (Wu et al., 2023). Free amino acids were determined using an HPLC system equipped with an AJS-02 amino acid analysis column (C18, 4.6 × 150 mm, 3 μm, Shimadzu, Kyoto, Japan) and a photodiode array (PDA) detector, following the protocol reported by Wen et al. (2024).

2.4. Analysis of volatile metabolites

Volatile compounds (VOCs) of Daqu were analyzed semi-quantitative using HS-SPME GC-MS. Briefly, 5.0 g of sample was added to 20.0 mL of saline (0.85 % NaCl, 1 % $CaCl_2$), ultrasonically treated at 0 °C for 30 min, and centrifuged at 3500 rpm for 20 min at 4 °C. Subsequently, 4 ml of the supernatant and 10 μL of internal standard (2-octanol, 125 mg/L) were transferred into a 20 ml headspace bottle vial containing 1.5 g of NaCl. VOCs were extracted from the headspace using DVB/CAR/PDMS fiber (50/30 μm × 2 cm) at 50 °C for 30 min under 250 rpm. The analysis was performed on an Agilent 8860/5977B (Agilent Technologies, Palo Alto, CA, USA) equipped with a DB-5MS column (30m × 0.25 mm × 0.25 μm, CA, USA), following a previously described method (Song et al., 2024). Compounds were identified by matching their mass spectra against the NIST 17 library.

2.5. High-throughput sequencing and data processing

Total genomic DNA was extracted following a previously reported protocol (Luo et al., 2023) with minor modifications. Briefly, DNA extraction: 0.3 g sample with glass beads was frozen in liquid N_2, ground, then mixed with DNA buffer (NaCl, sarcosine, Tris-HCl, EDTA), lysozyme/lyticase, and incubated at room temperature (30 min). After centrifugation, supernatant underwent RNAase treatment (37 °C, 30 min), followed by two PCI (25:24:1) extractions. DNA was precipitated with ice-cold isopropanol (−20 °C, 1h), washed twice with 70 % ethanol, air-dried, and dissolved in 50 μL nuclease-free H_2O. Quality was

verified by gel electrophoresis and Nano300 spectrophotometer. For bacterial, the primer pair 27F and 1492R was utilized to amplify the full-length 16S rRNA gene. For fungal, the primer pair ITS1F and ITS4R was applied to amplify the entire internal transcribed spacer (ITS) region. PCR amplification and sequencing were conducted utilizing the PacBio Sequel II instrument by Biomarker Technologies Corporation (Beijing, China). Bioinformatics analysis of the sequencing data was performed as previously described (Han et al., 2024).

2.6. Statistical analysis and data visualization

Differential analysis of physicochemical factors was performed using the "ggpubr", "tidyverse" and "rstatix" package. Alpha diversity indices and principal coordinates analysis (PCoA) were calculated using the "vegan" package in R. One-way ANOVA with Fisher's LSD multiple comparisons test was used to assess the differences in the α diversity index of the Daqu microbial community within the same craft at different fermentation times, while a paired *t*-test was employed to detect differences in the α diversity index of the Daqu microbial community across different crafts at the same fermentation time. The relative importance of stochastic and deterministic processes in the MTD microbial community assembly was evaluated using the "NST" package through the modified stochasticity ratio (MST). Significantly different taxa (LDA score >2) were identified using linear discriminant analysis effect size (LEfSe) using the "microeco" package. VOCs data were analyzed using Orthogonal Partial Least Squares Discriminant Analysis (PLS-DA) and VIP via the "ropls" package. Differential metabolites were obtained according to the standard of $p < 0.05$, VIP >1. Relationships between metabolite types and microbial communities, as well as between microbial communities and environmental factors, were examined using the Mantel test with the "linkET" package. The co-occurrence network and spearman correlation coefficient analysis ($|r| > 0.6$, $p < 0.05$) were done in R software, using the "psych" and "reshape2" package, and the network diagram was visualized by Gephi 0.9.1. Network topology measures were used to identify keystone taxa (Li et al., 2024). Redundancy analysis (RDA) was performed to elucidate the relationship among microorganisms, samples, and endogenous environmental factors (https://www.genescloud.cn). The random forest model was employed to assess the impact of endogenous factors on the community assembly pattern, using "randomForest" and "rfPermute" packages. Unless otherwise specified, all figures were plotted by R software (version 4.4.1). All data was presented as means ± standard (n = 3).

3. Results and discussion

3.1. Dynamic changes of physicochemical factors during Daqu fermentation

Temperature of Daqu was a bio-heat generated by microbial metabolic activities (Xiao et al., 2017). Based on the Qu-core temperature, the fermentation process was divided into three phases: the rising period, the stable period; and the cooling period (Fig. 1a). We found that the peak temperature of FD and NFD were 59.7 °C and 60.2 °C both on day 6 (the first flipping of Daqu), respectively. The temperature of FD was significantly lower that of NFD form days 15–25, after the second flipping of Daqu ($p < 0.01$), leading to a shorter stable period (7 days vs. 13days) than NFD. Similarly, the moisture of FD was significantly lower than that of NFD on days 9–18 ($p < 0.01$). Our results confirmed that flipping can modify the internal environment of Daqu by enhancing airflow within the fermentation room, reducing Daqu temperature, and accelerating moisture evaporation (Yang et al., 2021; Zhu et al., 2022). Similar peak total acidity (TA) was observed at 2.02 mmol/10g and 1.80 mmol/10g on day 4 in FD and NFD, respectively. However, at the end of fermentation, the TA of FD (0.55 mmol/10 g) was significantly lower than that of NFD (0.80 mmol/10 g, $p < 0.05$). As a result, the pH of FD

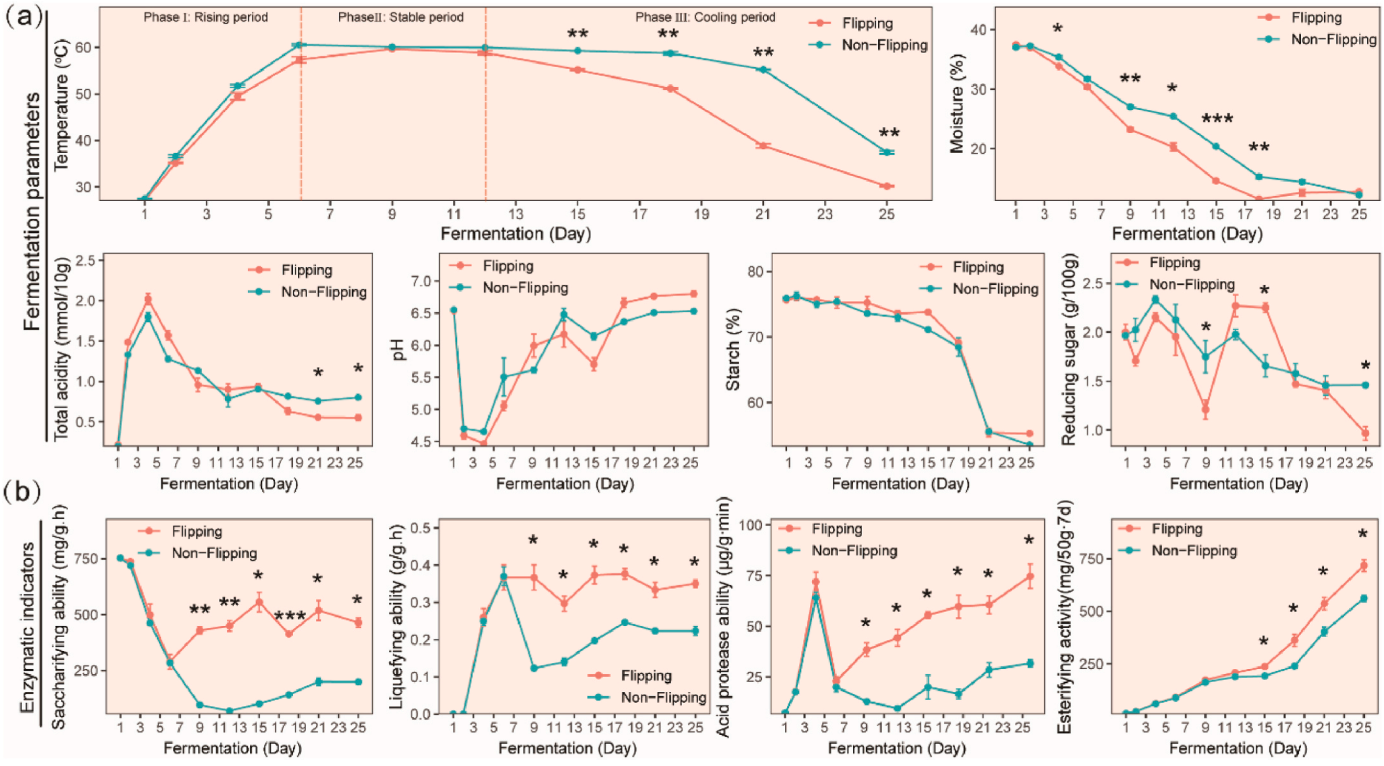

Fig. 1. Dynamics of physicochemical characteristics during MTD fermentation. (a) Changes in fermentation parameters, including temperature, moisture, total acidity, pH, starch content, and reducing sugar content. (b) Changes in enzymatic indicators, including saccharifying ability, liquefying ability, acid protease ability, and esterifying activity. *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

displayed higher than NFD. Moreover, the starch content of FD/NFD gradually decreased from 75.64 % to 75.92 %–53.54 % and 57.59 %, respectively. The content of reducing sugar showed a trend of fluctuation decreasing. As microorganisms secreted amylase and glucosidase to degraded the raw materials, we speculated that enzyme activities should differ between FD and NFD.

We then compared the activities of the key enzymes responsible for material fermentation, including saccharifying activity, liquefying activity, esterifying activity, and acid protease ability (Kang et al., 2022a; Shi et al., 2024). During the early stage of fermentation (days 1–6, prior

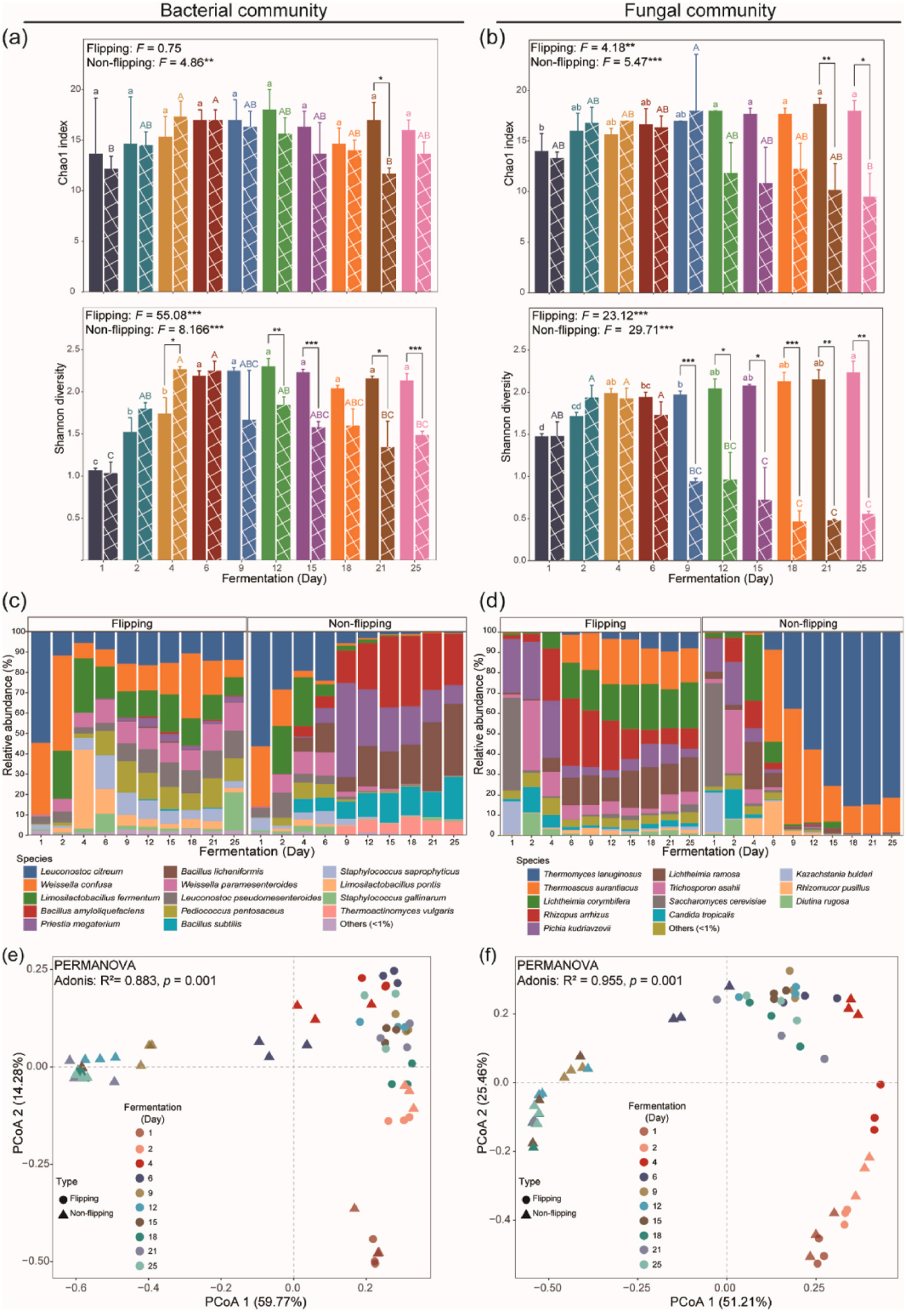

Fig. 2. Diversity and structure of the microbial community during MTD fermentation. Alpha diversity indices for bacterial (a) and fungal (b) communities at the species level. Dynamic changes in the bacterial (c) and fungal (d) communities at the species level, with species comprising less than 1 % relative abundance categorized as 'others'. PCoA analysis of bacterial (e) and fungal (f) communities based on the Bray-Curtis dissimilarity matrix at the species level. *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

to the first turning of the fermentation starter), there were no significant differences in the activities of four enzymes between FD and NFD. However, as fermentation progressed into the mid-to-late stages, the activities of all enzymes in FD became significantly higher than those in NFD ($p < 0.05$). Therefore, our results indicated that FD was more effective in producing substances associated with the functional properties of Daqu, besides the physicochemical factors variation between FD and NFD. More importantly, the Daqu quality evaluation results revealed that FD received significantly higher scores than NFD in both sensory characteristics and physicochemical properties. Therefore, FD was classified as premium grade, whereas NFD was rated as substandard (Table S1). These findings indicated that different flipping processes have a substantial impact on the quality of MTD. It can be inferred that variations in microbial community structure contribute to the observed quality differences between FD and NFD.

3.2. Microbial community diversity and succession during Daqu fermentation

To analyze the diversity and dynamics of the microbial communities

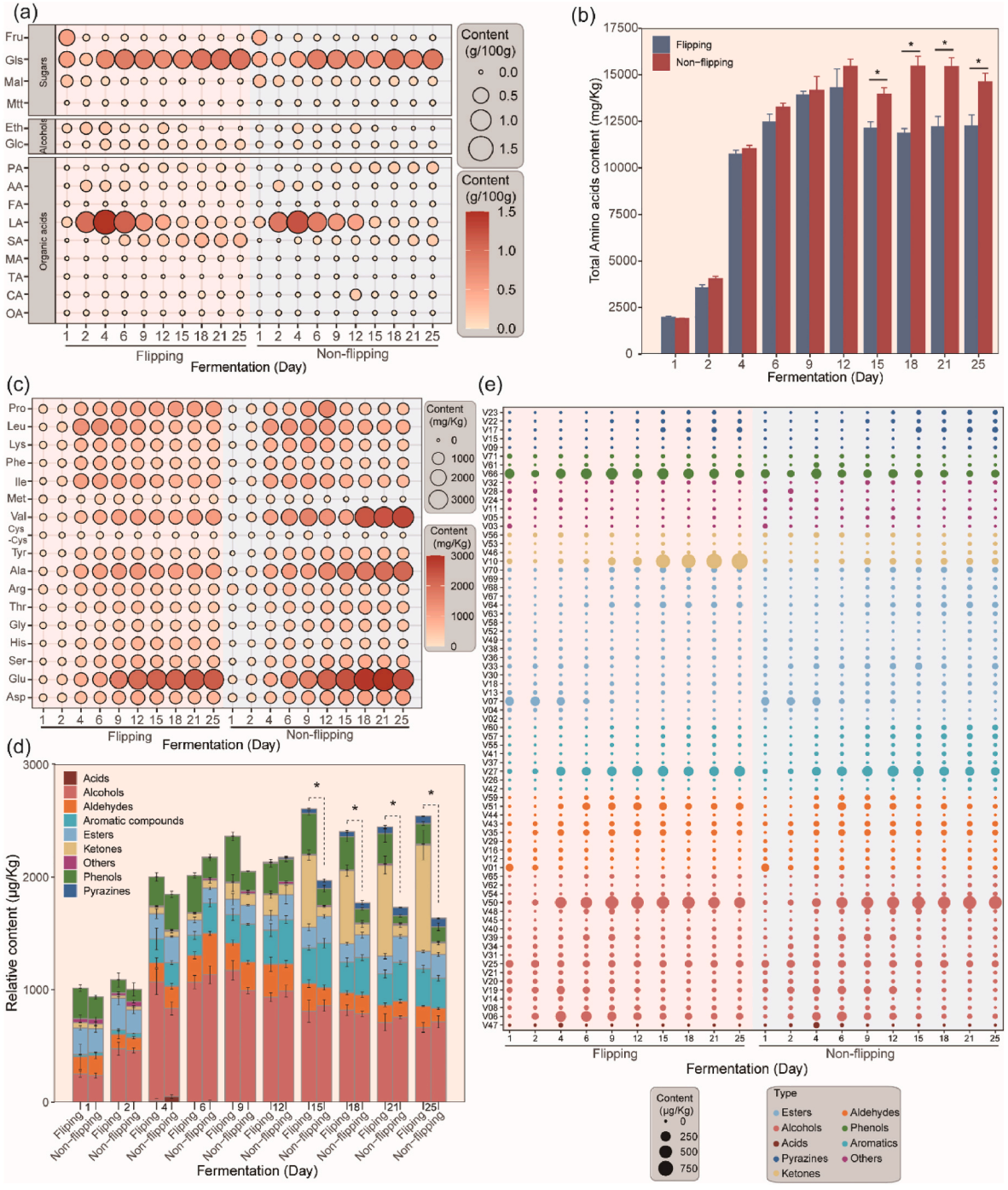

Fig. 3. Dynamics of metabolites during MTD fermentation. (a) Bubble charts depicting the temporal changes in the 4 sugars, 2 alcohols, and 9 organic acids. (b) Concentration changes in total amino acid content. (c) Bubble charts showing the temporal changes in the 17 free amino acids. (d) Concentration changes in 9 types of volatile compounds. (e) Bubble charts illustrating the temporal changes in the 71 volatile compounds. The asterisks above the bars within one group represent significant differences were detected between FD and NFD by paired *t*-test. *, $p < 0.05$.

in FD and NFD during fermentation, we performed full-length bacterial 16S rRNA gene and fungal ITS region amplicon sequencing on Daqu samples. The dilution curve demonstrated that the sequencing depth was sufficient to encompass the majority of fungal and bacterial taxa present in the samples (Fig. S2). We found that most of the microorganisms of both bacteria and fungi were widely distributed between FD and NFD during fermentation (Fig. 3a–d). The richness (Chao1 index) and diversity (Shannon diversity index) of the bacterial community in FD and NFD generally showed an increased trend, followed by a decreased trend during fermentation, with higher bacterial richness and diversity in FD than NFD ($p < 0.05$) at the end of fermentation (Fig. 2a). The fungal community in FD, however, continuously increased, exhibiting a different trend from that in NFD. (Fig. 2b). In addition, the richness and diversity of FD were significantly higher than NFD ($p < 0.05$) after the second flipping of Daqu. The results showed that the richness and diversity of microbial communities were significantly influenced by flipping crafts. Proper fermentation conditions promoted the expansion of microbial richness and diversity, while excessive temperature in NFD had a restrictive effect, with convergence becoming more pronounced as temperature and duration increased (Shi et al., 2024).

The microbial community dynamics of FD and NFD during fermentation were assessed at the species level. FD and NFD shared similar trends in the succession of both bacterial and fungal communities before first flipping of Daqu (days 1–6; Fig. 2c and d). Lactic acid bacteria (LAB) as the main bacteria producing organic acids and bacteriocins (Zou et al., 2018), dominated both FD and NFD, including *Leuconostoc citreum*, *Limosilactobacillus* spp. and *Weissella confusa*, with the relative abundance (RA) exceeding 86 %. Concurrently, *Pichia kudriavzevii* and *Kazachstania bulderi* are the dominant fungi, which preserved yeast diversity under the high RA conditions (Wen et al., 2024). After flipping, days 9–25, the bacterial and fungal communities in FD and NFD exhibited notable differences and reached relatively stable states, which is confirmed by a hierarchical clustering tree based on Bray-Curtis distances (Fig. S4). A clear temporal succession pattern was observed in both bacterial (ANOSIM: $R^2 = 0.883$, $p = 0.001$) and fungal community structures (ANOSIM: $R^2 = 0.955$, $p = 0.001$) (Fig. 2e and f). The microbial composition of FD was dominated by bacteria such as *Staphylococcus gallinarum*, *L. citreum*, *W. paramesenteroides*, *Leuconostoc pseudomesenteroides*, and *P. pentosaceus* (71.23 % RA on day 25), and fungi such as *Lichtheimia corymbifera*, *Lichtheimia ramose*, and *Thermoascus aurantiacus* (56.23 % RA on day 25). These microorganisms are generally not highly thermotolerant, consistent with previous findings (Yang et al., 2021). In contrast, the microbial community of NFD was dominated by thermophilic bacteria, primary *Bacillus* species, (*B. licheniformis*, *B. subtilis*, and *B. amyloliquefaciens*), with RA of 81.46 %. The fungal community in NFD was dominated by thermophilic species such as *Thermomyces lanuginosus* (81.39 %) and *T. aurantiacus* (17.02 %). Remarkably, *Bacillus* and *Thermoactinomyces*, known for their exceptional heat resistance, were the predominant bacterial genera in HTD (Niu et al., 2025; Shi et al., 2024; Zhang et al., 2022b). These results demonstrate that flipping operations induce temporal heterogeneity in microbial community succession between FD and NFD. The microbial compositions of FD and NFD conferred different functionalities to MTD. For instance, *S. gallinarum*, a key carboxylesterase-producing species in MTD related to acetic acid and amino acid formation (Yang et al., 2021), presented highly in FD but lowly in NFD. Similarly, higher abundance of *L. ramose* and *L. corymbifera* associated with saccharifying activity and acid protease ability was found in FD compared to NFD, converting starch into glucose and proteins into amino acids to supply essential substrates and energy for Daqu and Baijiu fermentation (Wang et al., 2018).

The ecological processes of microbial community assembly include stochastic processes and deterministic processes. Deterministic processes, driven by species interactions (e.g., competition, mutualism, and predation) and abiotic environmental conditions (e.g., pH, temperature, and moisture), are beneficial to food fermentation (Ban et al., 2024; Yang et al., 2023b). Therefore, understanding the microbial community assembly process is crucial for the targeted regulation of functional species. During fermentation, the bacterial and fungal communities in FD and NFD transitioned from being predominantly shaped by deterministic processes to stochastic processes and then reverted back to deterministic processes, with deterministic processes exerting a stronger influence on NFD by the end of fermentation (Fig. S5). Previous studies have identified fermentation temperature as a pivotal factor influencing deterministic processes, with higher peak-temperature strengthening the processes (Wen et al., 2024).

Our findings indicated that due to prolonged environmental stress, the microbial community in Daqu undergoes a complex assembly process, experiencing substantial species turnover, transitioning from complexity to simplification, and ultimately retaining adaptive microorganisms (Fig. 2c and d). Thus, the flipping crafts essentially affected the structure and function of microbial communities by changing the environmental conditions.

3.3. Dynamic variations of metabolites in FD and NFD

Daqu functions as a saccharifying agent, fermenter and aroma enhancer in Baijiu production, providing essential flavor and precursors for subsequent fermentation. Thus, a systematic analysis of its metabolites is highly significant. Using HPLC, 4 sugars, 2 alcohols, and 10 organic acids were monitored during fermentation (Fig. 3a). Sugars are a crucial as carbon sources for microbial growth and metabolism (Shang et al., 2022). On day 1, the primary sugars in FD and NFD were glucose (Gls), maltose (Mal), and fructose (Fru), accounting for over 99 % of the sugar content (Fig. 3a). Glucose, the predominant sugar in both FD and NFD, showed a consistent increase after day 2, peaking at 0.97 g/100 g and 0.92 g/100 g on day 25, respectively. Ethanol levels rose rapidly, peaking on day 4 (0.24 g/100 g in FD and 0.14 g/100 g in NFD), followed by a sharp decline, becoming undetectable after day 15. Ethanol production was attributed to yeasts metabolizing sugars under hypoxic conditions (Wang et al., 2019), while subsequent microbial utilization of ethanol contributed to flavor compound synthesis.

Organic acid plays a pivotal role in regulating pH, thereby influencing microbial community structure and product flavor. The primary organic acids were lactic acid (LA), acetic acid (AA), and succinic acid (SA), each exhibiting dynamic change during fermentation. LA and AA, which mirrored the total acids trends peaking on day 2 (1.50 g/100 g in FD and 1.24 g/100 g in NFD) before gradually. SA increased continuously, whereas oxalic acid (OA), citric acid (CA), tartaric acid (TA), malic acid (MA), and fumaric acid (FA) remained low without discernible trends.

Free amino acids in Daqu originate from protease-driven degradation in raw materials, serving as nitrogen sources for microbial growth and precursors for flavor compounds (Zhang et al., 2021). Total amino acid (TAA) content in FD and NFD increased rapidly on days 0–12, peaking at 14302.59 mg/kg and 15448.10 mg/kg, respectively (Fig. 3b). Subsequently, TAA levels decreased with fluctuations, with FD showing significantly lower levels than NFD ($p < 0.05$). The main amino acids, including Glu, Val, Ala, Asp, Pro, Leu, Lys, Ile and Ser, constituted 52.99–79.94 % of TAA (Fig. 3c). Notably, in the later stages, despite higher acid protease ability in FD, its lower TAA levels were attributed to differences in microbial communities, leading to the secreted proteases with varied catalytic mechanisms.

VOCs are critical indicators of Daqu quality during fermentation (Shi et al., 2024; Zhang et al., 2021). A total of 71 VOCs were initially identified, including 18 esters, 17 alcohols, 1 acid, 5 pyrazines, 9 aldehydes, 4 ketones, 3 phenols, 8 aromatic compounds, and 6 others (Table S2). VOC contents in FD and NFD exhibited a similar trend, initially increasing and then decreasing, peaking at 2606.12 μg/kg on day 15 for FD and 2175.96 μg/kg on day 6 for NFD (Fig. 3d). On days 12–25, the VOCs content in the FD group was higher than in the NFD

group. Alcohols, aromatics, ketones, and pyrazines showed an increasing trend during the fermentation process (Table S3). At the end of fermentation, FD exhibited higher levels of ketones, alcohols, and aromatics, accounting for 76.45 % of total VOC (Fig. 3d). VOCs differences were influenced by microbial fermentation in high temperature environment (Du et al., 2023). The most abundant VOCs included 2-phenylethanol, 2,4-Di-tert-butylphenol, benzaldehyde, 2-octanone, 1-pentanol, 2-ethyl-1-hexanol, 1-octen-3-ol, 2-phenylethanal, and 2-furanmethanol (Fig. 3e). These compounds, formed and transformed through microbial-mediated or chemical reactions (Yang et al., 2024), indicating the critical role of flipping process in VOCs formation during fermentation. For instance, 2-phenylethanol, contributing rosy and honey aromas, is a typical volatile metabolites of yeast (Fan et al., 2020), while 2,4-di-tert-butylphenol, with oxidized and lavender-like notes, is characteristic of LAB (Fan et al., 2020; Oliveira et al., 2020). 2-octanone, with cheese-like and mushroom-like aromas, is produced by *Staphylococcus* via incomplete β-oxidation of fatty acids (Yang et al., 2022). These key aroma compounds have been identified as vital aroma contributors in MTD (Du et al., 2023; Yang et al., 2021; Zhang et al., 2011) and N-Baijiu (Zheng et al., 2014).

3.4. Identifying the biomarkers and metabolic markers during MTD fermentation

To deeply understand the microbial characteristics due to flipping crafts, we applied LEfSe to compare the specific microbes in FD and NFD (LDA score >2, $p < 0.05$). A total of 6 bacteria (*W. confuse*, *L. fermentum*, *L. pontis*, *P. pentosaceus*, *W. paramesenteroides*, and *Staphylococcus saprophyticus*) and five fungal biomarkers (*Rhizopus arrhizus*, *L. corymbifera*, *P. kudriavzevii*, *Lichtheimia ramosa*, and *T. asahii*), were identified in ND. As a comparison, the biomarkers for NFD were predominantly thermotolerant microorganisms, including *B. amyloliquefaciens*, *Priestia megaterium*, *T. vulgaris* and *T. lanuginosus* (Fig. 4a–d). Our results highlighted the selective influence of high-temperature conditions on specialized microbial structures caused by flipping.

To further elucidate the differences between FD and NFD, OPLS-DA was used to construct a model analyzing metabolite composition corresponding to different flipping crafts. The OPLS-DA score plot demonstrated excellent stability and prediction ability ($R^2X = 0.721$, $R^2Y = 0.954$, $Q^2Y = 0.831$) (Fig. 4e). We identified a total of 40 different metabolites that differ between FD and NFD (VIP >1.0 and $p < 0.05$). Among these, substances with top five VIP values were 1,2-dimethoxybenzene (V41), glycerol (Glc), tridecane (V11), methyl anthranilate (V63) and oxalic acid (OA). Previous research has reported that bioaugmentation enhances 1,2-dimethoxy benzene level in MTD (Liu et al., 2024). This compound, a characteristic contributor to the "stale and musty" aroma of Pu-erh tea (Pang et al., 2019), is integral to forming unique flavor profiles. Glycerol plays a dual role in stabilizing yeast osmotic pressure and improving the lactic acid tolerance (Kang et al., 2022b), while also facilitating the release and perception of key flavor compounds in Baijiu (Wang et al., 2024a). During fermentation, most differential metabolites were detected in both FD and NFD but varied in concentrations. Consequently, these differences in metabolite composition become particularly evident during MTD fermentation and play a critical role in shaping the flavor profile of Daqu.

3.5. Correlation analysis between microorganisms and metabolites

As mentioned above, different crafts Daqu could be effectively characterized by metabolites generated during fermentation, which were primarily the result of microbial metabolic activity (Wang et al., 2021). Therefore, the metabolites between FD and NFD should be associated with microorganisms. To confirm this, we correlated the dominant microorganisms with metabolite types. In FD, aldehydes, amino acids, benzodiazepines, esters, ketones, and sugars compounds showed positive correlations with *P. pentosaceus* and *W. confusa* (r = 0.133–0.619, $p < 0.05$). Similarly, amino acids, benzodiazepines, and organic acids compounds were positively correlated to *L. fermentum* and *W. paramesenteroides* (r = 0.09–0.592, $p < 0.05$). Alcohols were positively associated with *L. fermentum*, *L. ramosa*, *P. kudriavzevii* (r = 0.156–0.276, $p < 0.05$), while pyrazines correlated positively with *B. amyloliquefaciens*, *P. pentosaceus*, *P. megaterium*, *W. paramesenteroides*, *L. corymbifera*, *L. ramose*, *R. arrhizus*, and *T. lanuginosus* (r = 0.09–0.260, $p < 0.05$). Bacteria primarily showed significant positive correlation with alcohols (e.g., V08, V48, and Glc), aldehydes (V12 and V43), and amino acids (e.g., Pyr, His, Ala), while fungi were primarily associated with alcohols (V14, V54, and V66), esters (V33 and V58), organic acids (OA and AA), and sugars (Mal). The results showed that the dominant microorganisms, including bacteria and fungi, synergistically contributed to the synthesis of specific differential metabolites (Fig. 5a and Table S4), which was further confirmed by the heatmap illustrating relationships between dominant microorganisms and all metabolites (Fig. S6a). However, in NFD, only fungi dominated the metabolite production (Fig. 5b and S6b) and were associated with more metabolites, particularly VOCs, with more negative associations, explaining the lower VOCs content in NFD than FD (Fig. 3d). The results indicated that variations in microorganisms within Daqu under different crafts were the fundamental reason for differences in metabolites.

3.6. Microbial interactions drive microbial community succession

Microbial interaction, as biotic factor, have been showed to drive Daqu community succession (Wen et al., 2024; Zhu et al., 2022), and they are a major cause of community fluctuations in multi-microbial co-fermentation systems (Tan et al., 2019). To elucidate potential microbial interactions and identify keystone species that significantly influence the community structure during fermentation, Spearson's rank correlation of ASVs was utilized to explore microbial co-occurrence patterns, including intra-domain (bacteria-bacteria and fungi-fungi) and inter-domain (bacteria-fungi) networks. Positive correlations predominated in the bacterial co-occurrence networks of both FD and NFD (Fig. 6a–d), with more interactions observed in the bacterial networks compared fungal networks, results consistent with previous reports in HTD (Shi et al., 2022). Similarly, positive correlations outweighed negative ones in fungal co-occurrence networks (Fig. 6b–e) and the inter-domain networks (Fig. 6c–f), where the inter-domain networks of FD and NFD exhibited more interactions than either bacterial or fungal networks. A similar phenomenon was observed in the solid-state fermentation system of Jianxiangxing Baijiu and peppers (Li et al., 2024; Zhang et al., 2022a). Positive correlations dominated all co-occurrence networks, suggesting potential coexistence of microbes through commensalism, cooperation, mutualism, or syntrophy (Canon et al., 2020), which likely contributes to the stability of the microbial community structure (Peng et al., 2021).

In FD, *P. pentosaceus* and *P. megaterium* were identified as keystone species in the bacterial network based on network node properties (Table S5), which were disparate to *L. pseudomesenteroides* and *R. kristinae* as keystone species in NFD. Similarly, *P. kudriavzevii*, *C. tropicalis*, and *R. microspores* were identified as keystone fungi in FD (Table S5), while *T. asahii*, *P. kudriavzevii*, *R. arrhizus*, and *T. lanuginosus* were identified in NFD. In FD, *P. kudriavzevii* was negatively correlated with *T. lanuginosus*, *R. microsporus* and *R. pusillus*, while other fungi exhibited positively correlation. In NFD, *T. lanuginosus* showed inverse associations with all other species, while the remaining keystone species were positively correlated with each other and with all other species except *T. lanuginosus*. Interestingly, *P. kudriavzevii* was negatively correlated with *R. pusillus* in FD but positively correlated in NFD, suggesting that habitat difference effect microbial interactions. In the bacteria-fungi inter-domain networks, fungi (*P. kudriavzevii*, *R. arrhizus*, and *R. microspores*) and bacteria (*L. citreum*, *L. pseudomesenteroides*, *L. pontis*, and *P. pentosaceus*) were identified as keystone taxa in FD. *P. kudriavzevii* was negatively correlated with all other microorganisms.

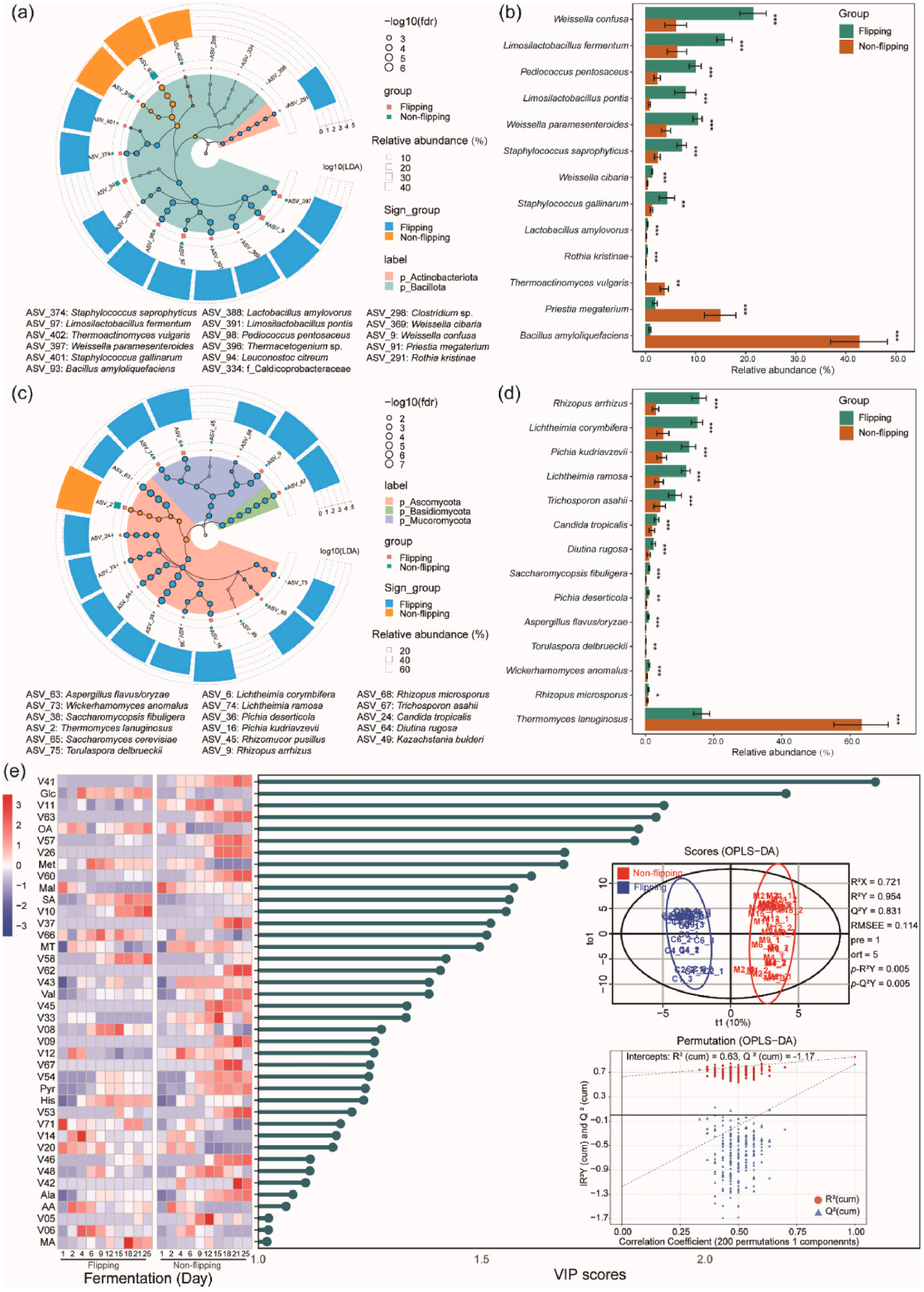

Fig. 4. Identifying the biomarkers and metabolic markers during MTD fermentation. Cladograms and LDA scores of bacteria (a) and fungi (c). Histograms of relative abundance differences for microorganisms between the two production processes: (b) bacteria and (d) fungi. Orthogonal partial least squares discriminant analysis (OPLS-DA) plots, cross-validation results, and heatmaps depicting differential compounds (e), including heatmaps of differential compounds during fermentation, OPLS-DA score plots, and OPLS-DA validation models with 200 permutation tests.

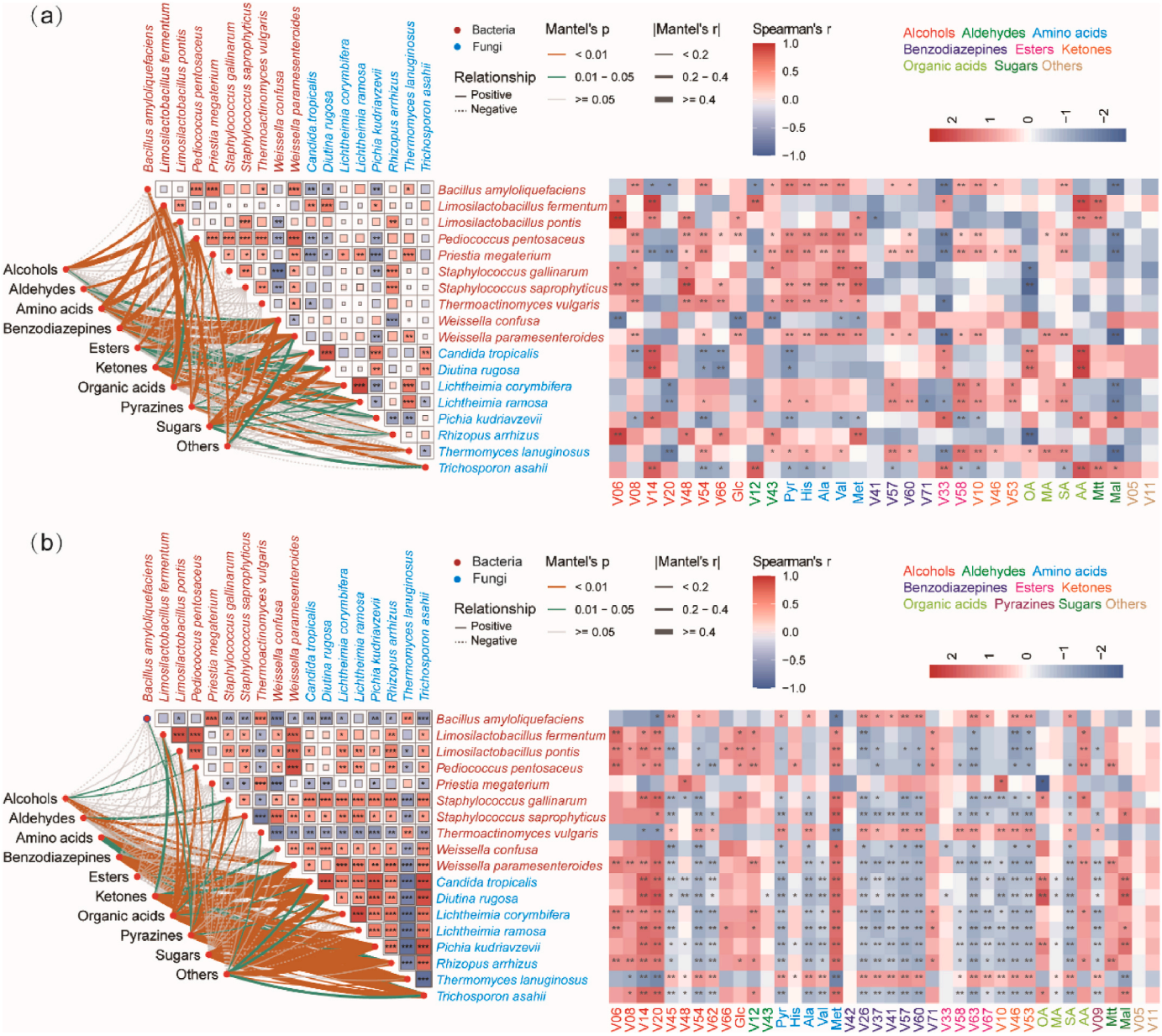

Fig. 5. Relationship between metabolite types and biomarkers (Mantel test, left) and correlations between biomarkers and differential compounds (heatmaps, right) in FD (a) and NFD (b). *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

In NFD, keystone taxa included bacteria (*R. kristinae*, *L. citreum*, and *L. pseudomesenteroides*) and fungi (*C. tropicalis*, *T. lanuginosus*, *W. anomalus*, *P. kudriavzevii* and *T. asahii*). All keystone bacterial taxa in NFD were positively correlated with keystone fungal taxa. Additionally, *R. pusillus*, *L. corymbifera*, and *L. fermentum* was positively correlated with inter-domain taxa in both FD and NFD. Compared to FD, the bacterial, fungal, and inter-domain networks of NFD exhibited greater complexity, with more edges and higher connectivity metrics, including higher average degree, clustering coefficient, degree centralization, and betweenness centralization (Table S5). The results showed that microbial interactions in NFD are stronger and its network structure was more complex. As keystone species within these co-occurrence networks, the interactions of these keystone species with other microbes may play a critical role in driving microbial community succession during fermentation.

Modularity, another key structural feature of networks (Newman, 2006), has been theoretically linked to stability in trophic networks (Thébault and Fontaine, 2010). The modularity indices of co-occurrence networks in FD exceeded 0.44 (0.64 for bacteria, 0.54 for fungi, and 0.58 for bacteria-fungi), while those in NFD were below 0.44 (0.28 for bacteria, 0.13 for fungi, and 0.24 for bacteria-fungi) (Table S6), suggesting a well-organized and functionally interrelated modular structure in FD microbial communities (Chen et al., 2020; Newman, 2006). High modularity enhances network stability by localizing disturbances within modules, preventing them from spreading and allowing the network to maintain its functions despite environmental changes (Wang et al., 2016). Communities in high-stress environments, such as those with extreme temperature and moisture, tend to exhibit lower network stability (Hernandez et al., 2021). Conversely, greater nutrient availability has been associated with more complex microbial networks (Naher et al., 2018) while weak interactions can promote stability in temporal ecological networks (Coyte et al., 2015). Overall, our results showed that the microbial co-occurrence network in FD exhibited less complexity and interactions but stronger stability than NFD.

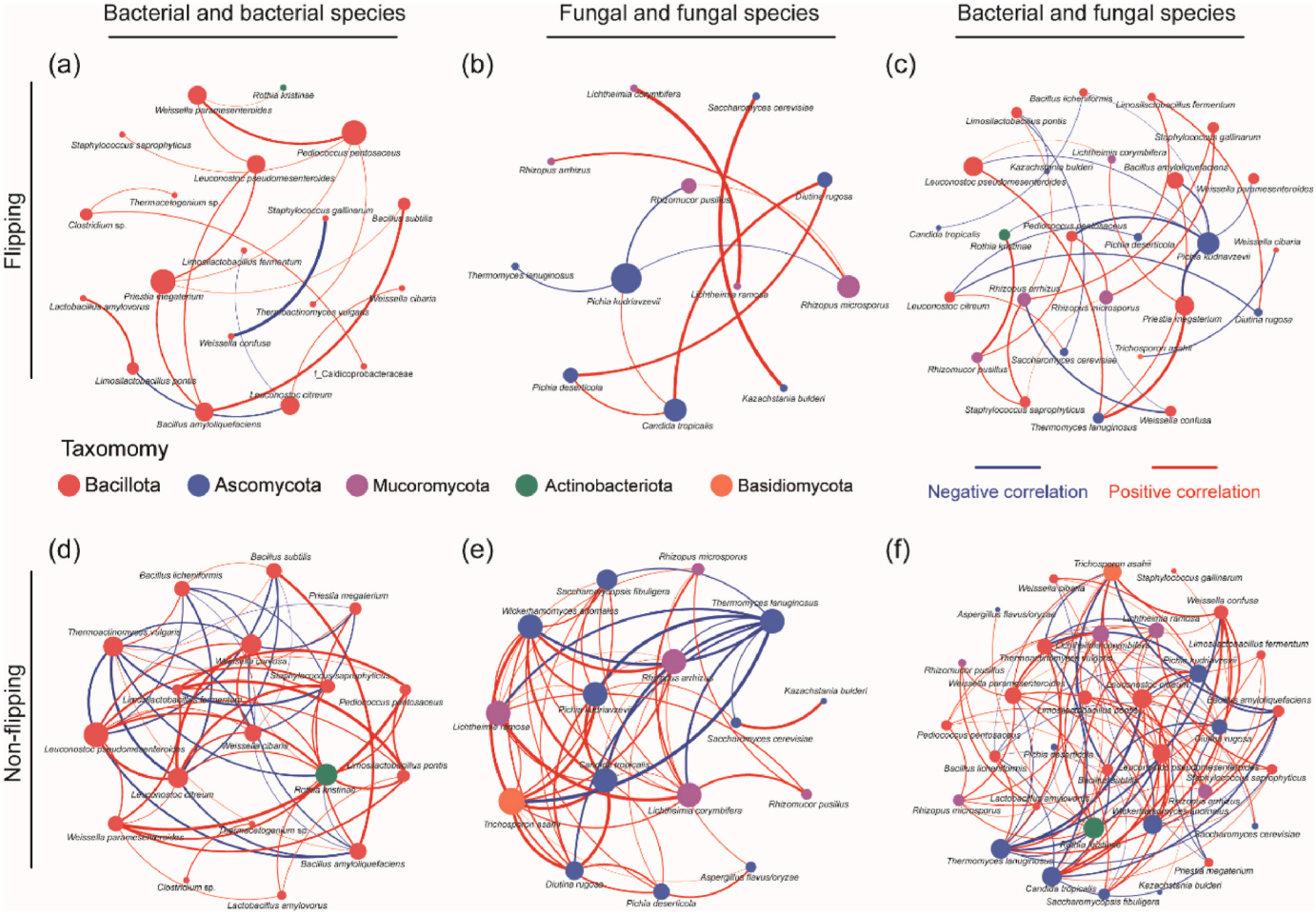

Fig. 6. Co-occurrence network analysis of microbial interactions at the species level in two craft-fermented Daqu. Bacterial-bacterial (a, d), fungal-fungal (b, e), and bacterial-fungal (c, f) communities during FD and NFD fermentation, respectively. ASVs with relative abundance ≥0.01 % and present in at least 20 % of the total samples were used to construct the microbial co-occurrence networks based on Spearman's correlation ($|r| > 0.6$, $p < 0.05$).

3.7. Endogenous environmental factors drive microbial community succession

During solid state fermentation, microbial community succession is affected by both biological factors and abiotic factors (Kang et al., 2022b; Li et al., 2024; Wang et al., 2024b). To investigate the impact of abiotic factors on microbial succession in Daqu, we used the Mantel test to analyze correlations between bacterial and fungal communities with their endogenous environmental factors during FD and NFD fermentation. As shown in Fig. 7a, d and Table S7, the bacterial and fungal communities in the both types of Daqu exhibited positive correlations with temperature ($r = 0.363–0.493$, $p < 0.01$), moisture ($r = 0.456–0.711$, $p < 0.01$), total acidity ($r = 0.419–0.688$, $p < 0.01$), pH ($r = 0.306–0.531$, $p < 0.01$), and total amino acids content ($r = 0.625–0.839$, $p < 0.01$). The results indicated that endogenous factors have potential effects on the microbial community succession during Daqu fermentation.

Redundancy analysis (RDA) further confirmed relationships among endogenous environmental factors, Daqu samples, and dominant microbiota. The first two axes explained 60.27 % (Figs. 7b) and 65.07 % (Fig. 7c) of the variance in bacterial communities, and 54.29 % (Figs. 7e) and 77.05 % (Fig. 7f) of the variance in fungi communities. Total amino acid, total acidity, pH, moisture, and temperature ($p < 0.01$) significantly affected both bacterial and fungal communities in FD (Fig. 7b and c; Table S8). In NFD, total acidity, total amino acid, pH, reducing sugar, moisture, temperature, and starch ($p < 0.01$) were significant drivers bacterial and fungal communities (Fig. 7e and f; Table S8). In the early stages of fermentation, total acidity, moisture, and starch exhibited positive correlations with *L. pontis*, *L. fermentum*, *W. confuse*, *T. asahii*, *P. kudriavzevii*, and *R. arrhizus* in FD, while with *L. fermentum*, *W. paramesenteroides*, *L. corymbifera*, and *L. ramose* in NFD. Dominant microorganisms such as LAB produced organic acids, which increased the acidity of the fermentation environment, creating stress conditions that inhibiting the growth of non-acid-tolerant microorganisms (Shanqimuge et al., 2015; Zhu et al., 2022). On the other hand, during the middle and late fermentation stages, total amino acids, pH, and temperature were significantly positively correlated with *P. pentosaceus*, *L. pseudomesenteroides*, *T. aurantiacus*, *L. corymbifera* in FD, while with *B. amyloliquefaciens, B. subtilis*, and *T. lanuginosus* in NFD. Dominant microorganisms such as *T. aurantiacus*, *L. corymbifera*, *B. amyloliquefaciens*, *B. licheniformis*, and *T. lanuginosus* exhibited significant correlations with enzyme activities, including saccharifying ability, liquefying ability, esterifying activity, and acid protease activity (Fig. S7). These enzymatic functions are critical for Daqu's role in breaking down macromolecular organic compounds, supporting microbial growth, and producing flavor compounds. Consequently, endogenous factors drive microbial community succession, and differences in these factors led to distinct microbial succession patterns between FD and NFD, ultimately resulting in functional differences.

Microbial succession is driven by a combination of ecological processes, including deterministic processes (such as environmental filtration and interspecies interactions) and stochastic processes (such as

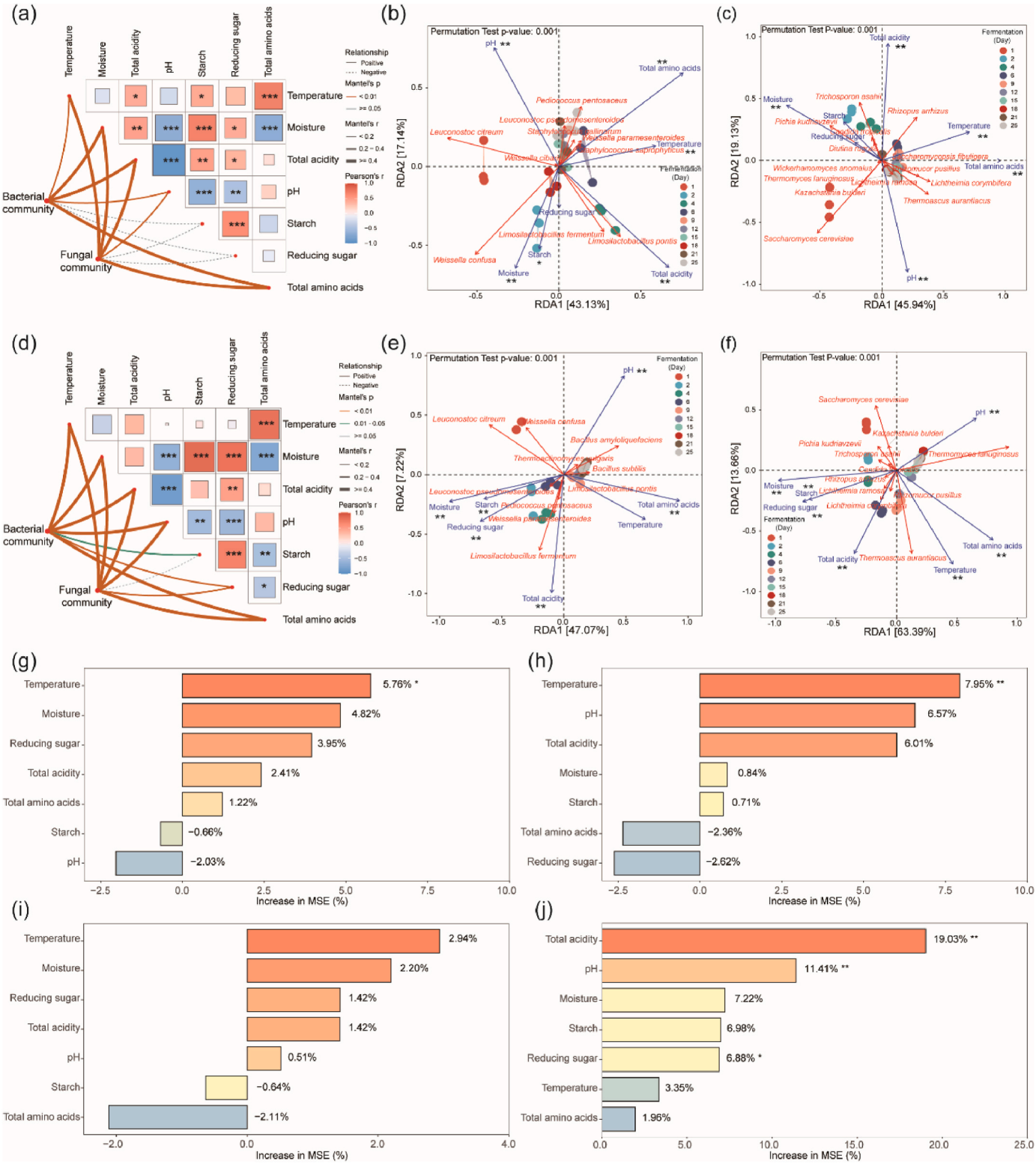

Fig. 7. Correlation analysis between endogenous environmental factors and dominant microbial communities, and their roles in driving community assembly pattern changes during Daqu fermentation. Mantel tests: dominant microbial communities and endogenous environmental factors in FD (a) and NFD (d). Redundancy analysis (RDA): dominant bacterial (b, e) and fungal (c, f) species associated with endogenous environmental factors in FD and NFD. Random forest models: endogenous environmental factors drivers of bacterial (g, i) and fungal (h, j) community assembly patterns in FD and NFD, respectively. *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

diffusion limits and random birth-death events) (Yang et al., 2023a). These varying community assembly patterns influence microbial composition and diversity, thereby driving the microbial succession (Shen et al., 2021). The relative importance of endogenous environmental factors on microbial community assembly pattern was assessed using a random forest model. The results showed that the microbial community assembly in different Daqu craft was affected by multiple endogenous factors (Fig. 7g–j). Specifically, temperature ($p < 0.05$ for bacteria and fungi) was primary driver of changes in bacterial and fungal community assembly patterns during FD fermentation (Fig. 7g and h). For NFD, total acidity ($p < 0.01$) and pH ($p < 0.01$) were significant drivers of the fungal community assembly patterns (Fig. 7i and j). Previous studies have shown that fermentation temperature was a key factor driving community assembly pattern by selectively enriching thermophilic microorganisms (Huang et al., 2024). Variations in temperature (e.g., Daqu types and different parts of Daqu) also increase the proportion of deterministic processes (He et al., 2021; Wen et al., 2024). Additionally, total acidity, primarily driven by lactic acid and acetic acid produced by LAB, inhibit non-acid-resistant microorganisms, thereby affecting microbial community assembly pattern. Thus, endogenous environmental factors indirectly affect microbial community succession by altering the balance between stochastic process and deterministic process during Daqu fermentation.

4. Conclusion

This study demonstrates that flipping crafts critically shape microbial community succession and metabolic outcomes during MTD fermentation. FD exhibited superior enzyme activity, volatile compound diversity, and microbial community stability compared to NFD, which can be attributed to the regulation of temperature and humidity during Daqu fermentation. Deterministic and stochastic processes jointly governed microbial community assembly in both fermentation processes during Daqu fermentation, with environmental factors (temperature and acidity) and species interactions driving the temporal dynamics of microbial succession. The simpler, yet modular, network structure of the microbial community in FD enhanced its resilience, whereas the increased complexity of the microbial network in NFD was associated with thermophilic dominance and a reduction in aroma diversity. These findings highlight the role of flipping crafts in balancing environmental conditions, promoting functional microorganisms, and facilitating flavor formation. The results provide feasible strategies for improving traditional Daqu production, ensuring quality consistency, and guiding mechanized fermentation processes. Future studies should aim to replicate the microbial-environmental synergies observed in FD within automated fermentation systems, thereby enhancing production scalability while preserving traditional quality attributes.

CRediT authorship contribution statement

Zhang Wen: Methodology, Investigation, Conceptualization, Data curation, Visualization, Writing – original draft. **Yu-Hua Wei:** Investigation, Methodology, Writing – original draft. **Da-Yong Han:** Methodology, Formal analysis, Funding acquisition. **Liang Song:** Methodology, Funding acquisition. **Hai-Yan Zhu:** Supervision, Conceptualization. **Liang-Chen Guo:** Investigation, Methodology. **Shen-Xi Chen:** Investigation, Methodology. **Bin Lin:** Investigation, Methodology. **Chao-Jiu He:** Investigation, Resources. **Zheng-Xiang Guo:** Investigation. **Pei-Jie Han:** Resources, Supervision, Funding acquisition, Project administration, Writing – review & editing. **Feng-Yan Bai:** Conceptualization, Supervision.

Declaration of competing interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2025.101063.

Data availability

Data will be made available on request.

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