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Application of indigenous *Saccharomycopsis fibuligera* for light-flavor Baijiu fermentation: Changes of microbial community and flavor metabolism

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ABSTRACT

Ethyl acetate, a characteristic flavor compound in light-flavor Baijiu (LFB), plays a pivotal role in defining its sensory profile. Insufficient concentrations in fermented grains may diminish the quality of Baijiu. Indigenous microbial bioaugmentation has proven effective in improving Baijiu quality by reshaping microbial community and modulating flavor metabolism within fermentation ecosystems. This study investigated the effects of *Jiuqu* fortified with an indigenous *Saccharomycopsis fibuligera* Y162 on the microbial community, physicochemical parameters, and volatile compound profiles throughout the fermentation of LFB. Results demonstrated a significant increase in ethyl acetate content in both original Baijiu and fermented grains, alongside pronounced fungal community restructuring. PacBio single-molecule real-time (SMRT) sequencing analysis showed that *Lactobacillus helveticus*, *Rhizopus* sp., *Saccharomyces cerevisiae*, and *Issatchenkia orientalis* became the dominant species at the end of fermentation. Correlation network analysis revealed that ethyl acetate was significantly related ($P < 0.05$, $|\rho| > 0.7$) to the *I. orientalis* and *Rhizopus* sp. (the main fungal biomarkers), which was driven by the functional *S. fibuligera*. Mantel test further highlighted acidity, temperature, and moisture as critical environmental factors influencing the microbial community ($P < 0.05$, $|\rho| > 0.7$). To summarize, biofortification with *S. fibuligera* exhibited positive effects in enhancing typical flavor metabolites by influencing fungal community structure in LFB. These findings imply that the indigenous strains have great application potential in improving the quality of Baijiu.

1. Introduction

As one of the six most popular distilled spirits in the world, Chinese Baijiu plays a vital role in Chinese people's spiritual and cultural life. In 2023, the annual yield of Chinese large-scale Baijiu enterprises was about 4.5 million kiloliters, and the total sales revenue of the industry reached 104.5 billion dollars (Kang et al., 2024). Chinese Baijiu is typically produced by spontaneous solid-state fermentation inoculated with different natural starter (*Jiuqu*), and grain saccharification and fermentation are simultaneously conducted (Jin et al., 2017). Distinct characteristics and styles of Baijiu flavor are affected not only by regional environmental factors, but also production techniques (raw materials, *Jiuqu* types, fermentation containers) (Li et al., 2022a; Tan et al., 2022; Tu et al., 2022). Differences in these abiotic factors result in unique microbiota and discrepancy in the synergetic microbial metabolism in the fermentation process of Baijiu (Tu et al., 2022). Consequently, microbiota play a pivotal role during the fermentation, and regulating the brewing microbiota to stabilize fermentation quality is an important measure to improve the Baijiu quality.

The traditional Baijiu production could be fell into two stages: the fermentation of *Jiuqu* and fermented grains (Zhang et al., 2024a). As the unique saccharification and fermentation starter of Baijiu, *Jiuqu* could provide key enzymes and microorganisms that have crucial impacts on the formation of Baijiu flavor (Zhang et al., 2023). Fermented grains are distilled to obtain original Baijiu, which is then stored and blended to produce the final product. However, the open fermentation environment

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and complex brewing technology adopted in Baijiu production have led to very complicated brewing microbial community, which has resulted in the instability of Baijiu production quality and low production efficiency (Zhang et al., 2024a). It has been proven that using single or varied microorganisms to regulate and strengthen Baijiu fermentation is an important approach to maintain the stability of Baijiu production (Li et al., 2023a; Pan et al., 2023; Xu et al., 2023). Notably, fortified *Jiuqu* by inoculating microorganisms that serve specific functions has been developed and applied to improve fermentation performance and Baijiu quality.

In recent years, a variety of functional strains have been bio-augmented to improve the flavor quality of Baijiu by regulating the microbial composition throughout the fermentation process. For instance, *Daqu* inoculation with *Bacillus subtilis* or *Bacillus licheniformis* altered its community structure and improved its flavor characters and enzyme activity (Yang et al., 2022; Wang et al., 2017). Chen et al. (2023) reported that light-flavor *Daqu* inoculated with *Lactobacillus brevis* influenced the composition of the dominant microbial community and offered more compounds for the flavor substance formation in the fermentation process. Moreover, yeasts are closely linked to the production of ethanol and key ester flavor, and thus adding functional yeasts to *Daqu* can change the content of flavor compounds in Baijiu (Pan et al., 2023; Li et al., 2020). Inoculation of *Wickerhamomyces anomalus* with *Daqu* increased the ethyl acetate content and led to variations in the levels of other flavor compounds during Baijiu fermentation (Wang et al., 2020). *Daqu* fortified with *Saccharomyces cerevisiae* changed microbial community composition in the fermentation of strong-flavor Baijiu, as well as improved the contents of ethyl hexanoate and other flavor substances (Pan et al., 2023).

Ethyl acetate, as one of the four main ethyl esters in Baijiu, which contributes a fruity (pears and bananas) and sweet aroma, is the primary aroma component of light-flavor Baijiu (LFB), and the quality of Baijiu can be improved by appropriately increasing the ethyl acetate content (Wang et al., 2019; Li et al., 2023b). Currently, functional yeasts were reported to be the major contributors to production of ethyl acetate in Baijiu fermentation, and mainly included *W. anomalus*, *Pichia kudriavzevii*, *Kluyveromyces marxianus*, and *Saccharomycopsis fibuligera* (Ni et al., 2022; Xiao et al., 2023; Zhang et al., 2020a; Xie et al., 2021). *S. fibuligera* could secrete amylase, β-glucosidase, and acid protease efficiently, and utilize carbohydrates in the fermented grains to generate ethanol and various flavor substances (Yuan et al., 2024). Previous studies showed that adding *S. fibuligera* and *S. cerevisiae* into *Jiuqu* fermentation process ultimately promoted the total alcohol and ester levels as well as the enzymatic activity of fermented grains (Su et al., 2020). Fortified *jiuyao* with *S. fibuligera* CY2111 and *Rhizopus microsporus* SM4 enhanced the alcohol content and elevated more pleasant esters such as isoamyl acetate, ethyl isovalerate, and ethyl caprylate in *huangjiu* (Zhu et al., 2023). However, the function of each strain and its contribution to Baijiu flavor could not be determined by multiple microbial fortifications. Single-microorganism-fortified fermentation systems enable comprehensive characterization of functional microorganisms' roles in shaping microbial succession and flavor metabolism dynamics. Notably, limited research has been conducted on biofortification using single *S. fibuligera*, and functional microbial enhancement influencing the fermentation process of LFB urgently need to be explored in depth.

Hereby, this study aimed to investigate the biofortification effect of the addition of *S. fibuligera* into *Jiuqu* on the fermentation process of LFB. To our knowledge, this report represented the first study on the impact of inoculation with the indigenous *S. fibuligera* on physicochemical properties, volatile flavor compounds, and microbial community in the solid-state fermentation process of LFB. Following this, the interrelationship among microorganisms, physicochemical factors, and flavor compounds was analyzed. This study will be beneficial to make better use of featured microbial resources, investigate their special roles in Baijiu fermentation, and provided valuable insights for improving the flavor quality of Baijiu.

2. Material and methods

2.1. Fortified Jiuqu preparation

The fermentation starters used in this study were the fortified *Jiuqu* (FQ) and traditional *Jiuqu* (TQ). The detailed preparation process of the traditional *Jiuqu* was described by Zhu et al. (2022). *Saccharomycopsis fibuligera* Y162 was isolated from the traditional *Jiuqu*. Fortified *Jiuqu* was made by exogenously inoculating with *S. fibuligera* Y162 based on the traditional *Jiuqu*. Briefly, crushed clay, rice bran, and *Zhongqu* were blended with water, in which 5‰ (w/w) of pure *S. fibuligera* Y162 bran (10^9 cells/g) was contained, and then were handmade to shape *Jiuqu* balls. After 7 days of cultivation, *Jiuqu* balls were dried and stored for 1 month for Baijiu brewing.

2.2. Baijiu fermentation and sample collection

Baijiu fermentation was performed according to the traditional light-flavor Baijiu production, including grain steeping, grain steaming, *Jiuqu* blending, saccharification, fermentation, and distillation described by a previous study (Hu et al., 2021). At the beginning of saccharification, steamed and cooled glutinous sorghum was mixed with 1.3% of mature traditional *Jiuqu* and fortified *Jiuqu*, respectively. After saccharification for 24 h, the saccharified grains were blended with the distilled grains at a weight ratio of 1:4 and put into fermentation tank, then anaerobically fermented for 9 days. After that, the fermented grains were distilled to obtain light-flavor original Baijiu. Thus, there were two fermentation systems fermented by traditional *Jiuqu* (TQ group) and fortified *Jiuqu* (FQ group), respectively.

According to five-point sampling method, five repeated sub-samples were collected from the center and four corners of the fermentation tank's middle layers at different fermentation times and thoroughly mixing them into one sample (Hu et al., 2021). The saccharified grains samples (250 g) were taken at the beginning and end of saccharification, and named as S0 and S1, respectively. 250 g of fermented grain samples were collected on day 0, 1, 2, 3, 4, 5, 7 and 9 during the fermentation, and named as D0, D1, D2, D3, D4, D5, D7, and D9, respectively. The saccharified grains and fermented grains were sampled in triplicate in each group. Finally, 60 samples were stored immediately at −80 °C until analysis.

2.3. Determination of physicochemical factors

The temperature of fermented grains was conducted in situ using a digital temperature sensor. A gravimetric method was performed by drying fermented grain samples at 115 °C for 3 h to determine the moisture content. 10 g of the fermented grain sample was mixed with 90 mL of distilled water and oscillated for 30 min. Then the mixture was filtered to obtain the supernatant. 20 mL of the supernatant was mixed with 30 mL of distilled water, and the acidity of the samples was measured by titration with 0.1 mol/L of NaOH to the endpoint of pH 8.2 with a pH meter (Li et al., 2024a). The reducing sugar of fermented grains was determined by 3,5-dinitrosalicylic acid (DNS) according to the prior method (Zhang et al., 2020b). All physicochemical factors of the fermented grains were measured in triplicate.

2.4. Microbial community analysis

Total genomic DNA was extracted from fermented grain samples using cetyltrimethyl ammonium bromide (CTAB) method. The DNA concentration and purity were evaluated by 1% agarose gel electrophoresis. For bacteria, the full length region of 16 S rDNA was amplified using 16 S primers pair (forward primer: AGAGTTTGATCCTGGCTCAG, reverse primer: GNTACCTTGTTACGACTT). ITS primers pair (forward primer: TACACACCGCCCGTCG, reverse primer: CCTSCSCTTANTDATATGC) were used to amplify the ITS full-length region of fungi. PCR

amplification procedures have been described in a previous study (Tang et al., 2024). After amplification, PCR products were purified and used to build SMRT Bell sequencing libraries. Subsequently, the library quality was evaluated using a Qubit@ 2.0 fluorometer (Thermo Fisher Scientific, USA). Finally, the library was performed on the PacBio Sequel II platform at Novogene Technology Co., Ltd. (Beijing, China).

The original sequences were processed to obtain Clean Reads by CCS (SMRT Link v7.0). Then, Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) was used to cluster the Clean Reads into operational taxonomic units (OTUs) with 97% similarity thresholds (Robert, 2013). Finally, fungal and bacterial representative sequences of OTUs with highest frequent sequence taxonomic classification were conducted by UNITE database (https://unite.ut.ee/) (Kõljalg et al., 2013) and Silva database (http://www.arb-silva.de/) (Quast et al., 2013), respectively.

2.5. Volatile compounds analysis

The main volatile compounds in the original Baijiu were detected by gas chromatography-flame ionization detector (GC-FID) conducted on the Agilent 7890 B GC system (CA, USA) according to the method previously depicted by Zhu et al. (2022). The absolute concentration of the main flavor compounds was calculated using the internal standard curve method.

The volatile components in fermented grains were determined by headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS) referring to the prior method with some modifications (Wang et al., 2024). Briefly, fermented grain sample (10 g), saturated sodium chloride solution (8 mL), and internal standard solution (20 μL) were added into a 20 mL headspace vial, simultaneously. Then the mixture was equilibrated at 50 °C for 5 min. The SPME fiber of 50/30 μm DVB/CAR/PDMS (Supelco Co., PA, USA) was inserted for microextraction at 50 °C for 40 min. Subsequently, the fiber of fermented grain sample was interposed into the injection port of the equipment and desorption was carried at 250 °C for 5 min. The extracts were examined using GC-MS performed on an Agilent 7890 B GC system equipped with an Agilent 5977 B mass selective detector (Agilent Technologies Inc., CA, USA). Volatiles were identified by comparing the mass spectral profiles with a matching mass of ≥80 with those available in the National Institute of Standards and Technology (NIST 20) library (Gaithersburg, MD, USA). The relative concentration of each compound was calculated based on the content of internal standards and their ratio to the peak area of volatile substances.

2.6. Statistical analysis

Microbial α-diversity, principal coordinate analysis (PCoA), and non-metric multidimensional scaling (NMDS) based upon the Bray-Curtis distance in fermented grain samples were calculated by QIIME (Version1.9.1). Linear discriminant analysis (LDA) effect size (LEfSe) was applied to identify the statistically significant biomarkers (LDA >4.0) (Zhang et al., 2024b). Differences of dominant species and volatile flavor compounds between groups were evaluated by Wilcoxon rank-sum test, with $P < 0.05$ indicating a significant difference. Redundancy analysis (RDA) utilizing the "vegan" and "plyr" package in R was performed to reveal the impact of physicochemical factors on microbial community, and the Monte Carlo permutation and Mantel tests were used to evaluate the significance of the physicochemical factors at the $P < 0.05$ level (Lin et al., 2022; Li et al., 2024b). Orthogonal partial least squares discriminant analysis (OPLS-DA) and variable importance in projection (VIP) were performed via SIMCA Version 14.1 (Umetrics AB, Umea, Sweden) (Qiu et al., 2024). Spearman's correlation coefficient (ρ) was conducted to characterize the interaction of microbial community and their correlation with differential flavor compounds, and visualized as a co-occurrence network with $|\rho| > 0.6$ and $P < 0.05$. All assays were conducted in triplicates, and the data results were presented as mean ± standard deviation.

3. Results

3.1. Evaluation of fortified Jiuqu at industrial scale light-flavor baijiu

21 batches of industrial scale sorghum-based fermentation were carried out to assess the impact of fortified *Jiuqu* (FQ) on the quality of light-flavor Baijiu. As shown in Fig. 1A,

FQ had a significant effect to improve the content of ethyl acetate compared to TQ. The average content of ethyl acetate in original Baijiu produced by FQ was 2.00 g/L, which was increased by 50.38% ($P < 0.0001$) compared with TQ. Furthermore, little difference was observed in the production of ethanol yield, acetaldehyde, methanol, n-propanol, and higher alcohols (Fig. 1A and B).

3.2. Changes in the physicochemical factors of fermented grains

In this study, the physicochemical factors of fermented grains including temperature, moisture, acidity, and reducing sugar were measured. During the saccharification and fermentation process, physicochemical factors of fermented grains using two types of *Jiuqu* showed the similar variation (Fig. 2). Temperature, moisture, acidity, and reducing sugar increased during the saccharification stage. The average temperature in FQ group was 16.4 °C at the beginning of fermentation (day 0) and increased quickly to the highest point of 31.1 °C in the first 4 days, then decreased slowly till the end of fermentation. Compared to FQ group, the temperature in TQ group was significantly higher from day 3 to day 9 (Fig. 2A). Moisture accumulated throughout the fermentation, which had a rapid growth in the first 4 days and then maintained fairly steady thereafter. Compared to TQ group, the moisture content at the beginning in FQ group was significantly higher, but showed an opposite trend at the end of fermentation (Fig. 2B). The acidity gradually increased from 0.28 to 0.45 mmol/10 g from day 0 to day 9 in FQ group, which increased from 0.27 to 0.49 mmol/10 g in TQ group. And the acidity at the end of fermentation in FQ group was significantly lower than that in TQ group (Fig. 2C). The reducing sugar content in the first 3 days in FQ group was significantly lower than that in TQ group, and there was no difference thereafter (Fig. 2D).

3.3. Microbial community analysis of fermented grains

PacBio SMRT sequencing analysis was used to study the microbial community of FQ group and TQ group. After filtering the low-quality sequences, the acquired bacterial and fungal clean reads in samples were 265,580 and 76,7165, respectively. Based on these high-quality sequences, effective tags with different phylogenetic OTUs for the microbial community were obtained from the samples using 97% sequence identity cutoff. Chao 1 and Shannon indices were applied to characterize the differences in microbial community richness and diversity, respectively. The bacterial α-diversity increased during saccharification stage, with the average Chao 1 index increasing from (47.43–53.59) to (58.21–77.28) and average Shannon index raising from (1.55–1.57) to (2.65–2.88) (Fig. 3A and B). While it decreased gradually from 0 to 9 days of fermentation, with the average Chao 1 index reducing from (81.82–97.48) to (22.60–39.5), and average Shannon index decreasing from (3.22–3.38) to (0.54–1.093). The dynamic changes of bacterial α-diversity in the saccharification stage and early fermentation stage were more intensity. This may be related to the abundant oxygen content, low alcohol and acidity contents in the fermented grains during this period, resulting in a greater variety and quantity of bacteria. While lactic acid bacteria dominated in the middle and late stages of fermentation, resulting in relatively gentle changes in bacterial α-diversity indices.

Conversely, the fungal Shannon index decreased significantly from (2.34–2.42) to (1.60–1.78) during saccharification stage (Fig. 3C and D). This may be due to the gradual increase of dominant fungal species leading to a decrease in fungal diversity. And fungal α-diversity indices

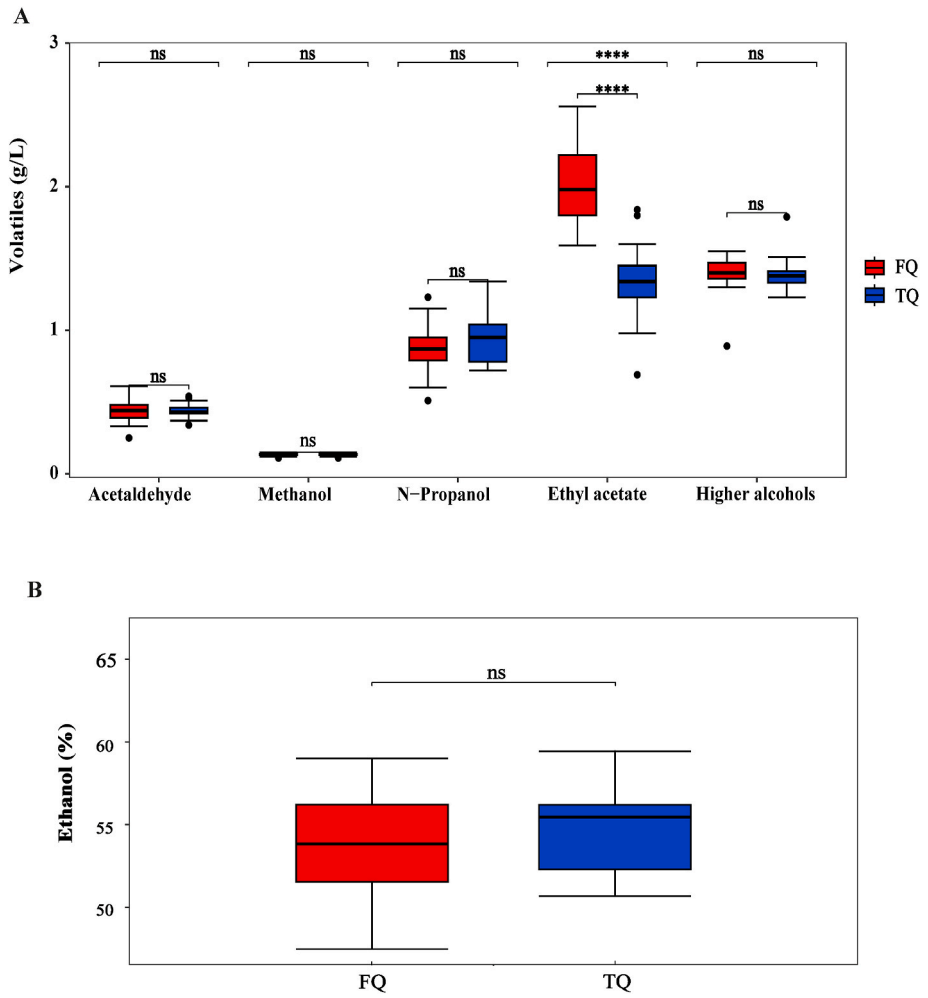

Fig. 1. Characteristics of fortified *Jiuqu* as measured in the industrial scale fermentation of light-flavor Baijiu. "ns" means no significance. ****, $P < 0.0001$.

remained relative stability during the fermentation. The average fungal Chao 1 index ranged from (35.40–49.37) to (26.94–41.57), and the average Shannon index ranged from (1.81–1.87) to (1.87–2.02) from day 0 to day 9. Fungal Shannon index were significantly higher ($P < 0.05$) during saccharification stage and in the early stages of fermentation in FQ group, which may be related to the strengthening of *S. fibuligera*. Futher, PCoA analysis explained 69.44% and 70.42% of the total variation in bacterial and fungal community, respectively (Fig. 3E–G). And only the fungal OTUs detected from two groups were significantly different examined by ANOSIM (Bacteria: R = - 0.0031, $P = 0.42$; Fungi: R = 0.1402, $P = 0.002$). These results were in line with NMDS analysis (Fig. 3F–H).

The top 16 dominant bacteria at the specie level for at least one time point (average relative abundances >1%) belonged to *Lactobacillus*, *Weissella*, and *Bacillus*, etc. (Fig. 4A). *Lactobacillus plantarum* and *Lactobacillus brevis* (80%) predominated at the beginning of saccharification, with the former gradually decreasing over time and the latter firstly increasing and subsequently decreasing. And their relative abundances were higher in FQ group than those in TQ group. More bacterial species were found at the end of saccharification and on day 0 and day 1 of fermentation. *Lactobacillus pontis* increased during the mid-fermentation stage, and its relative abundance was higher in FQ group than that in TQ group. *Lactobacillus helveticus* dominated in the late fermentation period, and its relative abundance (78.42%) on the last day of FQ group was lower than that (92.81%) of TQ group. *Lactobacillus* was the predominant bacteria throughout the fermentation process, with its relative abundance reaching over 90% after day 2.

For fungal community, 10 dominant species were detected including *Rhizopus* sp., *S. cerevisiae*, *Issatchenkia orientalis*, *Monascus ruber*, *S. fibuligera* and so on (Fig. 4B). The relative abundance of *Rhizopus* sp., *S. cerevisiae*, and *I. orientalis* ocuppied for more than 70%. *Rhizopus* sp. And *I. orientalis* increased during saccharification stage, and the former gradually decreased from day 0 to day 2 of fermentation, after which it stabilized. The relative abundance of *Rhizopus* sp. was lower in FQ group than that in TQ group. The relative content of *I. orientalis* remained relatively stable over the whole fermentation, and it was higher in FQ group than that in TQ group. *S. cerevisiae* decreased during saccharification stage and gradually increased throughout the fermentation process, the relative abundance of which was lower in FQ group than that in TQ group. The relative abundance of *S. fibuligera* with higher content in FQ group consistently decreased in the fermentation process, accounting for 0.22% and 0.15% on the last day of fermentation in FQ and in TQ groups, respectively.

Comparing relative abundances of these dominant species between FQ group and TQ group, *I. orientalis* (28.06% vs. 17.10%, $p < 0.001$) and *Aspergillus amstelodami* (3.27% vs. 1.82%, $p < 0.01$) had a significantly higher relative abundance in FQ group than in TQ group, while *Rhizopus* sp. (40.51% vs. 49.93%, $p < 0.05$), *La. helveticus* (18.37% vs. 26.46%, $p < 0.05$), *Apiotrichum loubieri* (0.09% vs. 0.23%, $p < 0.05$), and *Saccharomycopsis malanga* (0.10% vs. 0.27%, $p < 0.01$) had a significantly lower relative abundance in FQ group (Fig. 4C). LDA value was used to identify the statistically significant biomarkers (LDA >4) among FQ group and TQ group by LDA Effect Size (LEfSe) (Fig. 4D). *I. orientalis* and *A. amstelodami* were regarded as the biomarkers with higher abundance

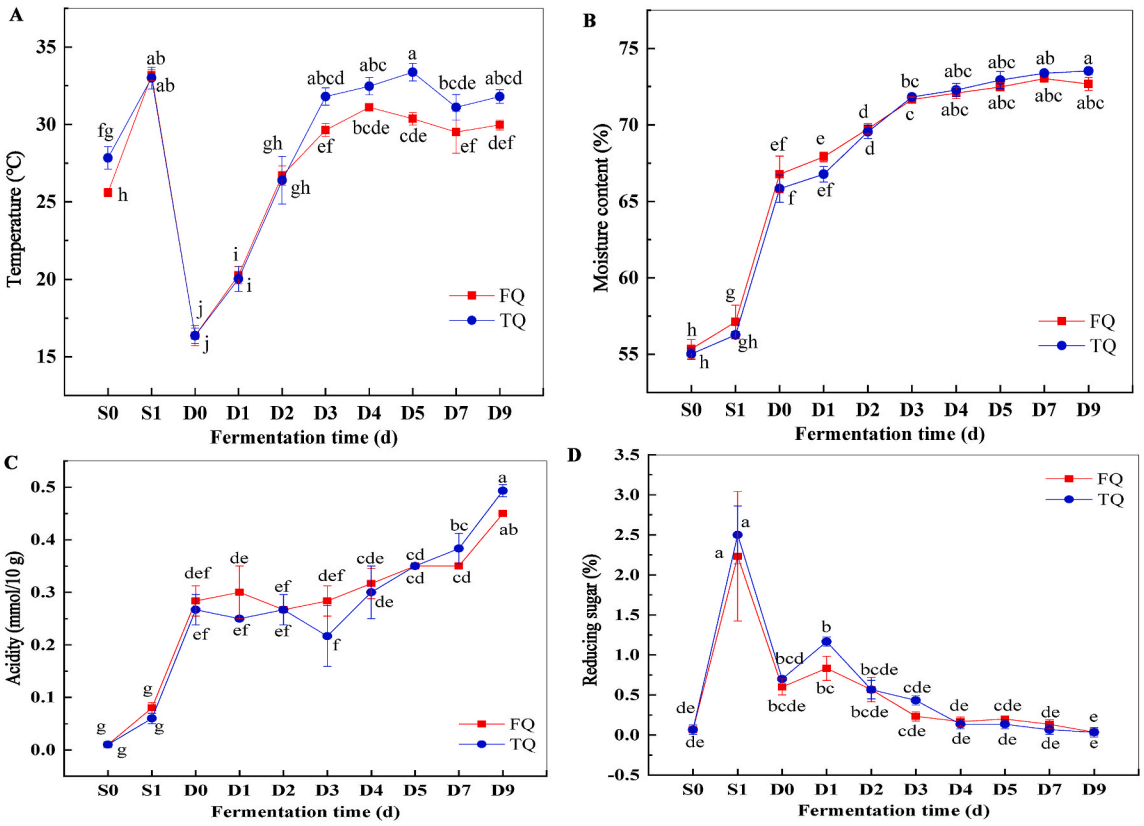

Fig. 2. Variations in physicochemical factors during the fermentation using two types of *Jiuqu*. A: Temperature. B: Moisture content. C: Acidity. D: Reducing sugar. FQ group: Representing the samples fermented using fortified *Jiuqu*. TQ group: Representing the samples fermented using traditional *Jiuqu*. Different lowercase letters indicate significant differences ($P < 0.05$) tested by Tukey's test. The same below.

in FQ group, and *Rhizopus* was a biomarker in TQ group.

3.4. Bioturbation effect on the flavor compounds in fermented grains

As we all know, the flavor characteristics of Baijiu are closely correlated with the volatile compounds of fermented grains, which are mainly produced by functional microbes during the fermentation. Here, the volatile profiles of fermented grains in the whole fermentation were detected via HS-SPME-GC-MS. As fermentation progressed, total contents of volatile flavor compounds increased (Fig. 5A). At the end of fermentation, esters (39.47–44.54%) were the main flavor compounds, followed by alcohols (30.35–30.67%) and acids (19.46–27.02%). The content of esters in FQ group was higher than that in TQ group. A total of 54 volatiles were identified in these two kinds of fermented grains, including 16 esters, 10 alcohols, 5 acids, 7 aldehydes, 10 aromatics, 4 phenols, and 2 others. The composition and content of volatile components were different in FQ group and TQ group (Fig. 5B). The PCA and OPLS-DA explained 93.14 % and 40.6 % of the total variation based on all detected volatiles, respectively. PCA visualized the volatile compounds similarity of FQ group and TQ group. Differences in volatile composition were further examined by ANOSIM ($R = 0.046$, $P = 0.026$) (Fig. 5C).

VIP of OPLS-DA was used to determine the potential differential flavor compounds. It can be seen that R^2X was 0.505, R^2Y was 0.902, and Q^2 was 0.853, indicating that the model fitting accuracy was good (Fig. 5D). As shown in Fig. 5E and F, there were 14 important flavor compounds with significant differences in volatiles in FQ group and TQ group, obtained by OPLS-DA (VIP >1) and Wilcoxon rank-sum test, including 3 esters (ethyl acetate, ethyl decanoate, and ethyl nonanoate), 3 alcohols (hexanol, octanol, and furfuryl alcohol), 2 acids (isobutyric acid and isovaleric acid), 4 aldehydes (phenylacetaldehyde, benzaldehyde, decanal, and trans-2-Decenal), 2 aromatics (2-ethylbutyl methacrylate and 3-propyltoluene). Among which, 12 important differential flavor substances had higher contents in FQ group than those in TQ group.

3.5. Microbial succession driven by physicochemical characteristics in fermented grains

RDA was conducted to clarify the impact of environmental factors on microbial community structures. RDA results suggested that the two axes explained 83.19% and 60.94% of the total variance for bacteria and fungi, respectively. Further, Monte Carlo permutation test showed that temperature, moisture, acidity, and reducing sugar were key factors determining microbial community structures in FQ group and TQ group ($P = 0.001$) (Fig. 6A and B).

For more details, the relationship between major microbial species and environmental factors was presented by the Mantel test (Fig. 6C and D). The results indicated that acidity had a strongly significant beneficial relationship with *I. orientalis*, *M. ruber*, *S. fibuligera*, *S. malanga*, *La. plantarum*, *Pediococcus acidilactici*, and *A. pasteurianus* in TQ group, while it revealed a significant positive connection with *I. orientalis*, *M. ruber*, *S. fibuligera*, and *La. plantarum* in FQ group ($P < 0.05$, $|\rho| > 0.7$). Correlation coefficient between reducing sugar and microbial species were less than 0.7 in FQ group and TQ group. The moisture module was significantly positively related to *A. amstelodami*, *Rhizopus microsporus*, *La. plantarum*, and *A. pasteurianus* in TQ group, while it only had a positive correlation with *La. plantarum* in FQ group ($P < 0.05$, $|\rho| > 0.7$). The temperature module was significantly positively related to *Bacillus ginsengihumi*, *Bacillus oleronius*, *B. subtilis*, and *Lactobacillus fermentum* in FQ group, while it had positive relationships with *Weissella confusa* and *Weissella paramesenteroides* in TQ group ($P < 0.05$, $|\rho| >$

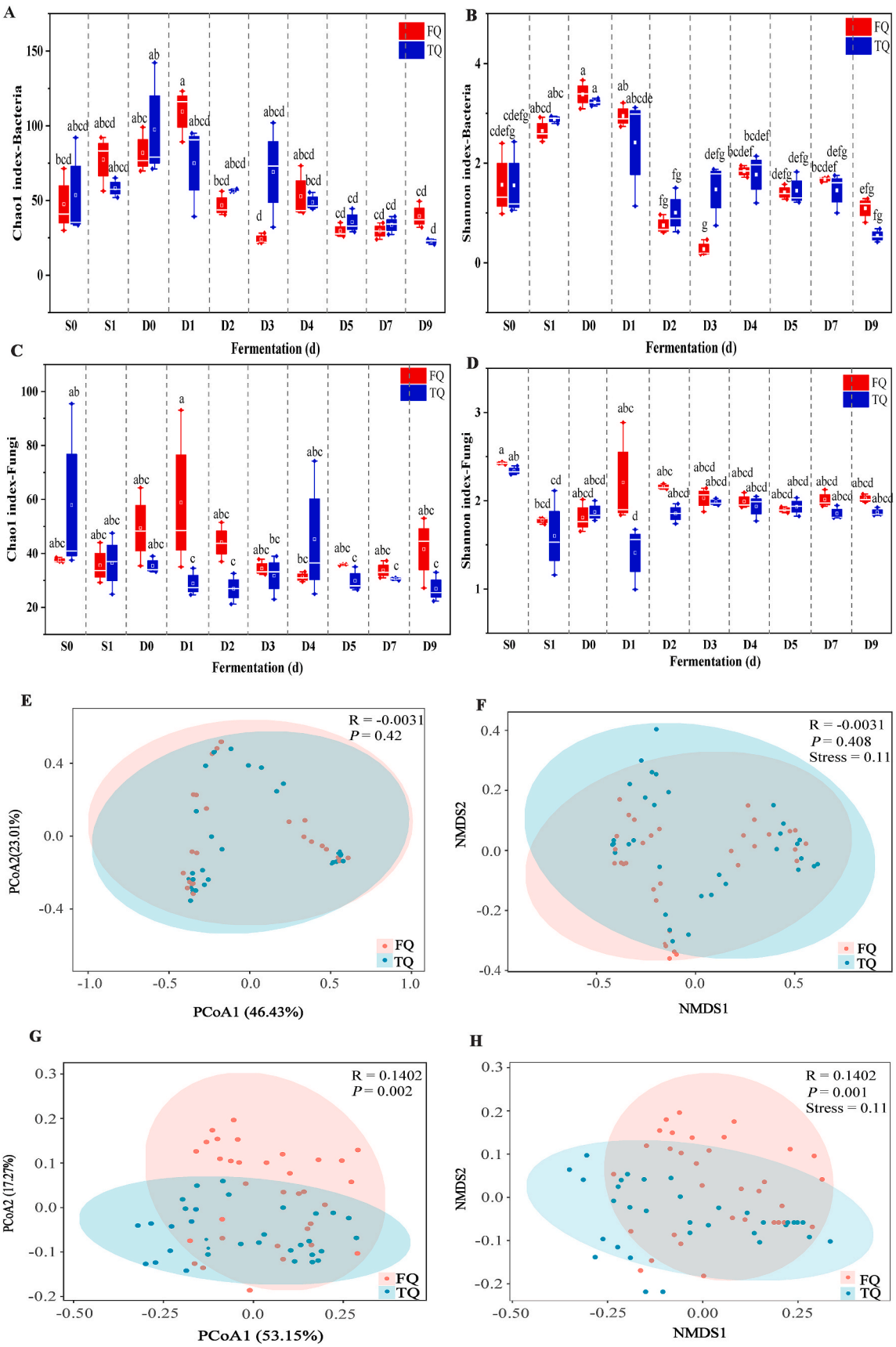

Fig. 3. Microbial community diversity difference in FQ and TQ groups. (A) The bacterial Chao 1 index; (B) The bacterial Shannon index; (C) The fungal Chao 1 index; (D) The fungal Shannon index; (E) The bacterial PCoA analysis; (F) The bacterial NMDS analysis; (G) The fungal PCoA analysis; (H) The fungal NMDS analysis.

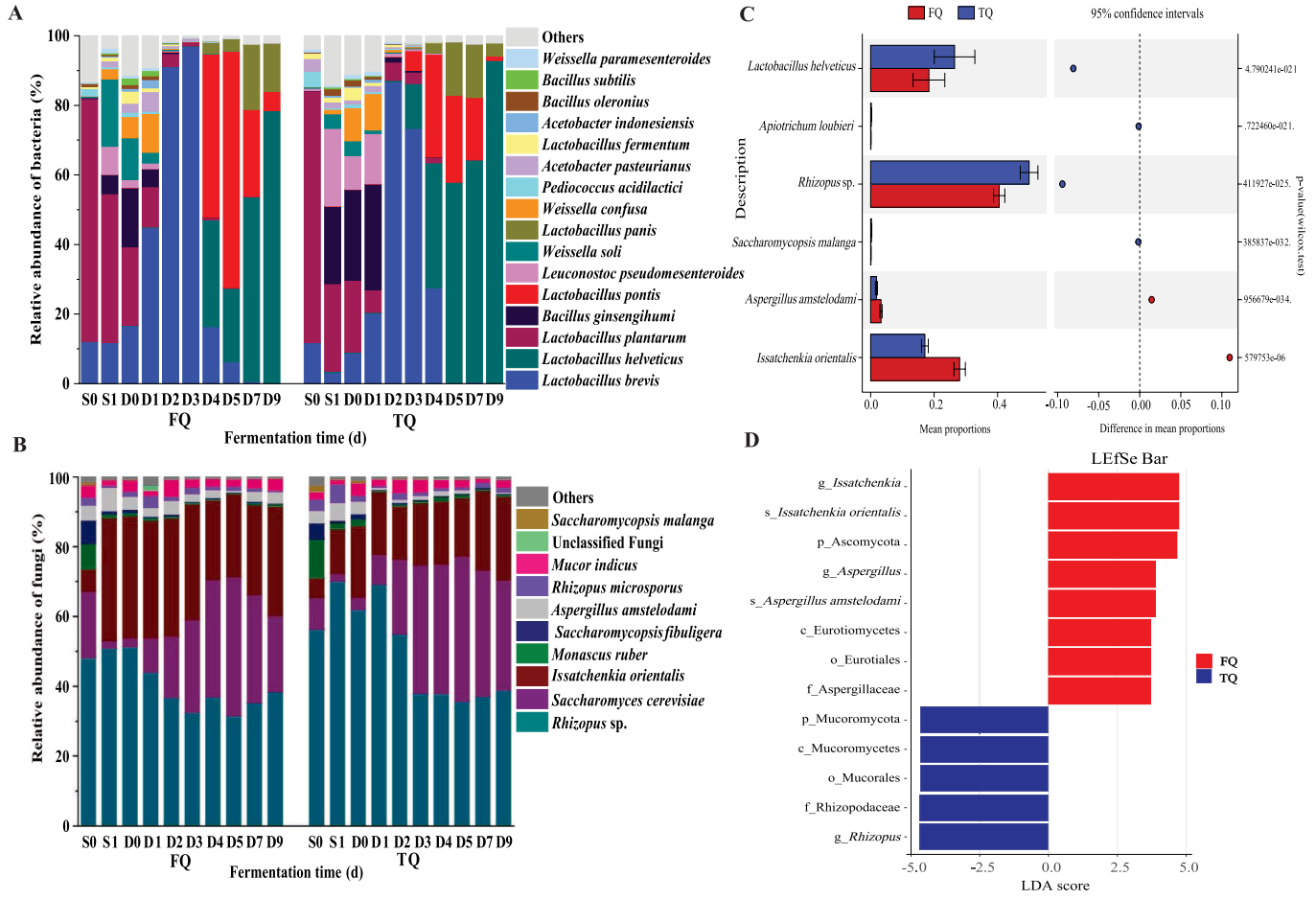

Fig. 4. The dynamics of bacterial (A) and fungal (B) communities at the species level in FQ and TQ groups. (C) Dominant species (relative abundance >1%) were compared. Data are represented as differences in mean proportions, calculated by the Wilcoxon rank-sum test between FQ group and TQ group, with 95% confidence intervals. Only microbial species with $P < 0.05$ are displayed. (D) Indicator fungi with LDA >4 in microbial communities (phylogenetic levels from phylum to genus associated with FQ and TQ). Different colored regions represent different constituents (red, FQ; blue, TQ).

0.7).

3.6. Interaction of microbial community and their relationship with differential flavor compounds

The network analysis based on Spearman's rank correlation ($|\rho| > 0.6$, $P < 0.05$) was preformed to examine the interactions among microbial dominant species (relative abundance >1%). The same microorganisms exhibited different associations when using different types of *Jiuqu* (Fig. 7A and B). For instance, *I. orientalis* showed significant correlations with some microorganisms in FQ group, while it had a negative association with *A. pasteurianus* in TQ group. In addition, *Rhizopus* sp. with a higher relative abundance in TQ group showed a stronger positive correlation with other microorganisms compared to FQ group.

142 co-occurrence networks including 48 positive and 94 negative ones were constructed between 24 microorganisms (8 fungal species and 16 bacterial species) and 11 important differential volatiles in FQ group (Fig. 7C). In TQ group, 7 important differential flavor compounds and 23 microorganisms (8 fungal species and 15 bacterial species) were found to form 60 co-occurrence networks including 28 positive and 32 negative ones (Fig. 7D). Among which, the abundances of *I. orientalis* and *La. helveticus* were significantly positively correlated with ethyl acetate ($P < 0.05$, $|\rho| > 0.6$). The relative abundance of *Rhizopus* sp. had a negative correlation with the content of ethyl acetate ($P < 0.05$, $|\rho| > 0.6$). *A. amstelodami* showed a significantly positive association with ethyl nonanoate ($P < 0.05$, $\rho = 0.70$) in FQ group, while it was negatively correlated with ethyl acetate and positive correlation with decanal ($P < 0.05$, $|\rho| > 0.6$) in TQ group. *A. loubieri* and *S. malanga* only showed positive correlations with decanal in TQ group.

4. Discussion

Recently, functional microorganisms have been applied for *Jiuqu* bioaugmentation to improve the Baijiu quality and reduce the presence of toxic and harmful substances in Baijiu. Fortified *Jiuqu* is typically inoculated into raw grains using functional microbial liquid or solid agents, and then molded, naturally fermented, and stored for later use. Several studies have shown that fortified *Jiuqu* can optimize the microbial community structure and metabolic function, leading to changes in Baijiu brewing (Zhang et al., 2023, 2024a). And the preparation procedure of fortified *Jiuqu* is relatively simple, which is suitable for Baijiu production enterprises. However, the mechanism of functional microorganisms on the fermentation performance of Baijiu remains unclear. The objective was to explore the application of high-yielding glucoamylase indigenous yeast (Table S1) to enhance the fermentation process to drive the microbial community succession and its impact on flavor metabolism, which will provide important references for the application of functional microorganisms in the actual industrial production of Baijiu to improve the quality of Baijiu.

The original Baijiu produced from two types of *Jiuqu* at industrial scale in this study has been evaluated by well-trained panelists (5 males and 5 females, 30 years old on average). Sensorial evaluation scores of the original Baijiu brewed by FQ was higher than that by TQ (Table S2). Wherein, the original Baijiu in FQ group showed a higher aroma and

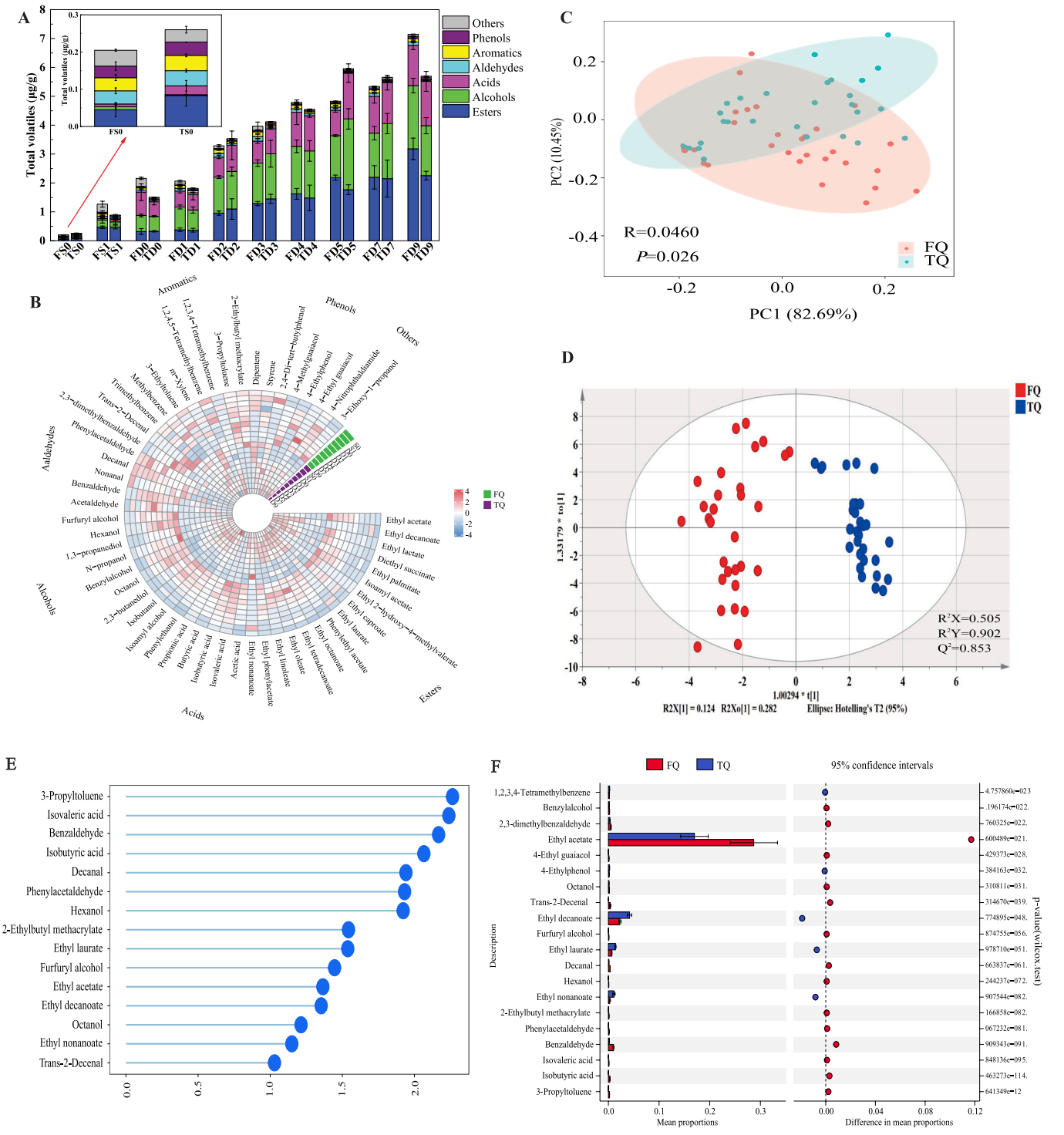

Fig. 5. The volatile flavor composition classification histogram (A), circo heatmap (B), principal component analysis (C), and OPLS-DA score diagram in FQ group and TQ group (D). Significantly different flavor substances by OPLS-DA analysis (VIP >1) (E). Wilcoxon rank-sum test bar plot on volatiles showing the significant differences in FQ group and TQ group (F).

there was no significant difference in other evaluation indicators. As shown in Fig. S1, the original Baijiu was characterized by a fresh fragrance, mellow and soft sweetness, and crisp aftertaste, while the original Baijiu produced by fortified *Jiuqu* exhibited a prominent ester fragrance. Further quantitative detection results indicated that functional strain fortified into *Jiuqu* significantly increased the ethyl acetate content in the original Baijiu. Ethyl acetate is the major characteristic aroma substance in light-flavor Baijiu and properly increasing its content can improve the Baijiu quality. Previous studies indicated that *W. anomalus* played a key role in ester synthesis, contributing significantly to the formation of ethyl acetate during the food fermentation, and biofortification with *W. anomalus* could increase the ester content in Baijiu (Wang et al., 2020; Li et al., 2024a; Liu et al., 2022; Fan et al., 2019). Our results showed that bioaugmentation with *S. fibuligera* could increase the content of ethyl acetate in light-flavor Baijiu. This was probably because that *S. fibuligera* could produce various high-activity enzymes such as amylase and glucoamylase and use carbohydrates in the fermentation substrate to improve the abundance of volatile

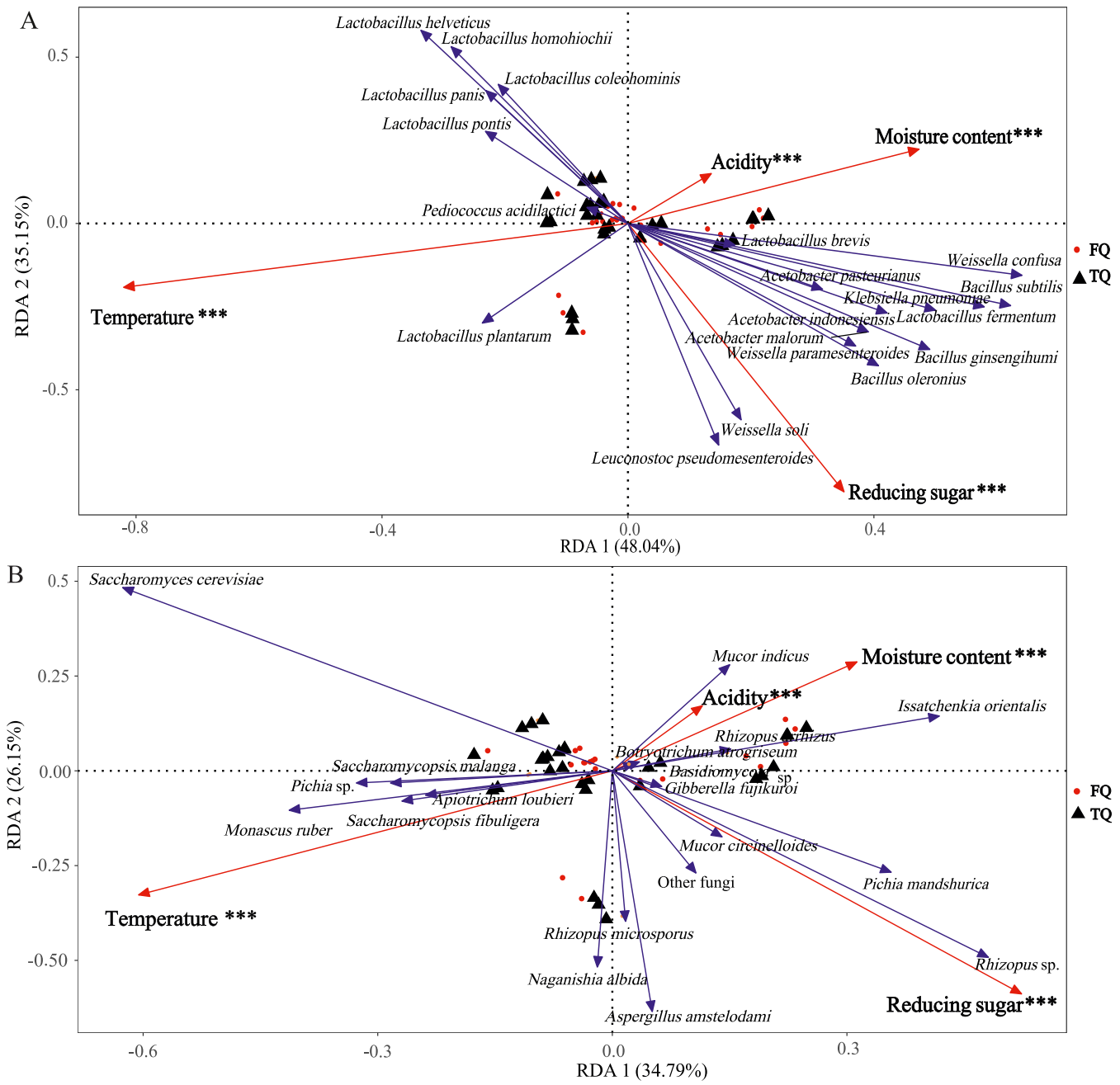

Fig. 6. Redundancy analysis of major bacterial (A) and fungal species(B) (relative abundance >1%) in FQ group and TQ group with respect to temperature, moisture, acidity, and reducing sugar. Mantel test of major bacterial and fungal species (relative abundance >1%) with physicochemical characteristics in FQ group (C) and TQ group (D).

compounds (Yuan et al., 2024).

In spite of the insignificant difference in the bacterial α-diversity and β-diversity of two groups ($P > 0.05$), the fungal α-diversity and β-diversity were significantly different ($P < 0.05$) (Fig. S2). The discrepancy may have been caused by adding *S. fibuligera*. In a previous study, similar microbial diversity was observed during fermentation process fermented with solid-state fortified *Jiuqu* inoculating with *Aspergillus flavus*, *Aspergillus niger*, *S. fibuligera*, and *PaeniBacillus* sp. (Wang et al., 2024). Su et al. (2020) found that bioaugmentation inoculating with *S. fibuligera* and *S. cerevisiae* had no significant effect on bacterial diversity. Similarly, *Daqu* fortified with *S. cerevisiae*, *Hyphopichia burtonii*, and *Clavispora lusitaniae* did not result in significant differences in bacterial community structure and significantly altered the fungal community compared to the unfortified *Daqu* (Li et al., 2020). Our results were line with these results, which might be due to the minimal impact of adding fungi on the bacterial community in *Jiuqu*.

Interestingly, the relative abundance of *S. fibuligera* did not significantly increase by bioaugmentation with *S. fibuligera*, while the relative abundance of *I. orientalis* significantly increased in FQ group. This was similar to the previous finding that *I. orientalis* could improve the proliferation of ester-producing yeast (Guan et al., 2023). *I. orientalis*, also known as *Pichia kudriavzevii*, is one of the important aroma-producing yeast in the solid-state fermentation of Baijiu (Xiao et al., 2023; Kurtzman and Robnett, 2003; You et al., 2021). Besides, it can effectively produce ethanol from fermented substrates due to its highly tolerance in acidic and high-temperature environment during fermentation (Li et al.,

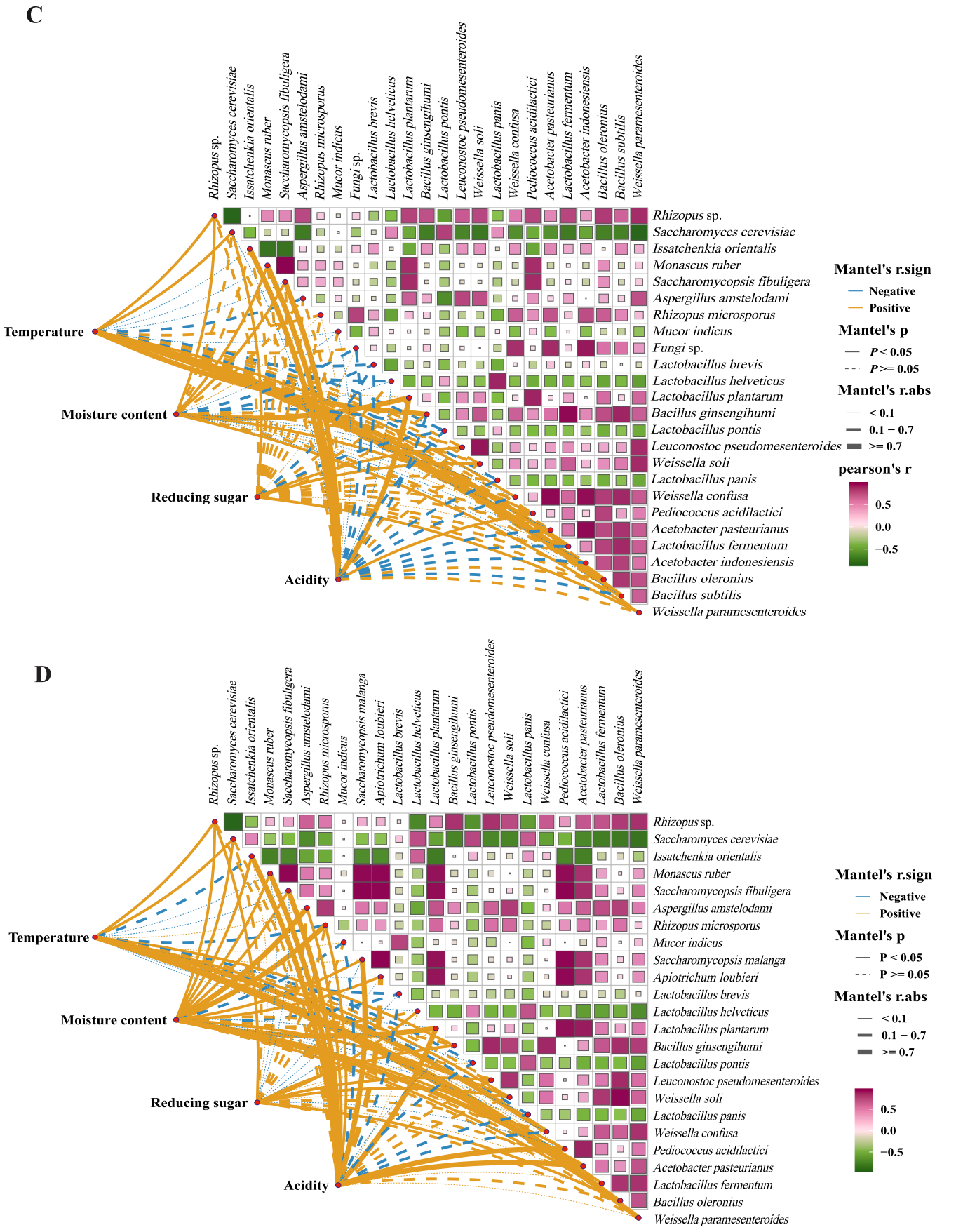

Fig. 6. (*continued*).

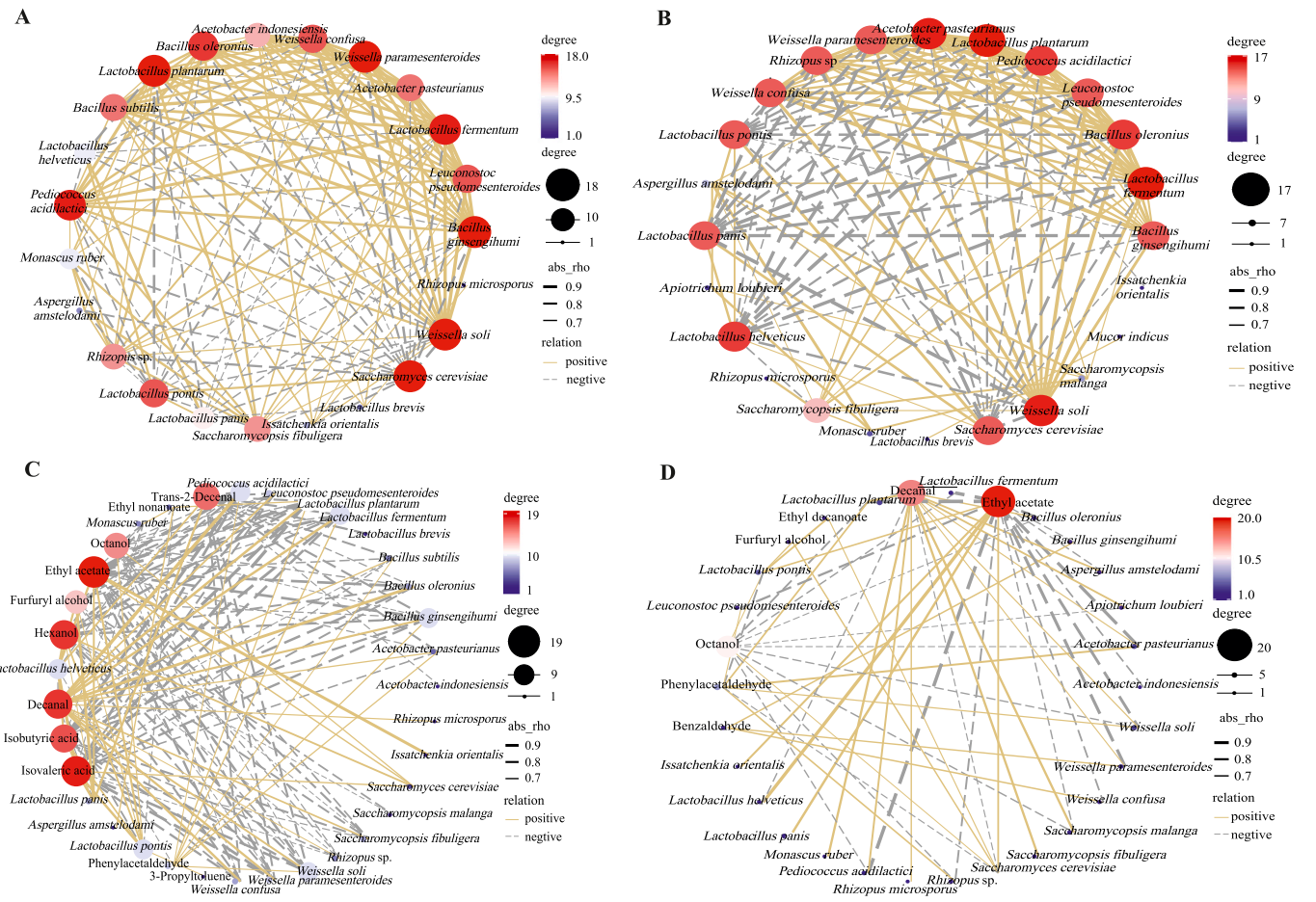

Fig. 7. Correlation analysis of microbial community in FQ group (A) and TQ group (B), major microbial species and important differential flavor compounds in FQ group (C) and TQ group (D).

2022b; Zhang et al., 2021; Lee et al., 2022). Compared to TQ group, the relative abundance of *Rhizopus* sp. significantly reduced in FQ group. Similarly, the abundance of *Rhizopus* sp. was much lower in the enhanced starter group (inoculating with *S. fibuligera* and *S. cerevisiae*) compared with that of the non-enhanced starter group (Su et al., 2020). *I. orientalis* and *Rhizopus* were the principal fungal biomarkers of FQ group and TQ group, respectively, indicating that these microorganisms might exert an influence on the quality of the fermentation process for different groups. Moreover, *Rhizopus* sp., *S. cerevisiae*, and *I. orientalis* dominated during the fermentation and the first two were also the most dominant fungi in the Hakka rice wine fermentation process (Qian et al., 2023).

ANOSIM analysis ($P < 0.05$) showed that there was marked difference in volatile flavor composition of fermented grains between FQ group and TQ group (Fig. 5C). The relative contents of esters and aldehydes were significantly higher in FQ group than those in TQ group ($P < 0.05$) (Fig. S3). The quality of Baijiu is determined by the amount and proportion of esters, which can generate the basic features of Baijiu, comprising flavor, aroma, grade, and style (Guan et al., 2023). Among them, ethyl acetate was the most abundant and significantly different important flavor compounds by OPLS-DA (VIP >1) and Wilcoxon rank-sum test. Bioaugmentation promoted the production of ethyl acetate, and its content was significantly higher in fermented grains of FQ than that in TQ ($P < 0.05$). The fermented grains are distilled to obtain the original Baijiu, so the flavor characteristics of Baijiu are closely linked to the volatile constituents of fermented grains (Liu et al., 2023). Thus, the high content of ethyl acetate in fermented grains of FQ led to a higher content of ethyl acetate in the original Baijiu. Additionally, 12 important differential flavor substances with higher contents in FQ group were observed, implying that biofortification of Baijiu fermentation could contribute to the high production of flavor metabolites (Wang et al., 2024; Qiu et al., 2024; Guan et al., 2023; He et al., 2020).

In this study, temperature, moisture, and acidity were identified as the main driving factors of microbial succession during Baijiu fermentation. Driven by physicochemical characteristics, totally 9 and 13 pairs of significant positive correlations ($P < 0.05$, $\rho > 0.7$) were identified from the microbial community in FQ group and TQ group, respectively (Table S3). Among them, the most significant is that 6 pairs of significant positive correlations (66.67%, $P < 0.05$, $\rho > 0.7$) existed in the bacterial community in FQ group, and the prominent significant was still the positive associations between bacteria (7 pairs, 53.84%, $P < 0.05$, $\rho > 0.7$) in TQ group. Compared to the fungal community, the bacterial community were more easily affected by environmental factors, which was similar to the reported results by Lu et al. (2024). Acidity was the most important regulatory factor in TQ group, significantly impacting 8 microbial species, while acidity and temperature regulated both 4 microbial species in FQ group ($P < 0.05$, $|\rho| > 0.7$). Among which, acidity showed a highly positive correlation with dominant biological marker (*I. orientalis*). A previous study stated a significantly positive relationship between acidity and *Issatchenkia* in biofortification group (Qiu et al., 2024). Acidity was sharply ($P < 0.05$) increased during saccharification stage, and then remained a slight increase, which was in consistent with the results of previously study (Qian et al., 2023).

Microbial interactions typically happen in the microbial community and significantly affect microbial succession and metabolism (Luo et al., 2023; Tan et al., 2019). Compared to TQ group, the relationships of microorganisms in FQ group were more complicated. A few substantial associations between *S. fibuligera* and more microorganisms such as *La.*

pontis, *L. pseudomesenteroides*, *W. paramesenteroides*, *B. ginsengihumi*, *Rhizopus* sp., and *S. cerevisiae* were found in FQ group, suggesting that biofortification may be beneficial for enhancing microbial interactions. Bioaugmented *Daqu* by inoculating *Bacillus velezensis* and *Bacillus subtilis* contributed to the enhanced interspecies interactions (Mu et al., 2023). Furthermore, *I. orientalis* (fungal biomarker) were only negatively correlated with *A. pasteurianus* in TQ group, while it showed significant positive correlations with more microorganisms after biofortification. 105 and 83 significant positive relationships between microorganisms were constructed in FQ group and TQ group, respectively. This indicated an increase in positive interactions and the complexity of interactions between microorganisms after biofortification. And the overall network structure of microbial community in microecosystems has undergone changes (Li et al., 2024a).

Correlation analysis showed that ethyl acetate in the fermented grains was positively correlated with *I. orientalis* and had a significantly negative association with *Rhizopus* sp. (Fig. 7C and D). This explained why there was a high content of ethyl acetate in FQ group. The increase of the *I. orientalis* abundance and the decrease of *Rhizopus* sp. abundance increased the content of ethyl acetate in FQ group. *S. fibuligera* showed a negative partnership with *S. cerevisiae*, while *S. cerevisiae* was significantly negatively correlated with *I. orientalis* in FQ group. Consequently, bioaugmentation with *S. fibuligera* contributed to the increase in the abundance of *I. orientalis*. It has been reported that *P. kudriavzevii* (*I. orientalis*) and *S. cerevisiae* showed a synergistic reaction to organic acid stress in the fermentation of Baijiu, which greatly improved microbial viability and the probability of using acid to generate esters (Deng et al., 2020).

5. Conclusion

This study comprehensively investigated the effects of bioaugmented *Jiuqu* on the dynamics of physicochemical factors, microbial community structure and diversity, and volatile compound profiles during LFB fermentation. The findings revealed that inoculating *S. fibuligera* effectively enhances key flavor metabolites. Biofortification with *S. fibuligera* markedly reshaped the fungal community, strengthened the interspecies interaction, and facilitated ethyl acetate biosynthesis. Microbial correlation analysis showed that ethyl acetate was significantly positively related to *I. orientalis* ($P < 0.05$, $|\rho| > 0.7$), which was driven by the functional *S. fibuligera*. These results demonstrated that regulating the microbial community based on bioaugmented *Jiuqu* is conducive to improving the community stability and flavor characteristics, which are helpful to promote the application of functional microorganisms and provide insights for the rational design of the initial community in Baijiu fermentation.

CRediT authorship contribution statement

Jie Tang: Investigation, Methodology, Data curation, Visualization, Funding acquisition, Writing – original draft. **Bin Lin:** Data curation, Writing – original draft. **Yimin Shan:** Data curation, Methodology. **Gang Zhang:** Investigation, Methodology, Data curation. **Liping Zhu:** Investigation, Data curation. **Wei Jiang:** Investigation, Data curation. **Qun Li:** Investigation, Data curation. **Lei Zhang:** Resources, Supervision, Validation. **Shengzhi Yang:** Resources, Supervision, Validation. **Qiang Yang:** Funding acquisition, Resources, Supervision. **Shenxi Chen:** Conceptualization, Supervision, Writing – review & editing. **Hai Du:** Resources, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jie Tang reports financial support was provided by Natural Science Foundation of Hubei Province (2023AFD026). Qiang Yang reports financial support was provided by Hubei Provincial Key Research and Development Program (2023BBB004). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2025.101008.

Data availability

Data will be made available on request.

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