



Spatial heterogeneity of microbiota and flavor across different rounds of sauce-flavor baijiu in Northern China

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ABSTRACT

Sauce-flavor baijiu (SFB) is a traditional Chinese distilled liquor crafted through a distinctive brewing process, involving seven rounds of stack fermentation (SF) and pit fermentation (PF). To date, there remains a knowledge gap regarding the microbial succession and flavor throughout all rounds of SFB with distinctive northern characteristics. Through LEfSe analysis, *Saccharopolyspora*, *Virgibacillus*, *Thermoascus* and *Thermomyces*, and *Lactobacillus* and *Issatchenkia* were found to be the most differentially representative genera in SF and PF, respectively. A total of 93 volatile flavor compounds were found in base baijius through the gas-chromatography mass spectrometry. Moreover, 29 volatile flavor substances with significant difference in base baijius of different rounds were revealed using the OPLS-DA model and VIP values and Spearman correlation analysis shows that bacteria have a greater impact on differential flavor compounds than fungi. This study provides a new perspective and insight into the brewing of northern SFB.

Introduction

Sauce-flavor baijiu (SFB) is a distinctive style of Chinese traditional fermented liquor, renowned for its characteristic roasted aroma resembling soy sauce, endowed by the intricate brewing technique employed and the complex microbial consortia involved in the fermentation process (Wang et al., 2021c). The production of SFB (Fig. S1) is a year-long process that comprises of seven rounds of stack fermentation (SF) and pit fermentation (PF). SF is an open process primarily aimed at recruiting microorganisms from the surrounding environment to synthesize sauce-flavor substances and precursors under the action of a variety of enzymes, which serves as a critical foundation for the subsequent fermentation process that takes place in the pit. PF represents the primary stage of baijiu fermentation, where significant transformations occur in microbial communities and their metabolic activities. Upon completion of PF, the fermented grains are subjected to distillation to yield seven base baijius, each possessing a distinct flavor profile corresponding to the respective fermentation round. The production process of SFB exhibits complexity, reflecting distinct traditional brewing

characteristics. The intricate craftsmanship involved in its production results in an exquisite aroma, a rich taste, and an enduring aftertaste (Duan et al., 2022).

Although Southern China is the main production area of SFB, other regions such as Beijing, Tianjin, and Shandong, etc., also produce SFB with distinctive northern regional characteristics, which has also aroused great interest of researchers. During the first to third rounds of PF in Beijing region, a notable succession in bacterial dominance is observed, transitioning from *Virginacillus* and *Oceanobacillus* to *Lactobacillus* as the fermentation process advances. Concurrently, the fungal composition is primarily characterized by *Issachenkia*, *Byssochlamys* and *Saccharomyces* (Xu et al., 2023a). Wang et al. conducted a comparative analysis of the microbial composition of the fifth and sixth rounds of SF and PF in the Beijing area (Wang et al., 2021c), revealing that the dominant bacteria in both rounds were *Lactobacillus*, *Oceanobacillus* and *Virgibacillus*. Moreover, it was observed that *Lactobacillus* exerted a dominance in the first, fourth, and seventh rounds of PF in Tianjin area. In addition, during the SF of the first to third rounds of SFB in Shandong Province, the core bacteria were *Lactobacillus*, *Acetobacter* and *Weissella*,

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and the core fungi were *Issatchenkia*, *Monascus* and *Pichia* (Zhang et al., 2023). These studies have provided preliminary understanding of microbial composition involved in the fermentation of northern SFB. However, the specific patterns of microbial succession and variations throughout the entire SFB fermentation process of both SF and PF in all rounds of northern area remain unexplored.

Microbial metabolism during the fermentation process lays an important foundation for shaping the flavor profile of the base baijiu (Duan et al., 2022). Some key flavor esters in Baijiu, such as ethyl acetate, showed significant positive correlation with *Lactobacillus*, *Saccharomyces* and *Byssoschlamys*, while benzoic acid ethyl ester was positively correlated with *Zygosaccharomyces* (Shen et al., 2022). In the fifth and sixth rounds of SFB fermentation in Beijing area, *Cladosporium* and *Diutina* were positively correlated with the biosynthesis of a series of ester substances (Wang et al., 2021c). Conversely, the contribution of bacteria to ester synthesis was relatively insignificant, with some bacteria even displaying a negative impact on ester synthesis (Xu et al., 2023a). Moreover, the investigation unveiled a noteworthy disparity wherein the sauce flavor in SFB produced in the northern region is comparatively less pronounced than its southern counterpart, which may be related to the contributions of microorganisms to the formation of flavor compounds during the fermentation process. Consequently, a more detailed exploration of the flavor composition and differences and relationship between flavor substances and the microorganisms involved in the fermentation process is imperative.

In this study, a comprehensive analysis comparing microbial succession and the impact of physicochemical factors across all rounds of SF and PF of SFB in northern China was represented for the first time. Additionally, the investigation delved into the composition and variances of flavor compounds in different rounds of the base baijiu, and the correlations between microorganisms and flavor profiles were analyzed. The present study provides valuable insights into the complex fermentation process of northern SFB, unravels the enigma surrounding its year-long fermentation, and helps to further understand the formation of the distinctive flavor.

2. Materials and methods

2.1. Reagents

Mass spectrometry grade sulfuric acid and sodium chloride were purchased from Merck (Darmstadt, Germany). Ethanol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Deionized water was purchased from a Millipore Elix 15 water purification system (Merck Millipore, Darmstadt, Germany). 2-methyl-2-butanol, acetic acid and lactic acid standard were purchased from Sigma Aldrich Chemical Co. (Steinheim, Germany). All other chemicals are analytical grade which from Thermo Fisher (Waltham, MA, USA).

2.2. Experimental design and sample collection

The experiment was carried out in a well-known SFB distillery (Beijing, China). The fermentation process was carried out in two distinct phases: SF and PF. Sampling was conducted throughout the complete production cycle in 2021, encompassing a total of seven fermentation rounds. The sampling period of the SF is 1 day, and the sampling points of the stacking are shown in Fig. S2A. The ABC points are respectively located in the inner, middle and outer layers of the same plane as the upper layer of SF, the DEF points are respectively located in the inner, middle and outer layers of the same plane as the stacking midpoint, and the GHI points are respectively located in the inner, middle and outer layers of the same plane as the lower layer of SF. Samples from points A, B, C, D, E, F, G, H, and I at the same stacking time in the same round were mixed evenly to reduce the heterogeneity, and then the mixed samples from the same round with different stacking times were mixed again, which was recorded as the SF samples for that

round. The sampling period of the PF are 5 days, and the sampling points are shown in Fig. S2B. Sampling points A, B, C, D, E, F, G, H, and I in the pit correspond to each location of SF. Consistent with SF, samples from points A, B, C, D, E, F, G, H, and I at the same PF time in the same round were mixed evenly to reduce the heterogeneity, and then the mixed samples from the same round with different PF times were mixed again, which was recorded as the PF samples for that round. After 30 days of PF, the fermented grains were distilled and the distillates (base baijiu) were collected for further analysis. Hence, we collected 21 samples of base baijiu from 7 rounds, 21 samples of stack fermented grains and 21 samples of pit fermented grains.

2.3. Fermentation parameters detection and analysis

To understand the SF and PF processes of different rounds, eight fermentation parameters, including starch, moisture, pH, titratable acidity, reducing sugar, ammonia nitrogen, acetic acid, and lactic acid were measured. The starch content of fermented grains was determined according to the international standard methods (ISO 5377). The moisture content, pH, and ammonia nitrogen were determined as described previously (Shen, 2007). Titratable acidity and reducing sugar were monitored by the method described by Tan et al. (Tan et al., 2019). Acetic acid and lactic acid were analyzed via high-performance liquid chromatography (HPLC, Waters 2695, Milford, U.S.A.) (Wang et al., 2018).

2.4. Volatile flavors compounds analysis

The base baijiu samples were subjected to dilution with redistilled water to achieve an ethanol content of 10 % (v/v). Subsequently, 10 mL of the diluted base baijiu, 3.0 g of NaCl, and 10 μ L of 2-methyl-2-butanol (utilized as the internal standard at a final concentration of 8.09 mg/L) were transferred into a 20 mL headspace vial. The vial contents were subjected to agitation at 50 °C and 280 r/min for a duration of 5 min. Volatile flavors in the samples were extracted using a DVB/CAR/PDMS fiber (50/30 μ m, Supelco, Bellefonte, PA, USA). Analysis of the volatile chemicals types and relative concentrations was performed using a Thermo Fisher headspace solid-phase microextraction gas chromatography-mass spectrometer (HS-SPME-GC-MS) TSQ 8000 Evo instrument (Thermo Fisher Scientific, Waltham, MA, USA). The instrument was equipped with a flame ionization detector and a DB-WAX column (60 m \times 0.25 mm \times 0.25 μ m, J&W Scientific, Folsom, CA, USA). The column temperature was initially set at 40 °C and maintained for 5 min. Subsequently, it was increased to 100 °C at a rate of 5 °C/min and held for 10 min. Following this, the temperature was further increased to 150 °C at a rate of 5 °C/min and held for 10 min. Finally, the temperature was raised to 250 °C at a rate of 5 °C/min and maintained for 15 min. Helium gas was employed as the carrier gas at a flow rate of 1 mL/min. MS analysis was performed using an electron impact with an ionization energy of 70 eV. The scan range for MS data acquisition was set from 30 to 400 amu. Identification of flavor chemicals was accomplished by comparing the obtained MS spectra with the NIST05 database.

2.5. Total DNA extraction, bacterial 16S rRNA gene amplicon and fungal ITS amplicon sequencing

Sample treatment and DNA extraction were performed according to the previously described method (Wang, Huang, et al., 2021). The bacterial 16S rRNA genes were amplified through PCR with the primer pair 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGAC-TACHVGGGTWCTAAT-3'). The fungal 18S rRNA genes were amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTCTTCATCGATGC-3'). The sequencing of PCR amplification was carried out on Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA). High-quality sequences were

obtained by eliminating chimeric sequences, and subsequently clustered into operational taxonomic units (OTUs) using UPARSE version 7.11 with a 97 % similarity cutoff. The taxonomic classification of each 16S rRNA and 18S rRNA gene sequence was performed using the RDP Classifier against the 16S rRNA and 18S rRNA database, respectively, at a confidence level of 70 % (Li et al., 2016). The sequencing data has been uploaded to NCBI Short Read Archive and the accession number is PRJNA994278.

2.6. Statistical analysis

All assays were conducted in triplicate and the results were presented as mean with standard deviations. Principle Coordinate Analysis (PCoA) was used to assess the ecological distances between different samples, employing unweighted UniFrac distances as the basis for comparison. The Spearman correlations were calculated using SPSS V22.0 (IBM, U.S. A) and visualize using TBtools. The statistical significant differences between SF and PF were processed using one-way analysis of variance (ANOVA) in SPSS ($p < 0.05$, Duncan's test).

Multivariate statistical analyses on volatile flavors were performed with the SIMCA-14.1 software (Umetrics, Sweden). Terms with large variable importance in the projection (VIP) > 1, are the most relevant for explaining the difference. And Spearman correlations between microbiota and flavors were calculated using IBM SPSS. Correlation coefficient with P -value < 0.05 between microbiota and flavors was visualized via Cytoscape (v3.9.1).

3. Results and discussions

3.1. Dynamics of physicochemical characteristics during the stack fermentation and pit fermentation process

During the fermentation process of SFB, physicochemical indicators such as starch content, moisture, ammonia nitrogen, total acidity, and reducing sugars change continuously with the progress of fermentation, which affects the metabolism of microorganisms and the formation of flavor compounds. Starch is not only the main energy source for microbial growth of baijiu brewing process, but also the key substance for alcohol production. During the seven rounds of SF and PF, there was an overall decreasing trend in the starch content of the fermented grains (Fig. 1A). This may due to that along with the fermentation, starch was gradually decomposed by amylase and further utilized. Reducing sugar in fermented grains is hydrolyzed from starch and other polysaccharides and used for microbial metabolism and flavor production. On the basis of SF, reducing sugar was further utilized in each round of PF, resulting in a significantly lower content of reducing sugar in PF than in SF (Fig. 1B). Moreover, as starch was gradually decomposed into reducing sugar, the reducing sugar content showed an obvious trend of increasing during the first to third round of SF, and then decreased due to microbial consumption. The acids in fermented grains were mainly produced by bacterial metabolism and could provide precursors for the synthesis of various esters (Chen et al., 2022). From the first to fifth round, acidity in fermented grains increased gradually and was significantly higher in PF than in SF (Fig. 1C), which was associated with a higher abundance of

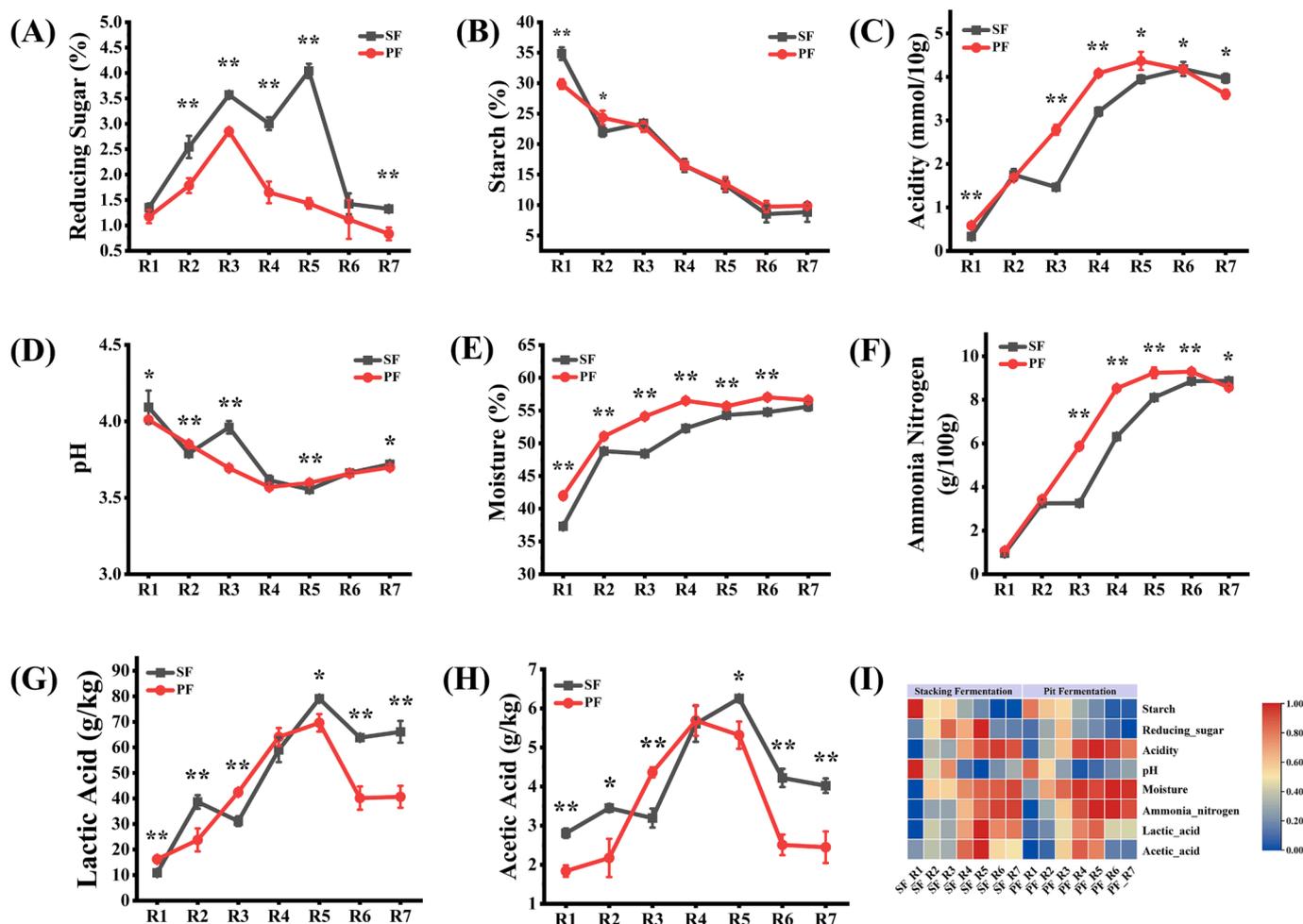


Fig. 1. The changes of physicochemical properties of fermented grains during the fermentation (A: reducing sugar, B: starch, C: acidity, D: pH, E: moisture, F: ammonia nitrogen, G: lactic acid, H: acetic acid). The heatmap regarding the changes in physicochemical factors during the fermentation (I).

acid-producing bacteria (Fig. 2B). Accordingly, the pH in different rounds of SF and PF showed a trend of first decreasing and then increasing (Fig. 1D). During the seven rounds of SF and PF, the overall moisture content showed a fluctuating upward trend, and the moisture content of fermented grains in PF under anaerobic environment was significantly higher than that in the open SF (Fig. 1E). The protein present in the raw material is metabolized by microorganisms to generate ammonia nitrogen, which serves as a direct nitrogen source that is required for the growth, reproduction, and metabolism of brewing microorganisms (Cai et al., 2021). Throughout the first to seventh rounds of fermentation, the content of ammonia nitrogen in the fermented grains exhibited an overall increasing trend. It was observed that the concentration of ammonia nitrogen was significantly higher in PF compared to the SF (Fig. 1F). In the fermentation process, lactic acid and acetic acid are important organic acids with high contents. From the first to seventh round, the contents of lactic acid and acetic acid showed an initial increase and a subsequent decrease (Fig. 1G, H). This may be attributed to the rapid accumulation and metabolism of acid-producing bacteria in the early stage of fermentation. Subsequently, organic acids react with ethanol to produce ethyl ester flavor substances in the later stage of fermentation (Yang et al., 2023).

3.2. Microbial diversity and dynamics during the stack fermentation and pit fermentation process

High-quality sequencing reads were classified at different taxonomic levels to investigate the microbial diversity and dynamics during the process of SF and PF. At the phylum level of bacteria, phylum Firmicutes was found to be dominant in both SF (relative abundance 68.15 %–83.48 %) and PF (66.40 %–99.47 %) (Fig. 2A). At the genus level, a total of 345 and 296 bacterial genera were detected in SF and PF, respectively. In SF, the genus *Virgibacillus* exhibited the highest abundance in the first to third rounds, followed by the genus *Oceanobacillus* (Fig. 2B). During fourth round to seventh round, there was a decrease in the relative abundance of *Lactobacillus*, from 34.23 % to 12.46 %, whereas the abundance of *Virgibacillus* exhibited a gradual increase, reaching 25.79 %. In PF, *Lactobacillus* was dominant in the first to third rounds, with an abundance of 79.90 %, 74.64 %, and 92.15 %, respectively (Fig. 2B). In the fourth round, it decreased to 33.17 %, while the abundances of *Oceanobacillus* and *Virgibacillus* increased to 16.56 % and 15.45 %, respectively. In the fifth to seventh rounds, the abundance of *Virgibacillus* decreased, while the abundances of *Lactobacillus* and *Rhodococcus* increased to 29.56 % and 29.78 %, respectively. The findings above shows that *Lactobacillus* is the main bacterial genus in the brewing process of SFB in Beijing region. Moreover, *Lactobacillus* is also the dominant bacterial genera in SFB of other cities in northern China (Wang et al., 2022). In contrast, the brewing process of Maotai Town in the southern region unveils a distinct microbial profile, characterized not only by the dominance of *Lactobacillus* but also by a significant presence of *Acetobacter*, playing an integral role in various metabolic pathways associated with the utilization of amino acids, terpenoids, and polyketides (Yang et al., 2023).

PCoA was performed to compare the differences in the bacterial community structure between the samples collected from SF and PF. As shown in Fig. 2C, the PCoA results based on the Bray-Curtis distance of the bacterial community datasets showed that 67.93 % of the variation in the total bacterial community was explained by the two axes (PCoA1 46.47 % and PCoA2 21.46 %). The bacterial communities of all samples collected from SF and PF were distributed separately, suggesting that the bacterial community participating in SF was clearly distinct from that in PF (Fig. 2C). Alpha diversity indexes were further calculated to evaluate the bacterial richness and diversity varied between SF and PF. As shown in Fig. 2D, similar ACE indexes were exhibited in two fermentation stage. In contrast, a significantly higher Chao index was observed in the SF compared with PF. LefSe analysis was used to analyze significantly different taxa between the microbiota in SF and PF, and 7 different taxa

were found in the SF and 3 in the PF, respectively. The SF had greater proportions of *Bacillales* and *Pseudonocardiales* belong to phylum Firmicutes and Actinobacteriota, respectively, whereas the PF had greater proportions of *Lactobacillales* belonging to Firmicutes (Fig. S3A). At the genus level, the abundance of *Saccharopolyspora* and *Virgibacillus* were much higher in the SF, whereas samples in PF featured a higher abundance of *Lactobacillus*. This is mainly attributed to the distinct roles played by microorganisms during the SF and PF. In SF, microbes primarily focus on the hydrolysis of polysaccharides, facilitating the breakdown of complex carbohydrates. During PF, their primary function shifts towards the production of ethanol and organic acids. For example, *Saccharopolyspora* and *Virgibacillus* have multiple saccharifying enzymes involving amylase, cellulase and hemicellulase, which can degrade starch in raw materials (Xia et al., 2023). *Lactobacillus* has the characteristics of acid resistance and acid production, and because of its facultative anaerobic characteristics, it can occupy a high position in the anaerobic PF process. These key representative bacterial taxa contributed to the differences in the assembly of microbial communities in SF and PF.

In addition to bacteria, fungi also assume a crucial role in the fermentation process, exerting their influence on the development of unique flavors in baijiu. At the phylum level of fungi, Ascomycota was the dominant phylum in both SF and PF, with an abundance of over 98 % (Fig. 3A), which is consistent with the results of previous study (Song et al., 2017). At the genus level, a total of 173 and 162 fungal genera were detected in SF and PF, respectively. In the first round of SF, *Saccharomyces* and *Byssoschlamys* had high abundance, accounting for 33.67 % and 32.19 %, respectively, followed by *Thermomyces* (15.45 %) and *Thermoascus* (7.90 %) (Fig. 3B). In the second round, *Issatchenkia* rapidly increased to become the dominant genus with an abundance of 48.79 %, which is similar with the findings observed in the fermentation processes of baijiu in other northern regions, such as Tianjin (Wang et al., 2022) and Shandong (Zhang et al., 2023).

During the third to seventh rounds, the temperature of fermented grains in SF increased obviously (Li et al., 2022), and *Thermoascus* that has thermotolerance and the ability to produce of numerous thermophilic enzymes, displayed an upward trend in its abundance (Fig. 3B). *Thermomyces* showed an initial increase followed by a subsequent decrease in abundance, while *Byssoschlamys* remained basically unchanged and *Aspergillus* increased slightly. Nevertheless, in the SF process of SFB in southern China, *Candida* emerges as the dominant fungal genus and showcases its capability in esterase production and actively participates in the metabolism of phenylalanine (Jin et al., 2019). Particularly, *Candida* employs the Ehrlich pathway to generate benzene ethanol, an important compound contributing to the distinctive aroma profile of SFB in Maotai town (Wang et al., 2021a). This may be one of the reasons for the difference in flavor between the north and the south. Similar to SF, in the initial round of PF (Fig. 3B), *Saccharomyces* (35.20 %) and *Byssoschlamys* (44.46 %) were found to be the most abundant genera, whereas *Issatchenkia* that produces various esters and aromatic substances emerged as the dominant genus in the second round with an abundance of 86.62 %. In the third round, *Issatchenkia* decreased (49.34 %), while *Byssoschlamys* (17.59 %) and *Monascus* (7.46 %) increased. In the fourth round, the abundance of *Saccharomyces* (25.01 %) and *Thermomyces* (26.61 %) increased and became the major genera. However, in the fifth and sixth rounds, unclassified_o_Saccharomycetales rapidly increased to 84.97 %, and then decreased to 13.70 % in the seventh round. At the end of fermentation, *Issatchenkia* became the dominant genus with an abundance of 65.58 %. These results are similar to those of previous studies (Wang et al., 2021c; Xu et al., 2023a).

Furthermore, the PCoA results of the fungal community datasets showed that 67.93 % of the variation in the total fungal community was explained by the two axes (PCoA1 46.47 % and PCoA2 21.46 %). The fungal communities of all samples collected from SF and PF were distributed separately, suggesting that the fungal community

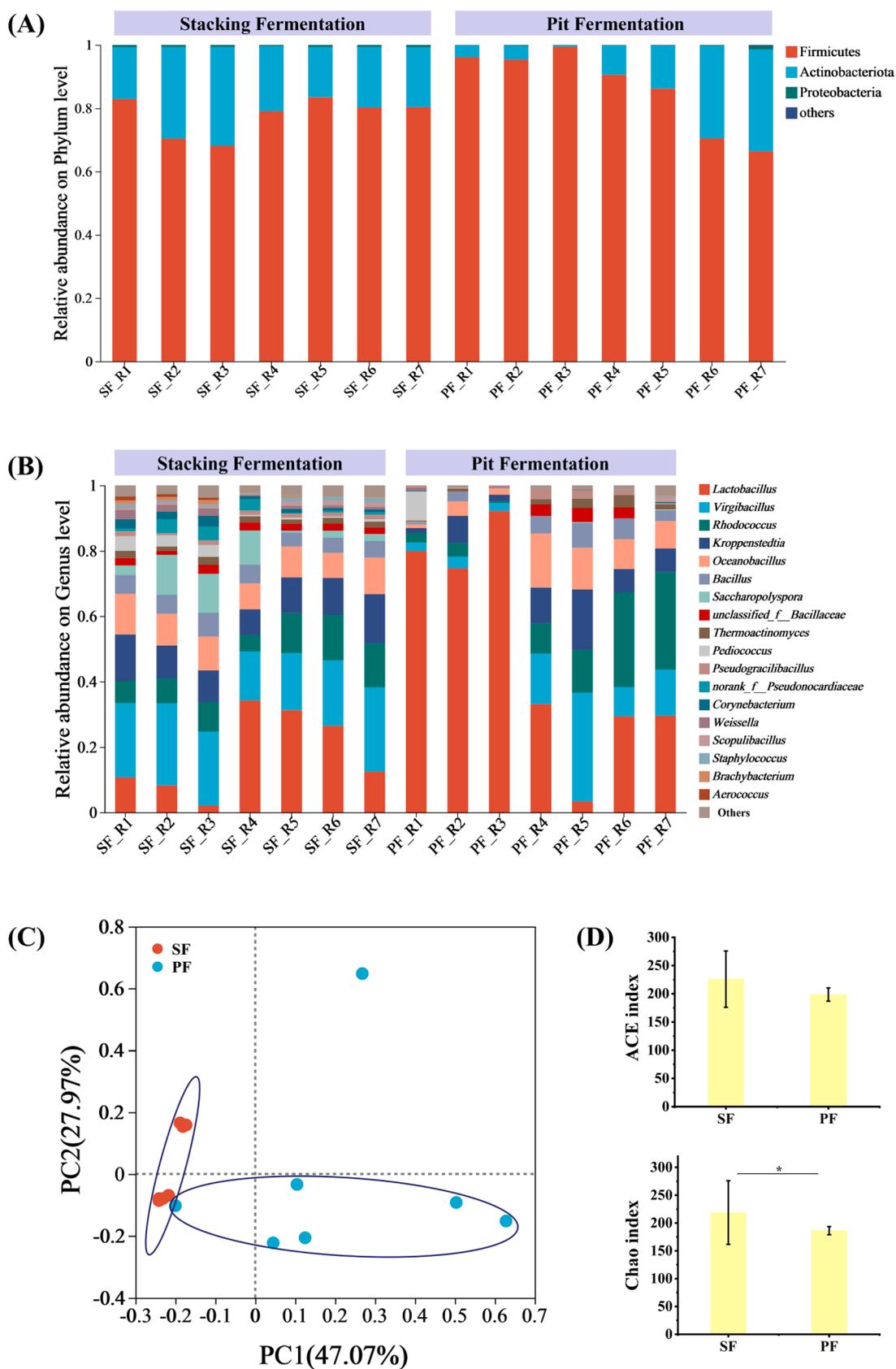


Fig. 2. Comparative analysis of bacterial differences between stack fermentation and pit fermentation (A) Changes in the relative abundance of bacterial community structure at the phylum and genus level during the stack fermentation and pit fermentation. (B) The group division of bacterial community of different fermentation stages according to PCoA and ANOSIM analysis. (C) Comparison of alpha diversity indices of bacterial community between stack fermentation and pit fermentation.

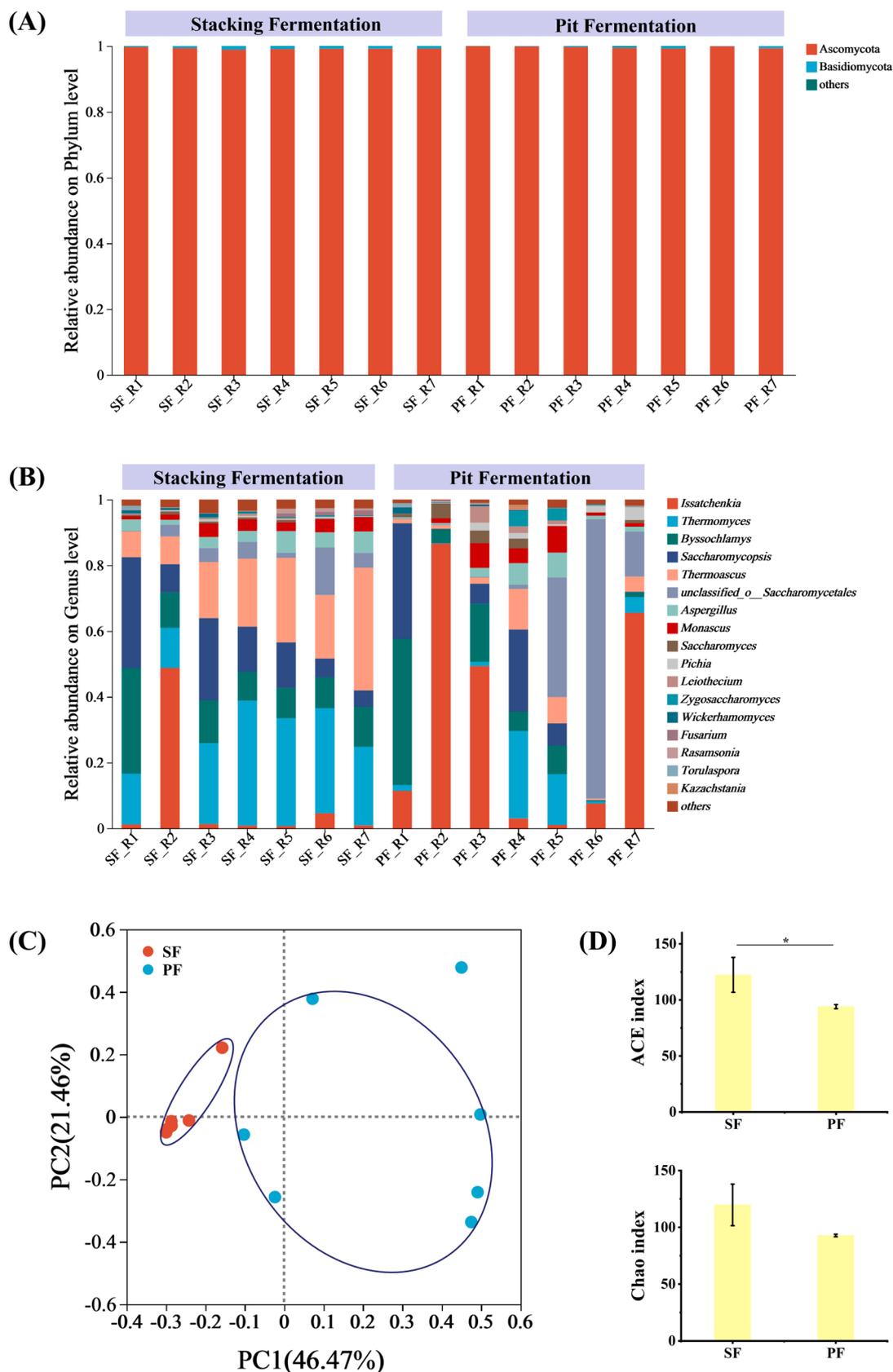


Fig. 3. Comparative analysis of fungal differences between stack fermentation and pit fermentation (A) Changes in the relative abundance of fungal community structure at the phylum and genus level during the stack fermentation and pit fermentation. (B) The group division of fungal community of different fermentation stages according to PCoA and ANOSIM analysis. (C) Comparison of alpha diversity indices of fungal community between stack fermentation and pit fermentation.

participating in SF was clearly distinct from that in PF (Fig. 3C). Similar Chao indexes were exhibited in two fermentation stage, while a significantly higher ACE index was observed in the SF compared with the PF (Fig. 3D). The SF had greater proportions of *Trichocomaceae* and *Thermoascaceae*, which belong to phylum Ascomycota, whereas the PF showed a greater proportion of *Saccharomycetales*, also belonging to phylum Ascomycota (Fig. S3B). Moreover, genera *Thermoascus* and *Thermomyces* were found to be the most differentially representative genus in SF, whereas *Issatchenkia* was the most differentially representative genus in PF. As the temperature in the SF process is notably higher than that in the PF process, this thermal environment fosters the growth

of thermo-tolerant *Thermoascus* and *Thermomyces*. They generate a diverse array of thermos-tolerant enzymes that play pivotal roles in facilitating the efficient degradation of plant materials utilized in the fermentation process (Xu, Wu, Niu, et al., 2023). In contrast, *Issatchenkia* was the dominant fungal genus in the PF process that can produce β -glycosidase, phenylethanol, various acids, ethyl ester, etc., differentiating it from the dominant presence of *Pichia* observed in the fermentation of southern region (Zhang et al., 2021a).

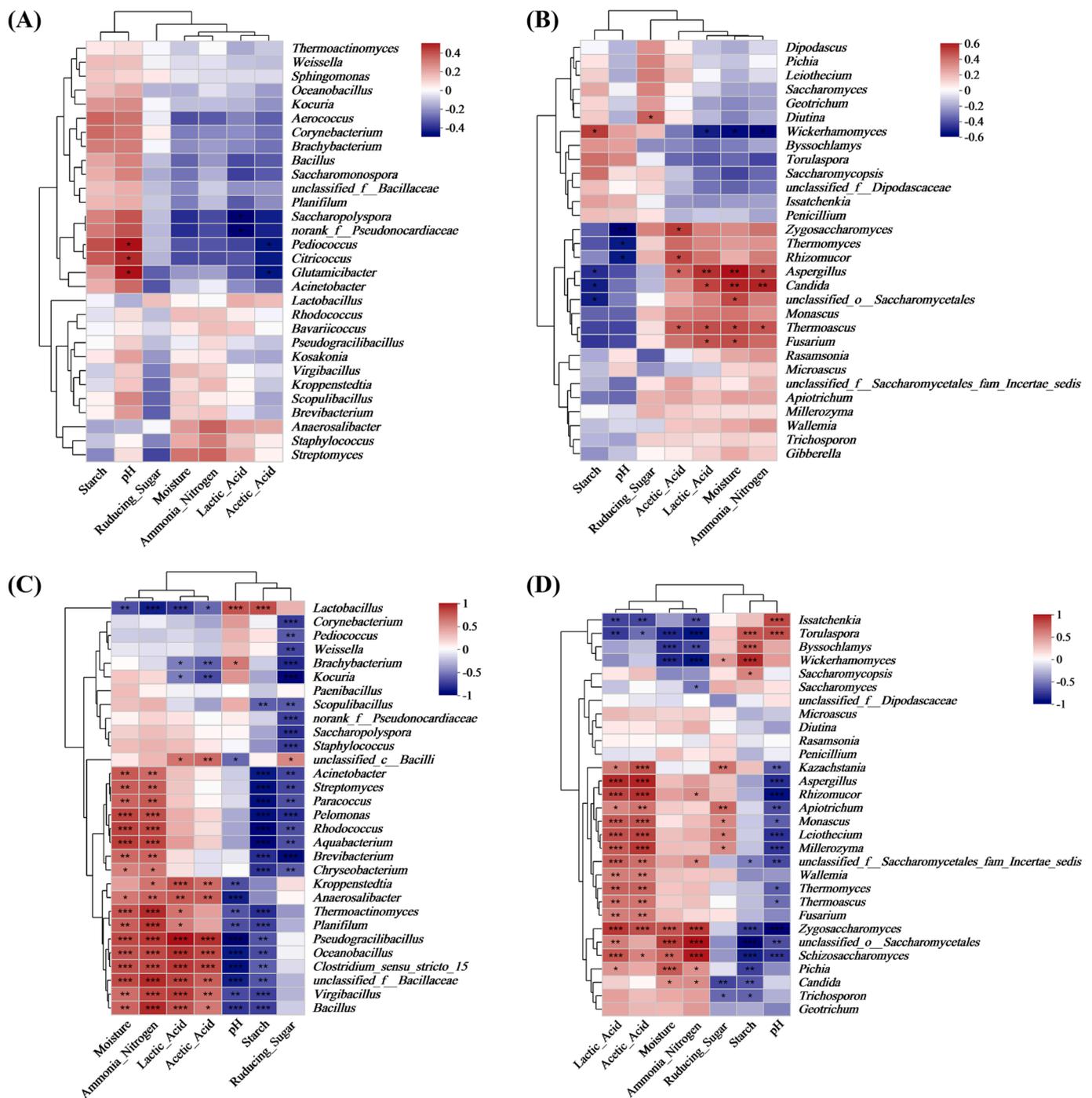


Fig. 4. Spearman correlation between physicochemical indexes and core genera (top 30 in abundance) in fermentation process. (A) Physicochemical indexes and the bacterial genera of stack fermentation. (B) Physicochemical indexes and the fungal genera of stack fermentation. (C) Physicochemical indexes and the bacterial genera of pit fermentation. (D) Physicochemical indexes and the fungal genera of pit fermentation. * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$.

3.3. Effects of physicochemical factors on core genera of stack fermentation and pit fermentation

Environmental factors play a pivotal role in driving microbial succession, exerting significant influence on the composition and dynamics of microbial communities. In this work, Spearman correlation analysis revealed notable disparities in the correlations between physical and chemical indexes and the core genera (top 30 in abundance) in the fermentation process ($|p| > 0.1, p < 0.05$) (Fig. 4). Specifically, in the PF, a higher number of significant correlations were observed between core genera and physicochemical factors. Among the core genera observed in the SF, a total of 34 significant correlations were identified with respect to various physicochemical factors (Fig. 4A, B). In contrast, among the core genera observed in the PF, a total of 190 significant correlations were identified in relation to physicochemical factors (Fig. 4C, D).

During SF, bacterial genera strongly correlated with physical and chemical indexes include pH, lactic acid and acetic acid (Fig. 4A). *Pediococcus* and *Glutamicibacter* were significantly positively correlated with pH ($p < 0.05$) and negatively correlated with acetic acid ($p < 0.05$), respectively, while *Saccharopolyspora* was significantly negatively correlated with lactic acid ($p < 0.05$). For fungi, the core genera exhibited highly significant correlations ($p < 0.01$) with pH, humidity, and lactic acid. Specifically, a notably significant negative correlation was observed between *Zygosaccharomyces* and pH ($p < 0.01$). Previous studies also found that *Zygosaccharomyces* can adapt to high acid environment and is crucial in SFB production (Wu et al., 2012). In addition, *Aspergillus* and *Candida* were significantly positively correlated with lactic acid and also moisture (Fig. 4B).

During PF, *Pelomonas*, *Rhodococcus*, *Aquabacterium*, *Thermoactinomyces*, *Planifilum*, *Pseudogracilibacillus*, *Oceanobacillus*, *Virgibacillus*, *Bacillus* showed significant positive correlation with moisture and ammonia nitrogen ($p < 0.001$) (Fig. 4C). *Acinetobacter*, *Streptomyces*, *Paracoccus*, *Pelomonas*, *Rhodococcus*, *Aquabacterium*, *Brevibacterium*, *Chryseobacterium* showed a significant negative correlation with starch and reducing sugar ($p < 0.001$). Besides, *Corynebacterium*, *Pediococcus*, *Weissella*, *Brachybacterium*, *Kocuria*, *Scopulibacillus*, *Saccharopolyspora*, and *Staphylococcus* also showed a significant negative correlation with reducing sugar ($p < 0.001$). The above abundant genera utilize sugars degraded by starch as the main carbon source and establish diverse microbial communities for PF. For fungi, lactic acid and acetic acid were significantly positively correlated with core genera, whereas pH exhibited a significant negative correlation (Fig. 4D). These fungal genera namely *Kazachstania*, *Aspergillus*, *Rhizomucor*, *Monascus*, *Leiothecium*, *Millerozyma*, and *Zygosaccharomyces* were also commonly found in acidic food, indicating that they can be naturally domesticated to be acid-tolerant during fermentation (Guan et al., 2020; Shang et al., 2022). The comparable phenomenon has also been observed in light-flavor baijiu (Kang et al., 2022) and strong-flavor baijiu (He et al., 2022). However, *Issatchenkia*, a distinctive fungal genus prevalent in the Beijing production area, exhibited a significant negative correlation with lactic acid and acetic acid ($p < 0.001$), along with a significant positive correlation with pH during PF ($p < 0.001$) (Fig. 4D), indicating that *Issatchenkia* in Beijing area were not able to tolerate excessive acidity. This observation presents a distinct contrast to the significant acid resistance exhibited by *Issatchenkia* originating from Maotai area (Wang et al., 2022). In this work, although acid to some extent inhibits the growth of *Issatchenkia*, a notable resurgence of *Issatchenkia* was observed when acidity levels decrease in the late fermentation, especially the seventh round (Fig. 3B). Thus, the predominant *Issatchenkia* plays an important role in the flavor of baijiu. Additionally, it should also be noted that environmental factors not only promote or inhibit the growth of microorganisms, but also to some extent cause the release of certain potentially harmful substances. For instance, it has been found that *Pediococcus* can produce biogenic amines in the presence of ethanol (Gao et al., 2022). However, during the baijiu brewing process, the microbial metabolic interactions can lead to the decomposition or

transformation of those metabolites. Moreover, the distillation process further refines baijiu, aiding in the purification of the final product.

3.4. Volatile metabolites in base baijius of different rounds revealed by SPME-GC-MS

As shown in Table S1, 93 volatile flavor substances were detected in base baijius from seven rounds, including 50 esters, 9 alcohols, 8 alkanes, 7 ketones, 6 naphthalenes, 5 aldehydes, 3 acids and 5 other flavor substances. Esters are the most important and abundant flavor compounds in base baijius, which is consistent with the previous studies (Wang et al., 2019). In the base baijius of southern SFB, ethyl acetate and ethyl lactate are the main esters (Cai et al., 2019). However, in this study, hexadecanoic acid ethyl ester and decanoic acid ethyl ester were the most abundant esters (Table S1). Previous study has revealed that hexadecanoic acid ethyl ester (fruit aroma) is a marker of sauce aroma of SFB (Wu et al., 2023). Furthermore, high-quality base baijiu is closely related to decanoic acid ethyl ester, and it contributes to the typical characteristics of SFB, specifically recognized as “a lingering aroma in empty glass” (Xu et al., 2023a). In this study, hexadecanoic acid ethyl ester exhibited the highest content in the first round of base baijiu, followed by a progressive decline in the second and third rounds, a subsequent increase in the fourth round, and ultimately the lowest content observed in the seventh round (Table S1). In contrast, the content of decanoic acid ethyl ester displayed a different trend, initially low in the first and second rounds, reached its peak in the third round at 35.582 mg/L, and decreased across subsequent rounds. Moreover, it has been reported that several short-chain fatty acids possess sweet and fruity characteristics (Wang et al., 2019). Benzeneacetic acid ethyl ester (rose and honey aroma) exhibited relatively lower content in the first and second rounds, followed by a rapid increase in the third round, reaching its peak in the fifth round, and then showing a slight decline. Similarly, butanoic acid ethyl ester (pineapple aroma), hexanoic acid ethyl ester (pineapple aroma), and heptanoic acid ethyl ester (pineapple aroma) also attained their highest levels in the fifth round. Nonanoic acid ethyl ester (grape and rose aroma) consistently maintained high levels across all rounds, while tetradecanoic acid ethyl ester (floral aroma) and undecanoic acid ethyl ester (coconut aroma) both showed a pattern of initial increase followed by a subsequent decrease. Ethyl acetate, an essential compound known for its fruity aroma, imparts a distinct fruity and floral fragrance to baijiu. Notably, in the southern base baijiu of SFB, the content of ethyl acetate exhibited pronounced prominence, reaching its highest level in the first two rounds, with concentrations up to 71.481 ± 16.235 mg/L (Wu et al., 2016). However, in the northern SFB analyzed in this study, the content of ethyl acetate in rounds 1 to 4 was notably higher compared to rounds 5 to 7, ranging from 3.784 ± 0.016 mg/L to 8.45 ± 0.127 mg/L. The specific microorganisms that produce the precursors of flavor substances may exhibit different growth and metabolic activity in distinct regional environments. Moreover, different chemical reactions occur between metabolites, which further leads to differences in the composition and contents of flavor substances in the base baijius.

Alcohols are key substances in baijiu, mainly produced by yeast through glycolysis during fermentation. In the southern base baijiu of SFB, 3-methyl-1-butanol stands out as a characteristic compound present in the third, fourth, and fifth rounds, with malty aroma, and has been identified as a crucial alcohol marker in SFB (Wu et al., 2023). Moreover, the content of 3-methyl-1-butanol in the fourth and fifth rounds was observed to surpass that in the third round (Wu et al., 2023). In contrast, in the northern base baijiu of SFB, the content of 3-methyl-1-butanol was higher in the third and fifth rounds compared to the fourth round. Furthermore, during the seven rounds, the content of 3-methyl-1-butanol displayed an ascending pattern followed by a declining trend. The first and seventh rounds exhibited the highest content at 3.705 ± 0.021 mg/L and 3.731 ± 0.022 mg/L, respectively (Table S1). Phenethyl alcohol is an important contributor to various food flavors

because of its outstanding flower fragrance and it has been demonstrated to be present at higher levels in high-quality base baijiu (Wu et al., 2023). In our study, the content of phenethyl alcohol reached its peak in the third round of base baijiu, providing some insight into the high quality of the baijiu obtained in the third round. Additionally, the relatively elevated content of esters and comparatively lower content of alcohols observed in rounds 3, 4, and 5 further enhance the overall flavor characteristics of the base baijiu (Wang et al., 2021b). Notably, some higher alcohols were also identified, including 1-nonanol, 5-decyl-1-ol, 1,6,10-dodecatrien-3-ol, 3,7,11-trimethyl-, 1-decanol and 1-hexadecanol, 2-methyl-, which are the important by products of baijiu brewing and play a pivotal role in enhancing the sweetness and after-taste of baijiu (Cai et al., 2019).

Aldehydes, being significant flavor compounds in baijiu, are formed through the oxidation of alcohols and play a crucial role in shaping the overall flavor profile (Zhang et al., 2021b). A higher proportion of aldehydes can contribute to the distinctive sauce flavor and impart unique quality to the final product. Previous studies have revealed that benzaldehyde and furfural are aldehydes with high contents in high-grade base baijiu, and they are considered typical flavor substances in southern base baijiu of SFB (Wu et al., 2023). Moreover, the content of furfural in rounds 1–7 exhibited a gradual increasing trend, with the highest relative concentration at 5.676 ± 0.610 mg/L. In this study, from the first to sixth rounds, the furfural content showed a steady increase and reached 5.539 ± 0.011 mg/L in the sixth round, comparable to the levels found in southern base baijiu samples (Table S1) (Wu et al., 2023). Different from furfural, the content of benzaldehyde in the base baijiu of southern SFB demonstrated an initial increase followed by a subsequent decrease, which is similar to that of northern SFB (Wu et al., 2016). However, it was observed that the benzaldehyde content in the base baijiu of northern SFB was significantly higher than that in the southern SFB. Specifically, the benzaldehyde content reached its peak at 3.954 ± 0.033 mg/L during the fourth round of fermentation in the northern SFB (Table S1), while in the southern SFB, the peak was much lower, at 0.050 ± 0.010 mg/L, occurring in the second round (Wu et al., 2016). Isovaleraldehyde is characterized by its malt, fruity, and cocoa-like aroma, and it serves as a significant contributor to the flavor formation of SFB (Cai et al., 2019). In the brewing process of southern SFB, core microorganisms (unidentified mitochondria) are found to regulate the production of isoamyl alcohol, consequently impacting the formation of isovaleraldehyde (Shen et al., 2022). However, during the fermentation of northern SFB, isovaleraldehyde was not detected in the base baijiu (Table S1), which may be related to the trace amounts of unidentified mitochondria and other microorganisms associated with the formation of isovaleraldehyde in the fermented grains (Fig. 2). The above results may be one of the reasons why the sauce flavor in the northern region is relatively light compared to the southern region (Xu et al., 2023a).

In order to further screen the key and significant contributing differential flavor substance in base baijiu of seven rounds, the difference of flavor substances were characterized by the OPLS-DA matrix (Fig. 6). The results showed that R2X and R2Y were 0.749 and 0.916, respectively, and Q2 of the model was 0.652, suggesting that the OPLS model was well fitted for analysis and prediction. Based on OPLS-DA of the volatile metabolites in base baijiu, it can be seen that the samples of the first round to seventh round can be well separated (Fig. 6A), indicating that there were differences in volatile metabolites among different rounds. Furthermore, VIP values were used to estimate the importance of each variable in the projection and major volatile compounds were screened as differential marker candidates in base baijiu of different rounds. Compounds with $VIP > 1.0$ and $P < 0.05$ were selected as the important contributors to the differentiation of samples (Fig. 6B, Table S2) and consequently 29 differential volatile flavor substances were screened, including fourteen esters, six ketones, two alcohols, two naphthalenes, one aldehydes and four other substances. Among all esters, ethyl ester accounted for 85.71 % and the top three substances with

VIP values were all ethyl esters. Z19 (ethyl 9-hexadecanoate) had the highest VIP value, with the highest content in the fourth round and the lowest in the seventh round of base baijiu (Table S1). Similarly, Z20 (ethyl 9-tetradecanoate), which exhibits the second-highest VIP value, displayed the highest content in the base baijiu of third and fourth rounds, while the lowest content was observed in seventh round. Z5 (ethyl 13-methyl-tetradecanoate), known for its fruity aroma, showed relatively elevated concentrations in rounds 3–6 (ranging from 10 mg/L to 13 mg/L), significantly higher than in other rounds (approximately 3 mg/L) (Table S1). Additionally, C9 (3-methyl-1-butanol) was also identified as a significant differential flavor substance, consistent with the flavor markers of southern SFB (Wu et al., 2023).

3.5. Spearman correlation between microbiota and differential volatile metabolites

Previous study has substantiated the crucial role of PF in the intricate process of flavor substance formation in base baijiu (Wang et al., 2019). Therefore, to illuminate the interactions among microbiota during first to seventh rounds of PF, the co-occurrence and co-exclusion patterns of microbial communities in PF based on Spearman correlations ($|\rho| > 0.5$ and $P < 0.05$) were explored (Fig. 5). A total of 513 pairs of robust and significant correlations were identified among 50 bacterial genera within the bacterial network (Fig. 5A). These correlations consisted of 5.07 % negative correlations and 94.93 % positive correlations, indicating a predominantly positive association among the bacterial genera. The node of genera were assigned to six types of bacterial phylum. Among these phyla, Firmicutes, Proteobacteria and Actinobacteria were widely distributed, accounting for 40 %, 32 % and 22 % of all nodes, respectively. Based on betweenness centrality scores (Fig. S4A), the top five genera identified as the keystone taxa were *Pseudoclavibacter*, *Brachybacterium*, *Stenotrophomonas*, *Isoptericola* and *Kosakonia*. In the fungal co-occurrence network, a total of 252 pairs with significant and strong correlations were found from the 48 genera with the highest relative abundance, including 82.54 % positive correlation and 17.46 % negative correlation (Fig. 5B). These genera were classified into four phyla, namely Ascomycota, Basidiomycota, Mucoromycota, and unclassified_K_Fungi, representing 81.25 %, 12.5 %, 4.17 %, and 2.08 % of the total genera, respectively. Topological analysis found that the top five genera with the highest betweenness centrality values in fungal networks were *Schizosaccharomyces*, *Aspergillus*, *Thermoascus*, *Zygosaccharomyces* and *Trichomonascus*, respectively (Fig. S4B). It is worth noting that *Aspergillus* not only has a high betweenness centrality value, but also has the highest degree, occupying an absolute dominant position in the network. *Aspergillus* exhibited positive correlations with 12 genera and a negative correlation with one genus, *Torulaspota* (Fig. 5B). Notably, the correlation coefficient between *Aspergillus* and *Monascus* was found to be the highest at 0.857 ($P < 0.001$), indicating a strong positive association between them. Previous investigations have revealed that *Monascus* and *Aspergillus* of baijiu possess the ability to biosynthesize esters through esterification with acids and ethanol as substrates (Xu et al., 2022; Zhao et al., 2023). Furthermore, *Aspergillus* presents proficient starch saccharification capabilities in the simulated solid-state fermentation of baijiu, generating products that can serve as carbon sources for the proliferation of other microorganisms within the sample. Additionally, the substantial contribution of *Monascus* in the production of flavor compounds across all samples highlights its pivotal role in the formation of flavor substances. Both *Aspergillus* and *Monascus* function synergistically, working in tandem to facilitate the synthesis of flavor compounds (Xu et al., 2023b). Moreover, no individual modules were found in the co-occurrence networks (Fig. 5), indicating that all bacterial and fungal genera were interconnected within their respective modules. This observation suggests that the presence of keystone taxa is essential for the cohesive functioning and stability of microbial communities throughout the PF process.

Next, the correlation analysis of volatile flavor substances with

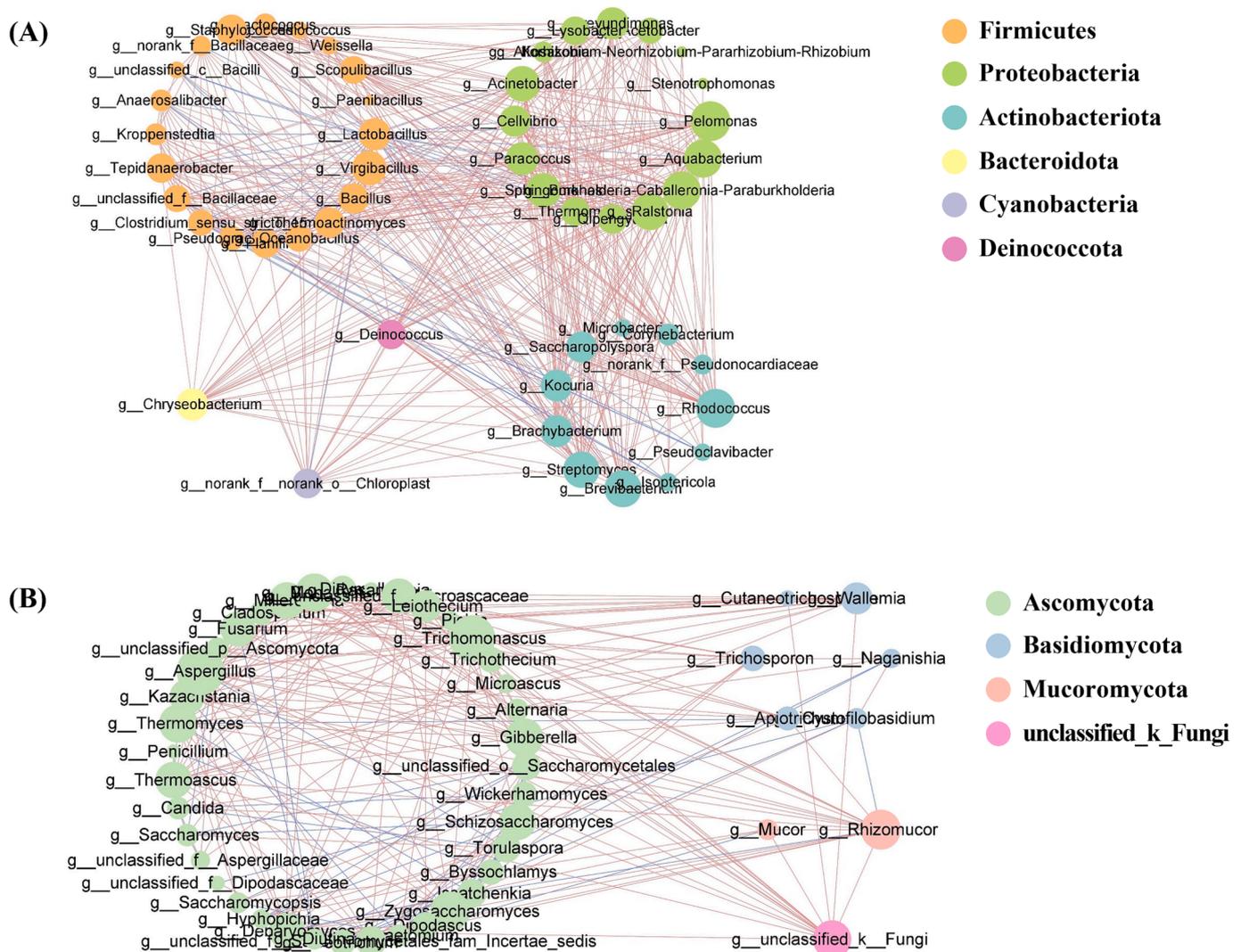


Fig. 5. Co-occurrence and co-exclusion patterns between bacteria (A) and fungi (B). Colors of the edges indicates negative (blue) or positive (red) significant correlations ($|r| > 0.5$ and $P < 0.05$). Size of each genera node is proportional to the number of connections. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant differences in base baijiu of seven different rounds was performed (Fig. 6C). The correlation network shows 1106 significant and robust correlations ($P < 0.05$) between 29 flavor substances and top 50 bacterial and fungal genera in abundance. Results showed that a total of 689 pairs of significant relationships between bacteria and flavor substances were identified, comprising 460 positive relationships and 229 negative relationships. Additionally, 417 significant relationships between fungi and flavor substances were observed, with 289 positive relationships and 128 negative relationships. Interestingly, there were more noteworthy correlations between bacteria and different flavor compounds compared to fungi. Among the bacteria, *Tepidanaerobacter* (degree = 25) and *Bacillus* (24) demonstrated the highest degrees of correlation, while among the fungi, *Schizosaccharomyces* (24) and *Torulaspota* (24) exhibited the highest degrees of correlation. The average degrees of bacteria and fungi were found to be 14.06 and 8.69, respectively, indicating that bacterial communities exert a greater influence on the synthesis of differential flavor substances in base baijiu of SFB. Esters were identified as the predominant differential flavor compounds among different rounds of base baijiu, and their correlation with microorganisms accounted for 46.75 % of all relationships. Among these microorganisms, *Schizosaccharomyces*, *Tepidanaerobate*, *Torulaspota*, *Bacillus*, *Thermoactinomyces*, etc., demonstrated the highest correlation

with ester substances, with positive relationships accounting for 66.54 % and negative relationships accounting for 33.46 %. Specifically, the flavor compound Z19 showed positive correlations with various fungi, including *Wickerhamyces*, *Kazakhstan*, and *Saccharomycopsis*, while exhibiting negative correlations with specific bacterial genera such as *Brevundimonas*, *Scopulibacillus*, and *Paracoccus*. Similarly, the formation of ethyl Z20 was found to be closely associated with certain fungi, particularly *Debaryomyces* and *Kazakhstan*, whereas bacteria, such as *Kosakonia* and *Isoptericola*, displayed a negative impact. Among other esters, including Z21 (benzeneacetic acid ethyl ester), Z44 (*cis*-5-dodecenoic acid methyl ester), and Z50 (dodecanoic acid ethyl ester), the most substantial positive correlation was observed with the genus unclassified_o_Saccharomycetales, displaying the highest correlation coefficient (Fig. 6C). Conversely, a significant negative correlation was found with the genus *Lactobacillus*. However, when exploring the correlation between flavor substances and microorganisms in the fermented grains from the first to third rounds of northern SFB, it was noted that Z21 and Z50 exhibited a positive correlation with *Lactobacillus* (Xu et al., 2023a). This discrepancy may be attributed to the significant differences in microorganisms and flavor substances between rounds 4–7 and rounds 1–3 (Fig. 2). Nevertheless, Z21 showed significant positive correlation with *Zygosaccharomyces* in both southern and

findings indicating their ability to synergistically enhance ester synthesis during baijiu brewing (Xu et al., 2022; Zhao et al., 2023). It's also worth noting that in our previous study of the fifth and sixth rounds of northern SFB brewing, bacterial contribution to ester synthesis was found to be not significant (Wang et al., 2021c). However, cross the entire brewing process, a more extensive analysis revealed that the number of significant correlations between bacteria and fungi and esters was 311 and 206, respectively. Moreover, there were more positive correlations between bacteria and esters. These findings strongly emphasize the crucial contributions of both bacteria and fungi to the production of flavor substances during the baijiu brewing process, with bacteria playing an even more substantial role in this regard.

4. Conclusions

In this study, a comprehensive investigation was conducted on microorganisms, physicochemical indicators, and flavor substances of SF and PF across all brewing rounds of northern SFB. *Saccharopolyspora*, *Virgibacillus*, *Thermoascus* and *Thermomyces* and *Lactobacillus* and *Issatchenkia* were found to be the most differentially representative genera in SF and PF, respectively. Besides, the dominant genus *Issatchenkia* in Beijing area was significantly positively correlated with pH, and negatively correlated with acetic acid, lactic acid, and ammonia nitrogen. Moreover, 93 volatile flavor substances were detected in base baijius from seven rounds, while isovaleraldehyde was not found, which may be one of the reasons for the lack of sauce flavor in northern SFB. Compared with fungi, bacteria had more significant influence on the 29 differential flavor compounds with high contribution values in different rounds of base baijius. The findings contribute to a deeper understanding of the northern SFB, providing valuable insights into the microbial contributions to the unique characteristics of SFB in the northern region.

CRedit authorship contribution statement

Yanfeng Wu: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Zhongfu Duan:** Methodology, Data curation, Investigation. **Jialiang Niu:** Investigation, Data curation. **Hua Zhu:** Investigation. **Chengnan Zhang:** Conceptualization, Methodology, Supervision. **Weiwei Li:** Conceptualization, Supervision. **Xiuting Li:** Supervision, Writing – review & editing, Funding acquisition. **Baoguo Sun:** Supervision.

Declaration of Competing Interest

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

Data availability

The sequencing data has been uploaded to NCBI Short Read Archive and the accession number is PRJNA994278.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2023.100970>.

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