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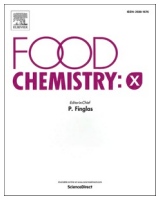

Multi-omics analysis of microbial spatiotemporal succession and metabolite differences in pit mud of varying cellar ages and spatial positions

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ABSTRACT

Microbes in pit mud (PM) are vital for the solid-state fermentation of strong-flavour *baijiu* (*SFB*), influencing the flavour and quality of *SFB* through metabolic activity. This study aimed to elucidate the differences in microbes and metabolites in PM at varying cellar ages and spatial positions. Microbes and metabolites in PM exhibited significant spatiotemporal variations, with temporal heterogeneity being more pronounced than spatial heterogeneity. Fourteen and 21 dominant genera were identified in 7-year and 50-year PM, respectively. Redundancy analysis suggested that pH, lactic acid, humus, and acetic acid drive microbial community succession. A total of 255 metabolites were identified, with acids, carbohydrates, and alcohols being the most prevalent. Significant positive correlations between the biomarkers and the main differential metabolites were revealed. Structural equation modeling demonstrated significant correlations between physicochemical factors, biomarkers, and the main differential metabolites. This study provides a foundation for future modifications of the quality and flavour of *SFB*.

1. Introduction

Baijiu is one of the most renowned distilled liquors worldwide and holds a special place in Chinese culture. It is categorised into three main and nine subcategories based on flavour characteristics (Zheng & Han, 2016). Strong-flavour *baijiu* (*SFB*) accounts for more than 70 % of the *baijiu* market and is widely consumed because of its potent aroma and long-lasting aftertaste (Wang, Chen, Wu, & Zhao, 2022). Generally, *SFB* is brewed using grain mixtures, such as sorghum, rice, wheat, and corn. The recycling fermentation process for *SFB* takes place over 60–90 days in a cellar lined with pit mud (PM) (Ren et al., 2023). During fermentation, the synergistic effects of *Daqu*, PM, Huangshui, and fermentation microbes drive the formation of the unique flavour of *baijiu* (Xia et al., 2024).

PM is a fermented clay rich in anaerobic microbes that serves as a medium for microbial growth and proliferation and is key to synthesising flavour compounds in *SFB* (Lu et al., 2021). The anaerobic habitat of PM is a symbiotic system comprising bacteria and fungi (Ren, Gu, Du, & Xu, 2018). The microbial balance regulated by the type, relative abundance, community structure, and metabolic activity of microbes in PM affects the quality and flavour of the liquor (Hou et al., 2022). Microorganisms in the PM break down macromolecular organic compounds into various metabolites, which combine to form distinctive flavour compounds in *SFB* (Gao et al., 2021). Microbes in PM significantly affect fatty acid production, which is vital for creating flavour compounds. Ethyl esters, such as ethyl caproate, ethyl butanoate, and

Abbreviations: PM, pit mud; *SFB*, Strong-flavour *baijiu*; AN, ammonium nitrogen; HS, humus; AP, available phosphorus; AA, acetic acid; BA, butyric acid; LA, lactic acid; CA, caproic acid; UWPM, upper-wall pit mud; MWPM, middle-wall pit mud; DWPM, down-wall pit mud; BPM, bottom-pit mud; PCoA, principal components analysis; LDA, linear discriminant analysis; FC, Fold change; KEGG, Kyoto Encyclopaedia of Genes and Genomes; VIP, Variable Importance in Projection.

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ethyl lactate, are found in different types of *baijiu* (Wu, Zhu, Fang, Wijffels, & Xu, 2021; Zhou et al., 2023). *Clostridia* is a major microbial group synthesising short- and medium-chain fatty acids, such as butanoic and hexanoic acids (Gao et al., 2021). To deepen our understanding of the complex mechanisms underlying the formation of flavour profiles, a thorough investigation of the microbes present in PM remains imperative.

Cellar age and spatial location are critical factors for the microbial community in PM during fermentation (Zhang et al., 2020; Zhang et al., 2020). Bacterial diversity in PM with varying cellar ages increases as the cellar age increases (Tao et al., 2014). Fungal diversity in PM increases as the cellar depth increases (Cai et al., 2022). The difference in microbes in PM is associated with cellar ages and spatial locations owing to variations in brewing technology, geographic location, manufacturer, raw materials, and other factors. However, the mechanisms underlying this association remain unclear. Additionally, environmental factors, such as pH, water content, ammonium nitrogen (AN), and available phosphorus (AP), also affect microbial community assembly and are important indices for the initial assessment of PM quality or degree of ageing (Liu et al., 2018). Xia et al. (2024) suggested that AP, NH_4^+-N, and moisture contents are key factors influencing bacterial communities, whereas AP and acetic acid (AA) contents are key factors affecting fungal communities. Furthermore, pH and AN contents were positively correlated with *Sedimentibacter* and negatively correlated with *Lactobacillus* (Zhang, Meng, et al., 2020). Overall, the microbial community in PM can be influenced by physicochemical factors, cellar age, and spatial position, which affect metabolite contents and metabolic pathways, ultimately affecting the flavour and quality of liquor.

In China, Sichuan, Jianghuai, and Northern China are home to most SFB distilleries (Hong et al., 2021). Jinhui liquor is produced in Longnan City, Gansu, China, at the southern base of the Qinling Mountains in the upper reaches of the Yangtze River and is a significant product and well-known brand in China (Fig. S1a). Along with Wuliangye and Luzhou Laojiao, it is brewed in Sichuan style. Most studies of Jinhui PM have focused on microbial communities and physicochemical factors, with little research on metabolites (Fig. S1b). Multidimensional research on microbes and metabolites in PM can aid in understanding the mechanism of flavour generation and evaluating PM quality.

This study aimed to compare microbial spatiotemporal succession and metabolites in Jinhui PM samples with varying cellar ages and spatial positions. This study aimed to (i) compare the differences in physicochemical parameters, microbes, and metabolites in Jinhui PM; (ii) explore the physicochemical factors driving microbial community succession; and (iii) elucidate the complex interactions between physicochemical indices, microbes, and key differential metabolites in the PM. These results offer a foundation for the application and maintenance of PM, improving the capacity of PM to produce flavour substances and highlighting the physicochemical factors, dominant microbes, and important differential metabolites of high-quality PM.

2. Materials and methods

2.1. Sample collection

Twenty-4 PM samples were obtained from the fermentation pit at Jinhui Liquor Co. Ltd. in Longnan, Gansu Province, China (east longitude 105°95′, north latitude 33°81′) at four spatial positions, with PM ages of 7-year and 50-year. PM samples were collected from the midpoints of four cellar walls and combined to form upper-wall (UWPM), middle-wall (MWPM), and down-wall pit mud (DWPM) samples (Fig. 1). Using a five-point sampling method, PM was collected from the cellar bottom and combined to form a bottom-pit mud (BPM) sample (Xia et al., 2024). To analyze physicochemical factors, microbial metagenomics, and metabolites, each mixed sample was split into three portions and stored in sterilised bags at 4 °C, −20 °C, and − 80 °C, respectively.

2.2. Determining the physicochemical properties of PM

Previously described methods were used to determine the pH and moisture content (Tao et al., 2014). The molybdenum blue method was used to measure the AP content (Zheng et al., 2020). The AN content was quantified using colourimetry by measuring absorbance at 425 nm (Zhou et al., 2023). The humus (HS) content was determined using the potassium dichromate oxidation method (Snyder & Trofymow, 2008). High-performance liquid chromatography (Wooking K2025 HPLC system) was employed to measure the organic acid contents, including lactic acid (LA), AA, caproic acid (CA), and butyric acid (BA).

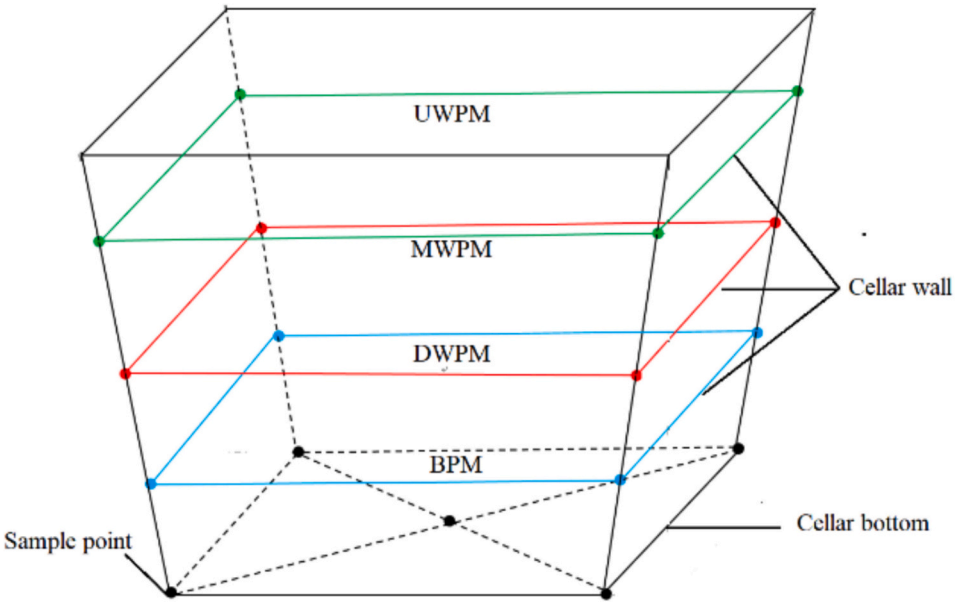


Fig. 1. Sampling of PM with varying spatial locations.

2.3. DNA extraction, PCR amplification, and Illumina NovaSeq sequencing

The hexadecyl trimethyl ammonium bromide method was used to extract genomic DNA from PM samples. The concentration and purity of the DNA were determined using 1 % agarose gel. The V3–V4 regions of the bacterial 16S rRNA genes and the internal transcribed spacer (ITS) regions of the fungal rRNA genes were amplified using the following specific primers: 341F (5'-CCTAYGGGRBGCASCAG-3′)/806R (5'-GGACTACNNGGGTATCTAAT-3′) and ITS1-1F-F (5'-CTTGGTCATTTAGAGGAAGTAA-3′)/ITS1-1F-R(5'-GCTGCGTTCTTCATCGATGC-3′) with a barcode. The conditions for PCR amplification were as previously described (Guan, Yang, Ou, & Zhang, 2021). The PCR products were mixed with an equivalent volume of 1× tris acetate-EDTA buffer, purified using a Qiagen Gel Extraction Kit, and electrophoresed on a 2 % agarose gel for detection. Sequencing libraries were prepared using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), and index codes were added. The quality of the library was evaluated using a Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific). Finally, sequencing was conducted on the Illumina NovaSeq PE250 6000 platform, following a Qubit@ 2.0 Fluorometer evaluation of the built library (Ren et al., 2023).

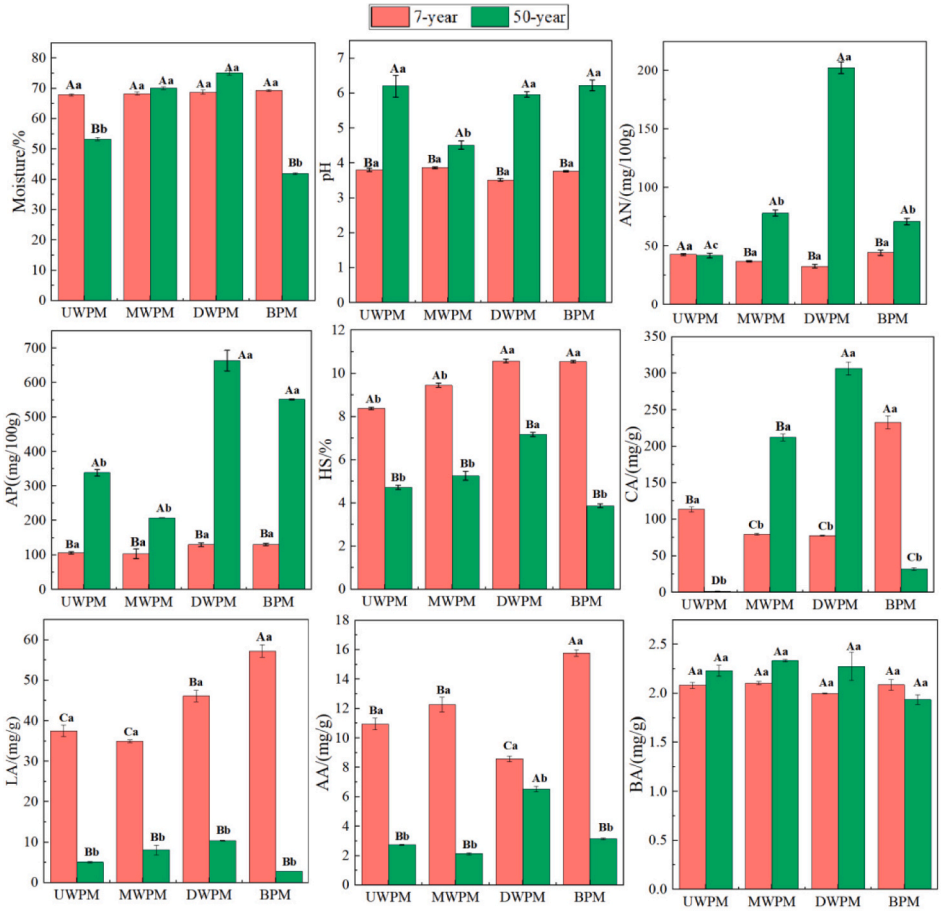
2.4. Metabolomic analysis

Gas chromatography–mass spectrometry was used to distinguish differential metabolites using an untargeted metabolomic analysis. After treatment with 1000 μL of extraction solution (methanol:isopropanol: water = 3:3:2 [v:v:v]), a 500-mg PM sample was vortexed and cooled in an ultrasonic ice-water bath. After centrifuging the extract at 12000 rpm for 15 min at 4 °C, the supernatant was added to a vial with 20 μL of an internal standard and evaporated under nitrogen flow. For quality control, 10 μL of the supernatant was obtained from each sample, and the residual was used for additional derivatisation and metabolite detection (Luo et al., 2022). An Agilent 8890 gas chromatograph connected to a 5977 B mass spectrometer equipped with a DB-5MS column was utilised for gas chromatography–mass spectrometry analysis.

3. Results

3.1. Differences in physicochemical factors in PM

The pH, moisture, AN, HS, AP, CA, LA, AA, and BA contents of different PM samples were compared (Fig. 2). Notably, the pH, moisture, AN, and AP contents exhibited significant differences among the 50-year PM from different spatial positions ($p < 0.05$). In contrast, HS content differed significantly among the 7-year PM samples from



Note: Significant differences between different spatial locations were indicated by different uppercase letters

(p<0.05). Significant differences between cellar ages were shoeed by different lowercase letters (p<0.05).

Fig. 2. Differences of physicochemical factors in different PM. Note: Significant differences between different spatial locations were indicated by different uppercase letters ($p < 0.05$). Significant differences between cellar ages were showed by different lowercase letters ($p < 0.05$).

different spatial positions. As the cellar age increased, the pH, AN, and AP contents increased, whereas the HS content decreased. Additionally, the moisture content in the 50-year UWPM and BPM was significantly lower than in the 7-year UWPM and BPM, which may imply that there were differences in the microbial metabolic activity. Organic acids are significant ester precursors in *SFB* and affect their quality (Ren, Cai, et al., 2023). CA was the most prevalent organic acid, followed by LA, AA, and BA. CA is produced by microbial fermentation, and high CA content indicated that the PM contained more numerous microorganisms producing CA (Wang et al., 2020). The CA contents in the 50-year UWPM and BPM were higher than those in the 7-year UWPM and BPM. The LA and AA contents decreased as the cellar age increased (Fig. 2), possibly owing to differences in LA and AA metabolism during microbial community succession. The CA, LA, and AA contents in the 7-year UWPM, MWPM, and DWPM were significantly lower than those in the 7-year BPM, likely owing to the gradual sinking of organic acids and Huangshui to the cellar bottom. Moreover, the LA, CA, and AA contents in the 50-year UWPM, MWPM, and BPM were substantially lower than those in the 50-year DWPM, which contradicts previous research findings (Ren, Liu, et al., 2023). Furthermore, no discernible spatiotemporal differences were observed in the BA content ($p > 0.05$). Generally, the temporal heterogeneity of physicochemical factors was more significant than their spatial heterogeneity.

3.2. Microbial community diversity in PM

As the cellar depth increased, the bacterial diversity and richness increased in the 7-year PM, whereas they decreased in the 50-year PM (Fig. S2a and S2b). In contrast, the bacterial diversity and richness in the cellar wall PM (UWPM, MWPM, and DWPM) increased as the cellar age increased, whereas they decreased at the cellar bottom. In the 7-year PM, the fungal diversity and richness decreased as the cellar depth increased, whereas they increased in the 50-year PM (Fig. S2c and S2d). Overall, bacterial and fungal diversity and richness in the 50-year PM were greater than those in the 7-year PM, possibly because of better microbial adaptation to the environment in the old PM.

Forty bacterial phyla and 307 bacterial genera were identified in PM (Fig. S3a and Fig. 3a). Firmicutes (49.05 %), Euryarchaeota (6.58 %), Actinobacteria (1.60 %), Bacteroidetes (19.00 %), and Proteobacteria (20.36 %) were the dominant bacterial phyla. Firmicutes predominated in the 7-year and 50-year PM, consistent with previous findings (Liang, Luo, Zhang, Wu, & Zhang, 2016). Proteobacteria and Actinobacteria were predominant in the 7-year PM, whereas Euryarchaeota and Bacteroidetes were the most abundant in the 50-year PM. Among the 307 bacterial genera, the most prevalent were *Lactobacillus* (21.86 %), *Ruminococcus* (7.09 %), *Pseudomonas* (5.59 %), *Petrimonas* (4.62 %), *Clostridium* (3.22 %), *Methanobacteria* (3.14 %), *Prevotella* (2.54 %), *Caloramator* (2.39 %), *Methanosarcina* (2.04 %), *Sedimentibacter* (1.81

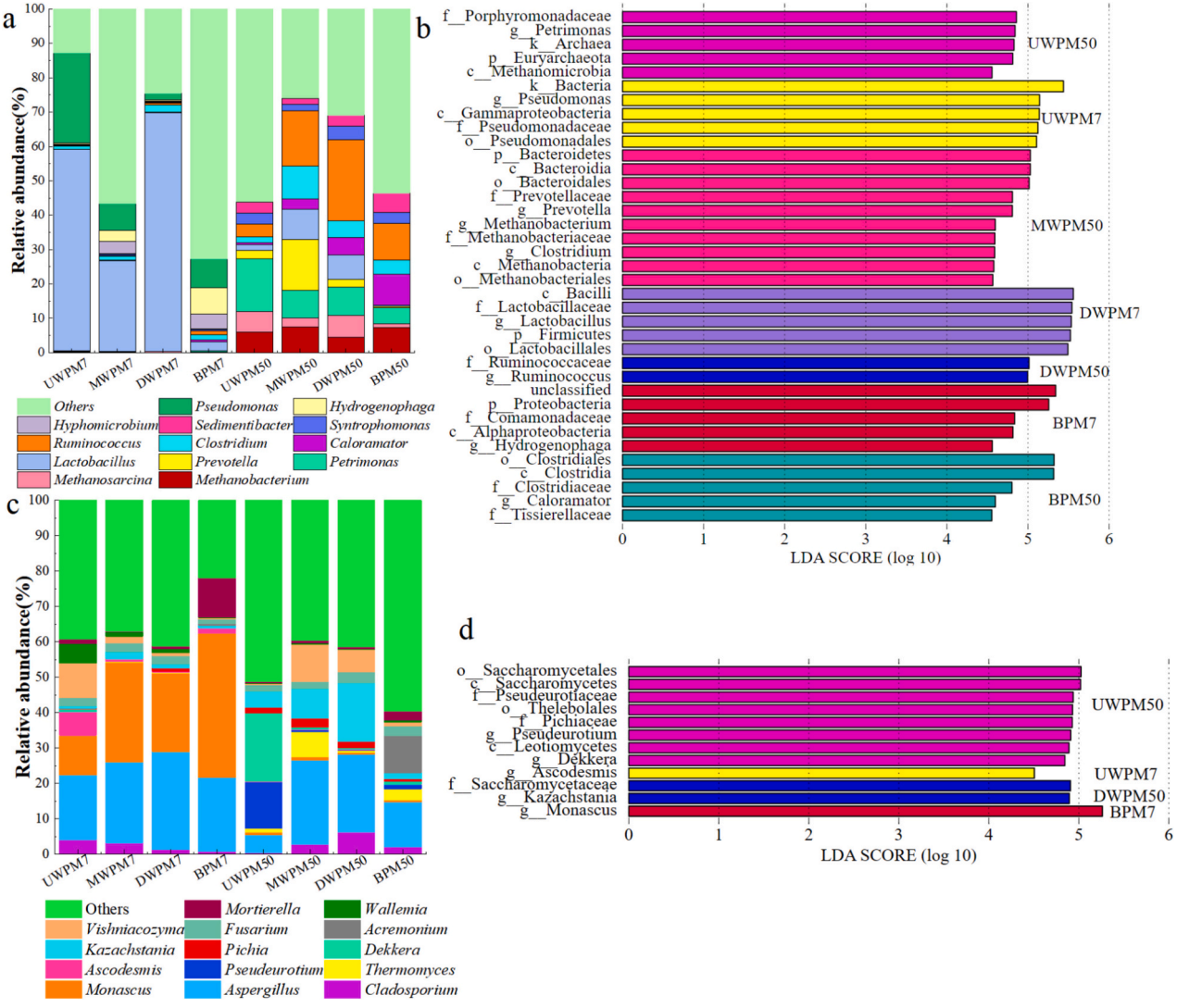


Fig. 3. Bacterial (a) and fungal (c) microbial compositions at the genus level, as well as the biomarkers of bacterial (b) and fungi (d) in PM.

%), *Syntrophomonas* (1.67 %), *Hydrogenophaga* (1.47 %), and *Hyphomicrobium* (1.04 %), accounting for 58.48 % of the total abundance. In the 50-year PM from different spatial locations, the relative abundances of the dominant genera significantly differed ($p < 0.05$), whereas that in the 7-year PM from different spatial locations showed no significant variation except for *Lactobacillus* and *Pseudomonas* ($p > 0.05$). In the 7-year UWPM, MWPM, and DWPM, the relative abundance of *Lactobacillus* was significantly higher than that in the 7-year BPM, whereas the relative abundance of *Pseudomonas* in the 7-year UWPM, MWPM, and BPM was significantly higher than that in the 7-year DWPM ($p < 0.05$). Compared with the 50-year MWPM, DWPM, and BPM, *Ruminococcus, Clostridium,* and *Caloramator* were more abundant than in the 50-year UWPM. Compared to the 50-year BPM, the relative abundance of *Petrimonas* in the 50-year cellar wall PM was higher ($p < 0.05$). As the cellar age increased, the relative abundances of *Lactobacillus* and *Pseudomonas* significantly decreased, consistent with previous reports that *Lactobacillus* is prevalent in young PM (Tao et al., 2014). Conversely, the relative abundances of *Caloramator, Petrimonas, Clostridium,* and *Ruminococcus* significantly increased as the cellar age increased. The predominant bacterial phyla and genera in PM showed significant differences among the different cellar ages but were similar among the various spatial positions.

Nineteen fungal phyla and 486 fungal genera were identified in PM (Fig. S3b and Fig. 3c). Ascomycota (67.04 %), Basidiomycota (12.91 %), Mortierellomycota (2.37 %), and Rozellomycota (1.86 %) were the dominant phyla. Ascomycota and Basidiomycota were predominant in 7-year and 50-year PM, which supports the findings of Cai et al. (2022). Among the 486 genera identified, the 14 dominant genera were *Aspergillus* (19.19 %), *Thermomyces* (13.56 %), *Monascus* (13.10 %), *Kazachstania* (4.45 %), *Vishniacozyma* (3.94 %), *Dekkera* (2.74 %), *Cladosporium* (2.54 %), *Fusarium* (2.25 %), *Mortierella* (2.22 %), *Pseudeurotium* (1.87 %), *Acremonium* (1.38 %), *Ascodesmis* (1.21 %), *Wallemia* (1.12 %), and *Pichia* (1.00 %), accounting for 70.57 % of the total abundance. *Aspergillus* and *Fusarium* were the two most dominant fungal genera in the PM. In the 7-year UWPM, MWPM, and BPM, the relative abundance of *Aspergillus* was significantly lower than that in the 7-year DWPM, whereas that in the 50-year MWPM and DWPM was higher than that in the 50-year UWPM and BPM. The relative abundance of *Fusarium* in the 7-year BPM was significantly lower than that in the 7-year cellar wall PM, whereas that in the 50-year cellar wall PM was significantly higher than that in the 50-year BPM. The relative abundances of *Monascus* and *Mortierella* in the 7-year cellar wall PM were significantly lower than those in the 7-year BPM. In the 7-year MWPM, the relative abundance of *Thermomyces* was significantly higher than that in the 7-year UWPM, DWPM, and BPM. In the 50-year UWPM, MWPM, and BPM, the abundance of *Kazachstania* was significantly lower than that in the 50-year DWPM ($p < 0.05$). Conversely, the relative abundances of *Aspergillus* and *Fusarium* decreased as the cellar age increased. Compared to the 7-year PM, *Monascus* was more abundant in the 50-year PM, whereas the relative abundance of *Kazachstania* exhibited the opposite trend. Furthermore, the relative abundances of unclassified fungal genera varied from 13.16 to 40.37 %, suggesting that these species were either unidentified or could not be identified using the current database. Therefore, the dominant fungal phyla and fungal genera demonstrated a consistent pattern in PM from different spatial positions, whereas they were significantly different in PMs of different cellar ages.

The default linear discriminant analysis (LDA) threshold score of 4.50 was used for linear discriminant analysis effect size to determine biomarkers and potentially distinguishable taxa in PM. Thirty-seven bacterial biomarkers were identified in the PM (LDA > 4.5) (Fig. 3b). The 7-year and 50-year UWPM, DWPM, and BPM had a single biomarker, whereas two and three biomarkers were present in the 7-year and 50-year MWPM, respectively. *Pseudomonas, Lactobacillus,* and *Hydrogenophaga* were biomarkers in the 7-year PM, whereas *Caloramator*, *Ruminococcus, Methanobacteria, Clostridium*, *Petrimonas,* and *Prevotella* were biomarkers in the 50-year PM. Twelve fungal biomarkers were detected in the PM (LDA > 4.5) (Fig. 3d). Genera biomarkers in the 50-year MWPM and DWPM were absent, suggesting that the dominant fungi had not yet emerged, and stable fungal biomarkers had not developed during domestication (Ren, Cai, et al., 2023). The biomarkers in the 7-year PM included *Ascodesmis*, *Rhizomucor*, and *Monascus*, whereas *Pseudeurotium*, *Dekkera*, and *Kazachstania* were the biomarkers in the 50-year PM. Importantly, *Lactobacillus*, *Ruminococcus*, *Prevotella*, *Pseudomonas*, *Petrimonas*, *Dekkera*, *Kazachstania*, *Pseudeurotium*, and *Monascus* exhibited an LDA ≥ 5, indicating that these microbes were responsible for the highly significant microbial differences ($p = 0.001$) in the PM (Cai et al., 2022).

3.3. Relationships between the dominant genera

Correlation analysis suggested 40 positive and 33 negative correlations among the bacterial genera in PM (Fig. S4a). Among these, eight bacterial genera (*Petrimonas*, *Clostridium*, *Methanobacterium*, *Prevotella*, *Caloramator*, *Methanosarcina*, *Sedimentibacter*, and *Syntrophomonas*) were positively correlated with *Ruminococcus*, which was a positive correlation hub. Conversely, *Ruminococcus* was negatively associated with *Pseudomonas*, *Hydrogenophaga*, and *Hyphomicrobium.*

Correlation analysis revealed 13 positive and 10 negative correlations between fungal genera in PM (Fig. S4b). Among them, *Monascus* was a negative correlation hub, positively associated with *Ascodesmis,* and negatively correlated with five fungal genera: *Kazachstania, Dekkera, Pseudeurotium, Thermomyces,* and *Aspergillus.* Additionally, the network complexity among the bacterial genera was higher than that among the fungal genera (Fig. S4a and S4b). Furthermore, the co-occurrence patterns of the bacterial and fungal genera were examined using strong and significant correlations. A total of 27 nodes and 178 edges were identified between the genera. Co-occurrence was primarily observed among 13 bacterial and 7 fungal genera (Fig. S4c). Most genera were positively correlated with *Ruminococcus*, *Petrimonas*, *Clostridium*, and *Methanobacterium.* Co-occurrence patterns demonstrated the possibility of niche space sharing and synergistic relationships within PM microbial communities (Du, Liu, Wang, & Xu, 2017). The relative abundances of dominant genera increased as the cellar age increased, suggesting that these genera may be crucial for the stability of the ecosystem (Zheng et al., 2020).

3.4. Analysis of differential metabolites in PM

As shown in Fig. S5a, 255 metabolites were identified in the PM, with the primary metabolites being acids, carbohydrates, alcohols, heterocyclic compounds, and lipids, comprising approximately half of the total metabolites. PCoA ($p = 0.001$) showed that the metabolite contents significantly differed in PM with different cellar ages and spatial positions (Fig. S5b-d). To determine the differential metabolites in the 7- and 50-year-old PM, in accordance with fold change (FC) ≥ 2, FC ≤ 0.5, and variable importance in projection (VIP) > 1, standards were obtained. In 7-year vs. 50-year-old PM, 109 differential metabolites were identified, of which 55 were upregulated and 54 were downregulated (Fig. 4a). The differential metabolites in 7- and 50-year-old PM from different spatial positions were identified in accordance with VIP > 1 and $p < 0.05$. One hundred and thirty-four and 128 differential metabolites were screened in UWPM7 vs. MWPM7 vs. DWPM7 vs. BPM7 and UWPM50 vs. MWPM50 vs. DWPM50 vs. BPM50, respectively (Fig. 4b). Additionally, the Venn diagram showed that 67 metabolites were screened as common differential metabolites in UWPM7 vs. MWPM7 vs. DWPM7 vs. BPM7 and UWPM50 vs. MWPM50 vs. DWPM50 vs. BPM50, with 67 and 61 unique metabolites screened, respectively.

The top ten metabolites with the highest VIP values were used to screen for key differential metabolites (Fig. 4c–e). The results indicated that 1-tetracosanol 2, D-mannitol 1, neophytadiene, 6-benzylquinoline, D-galactose 2, glycerin, 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, 4-amino-1-methyl-1H-benzotriazole, methyl (2*R*,3*R*,4*S*)-2,4-

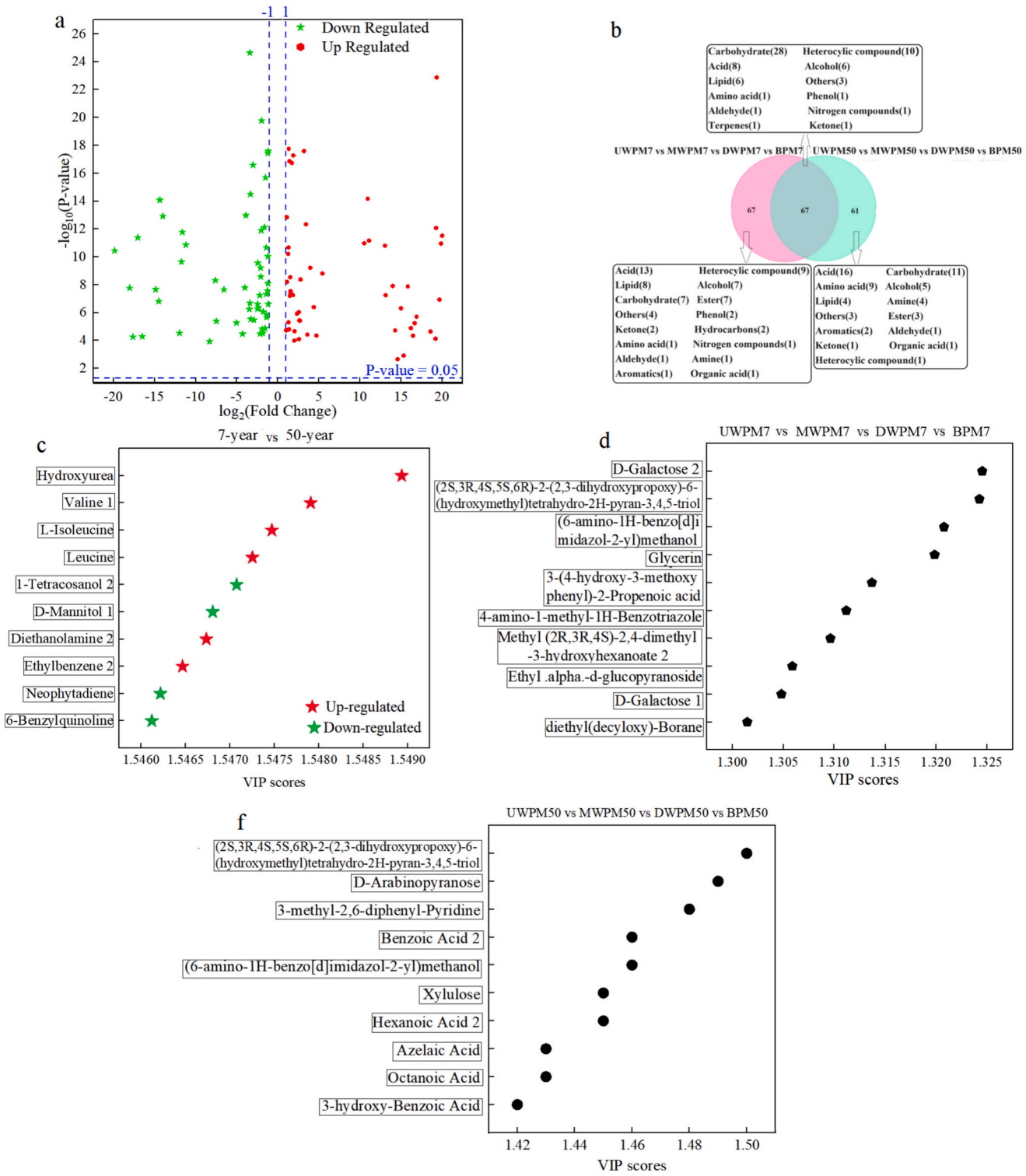


Fig. 4. Volcano maps of differential metabolites in 7-year vs. 50-year (a). The Venn diagram analyze of all differential metabolites at different spatial locations in 7-year and 50-year PM (b). Analysis of the difference of top 10 metabolites based on VIP scores >1 in PM: the difference of 7-year vs. 50-year (c); the difference of UWPM7 vs. MWPM7 vs. DWPM7 vs. BPM7 (d); the difference of UWPM7 vs. MWPM7 vs. DWPM7 vs. BPM7 (e).

dimethyl-3-hydroxyhexanoate, ethyl alpha-d-glucopyranoside, D-galactose 1, and diethyl(decyloxy)-borane were the key differential metabolites in the 7-year PM (Fig. 4d). Hydroxyurea, valine, L-isoleucine, leucine, diethanolamine, ethylbenzene 2, 3-hydroxy-benzoic acid, octanoic acid, azelaic acid, hexanoic acid, xylulose, benzoic acid, 3-methyl-2,6-diphenyl-pyridine, and D-arabinopyranose were the key metabolites in the 50-year PM (Fig. 4e). Therefore, a significant difference was observed in the key metabolites between 7- and 50-year-old PM.

3.5. *Predicting metabolic pathways*

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) was used to annotate enriched differential metabolites. The top 20 metabolic

pathways that were significantly correlated with the different cellar ages and spatial positions were mapped to create KEGG enrichment scatter plots (Fig. 5). In the 7-year vs. 50-year PM, the differential metabolites were mainly enriched in the two-component system, biosynthesis of unsaturated fatty acids, pentose phosphate, and other pathways (Fig. 5a). The differential metabolites in UWPM7 vs. MWPM7 vs. DWPM7 vs. BPM7 were primarily enriched in the biosynthesis of nucleotide sugars, degradation of aromatic compounds, biosynthesis of phenylpropanoids, and other pathways (Fig. 5b). In the UWPM50 vs MWPM50 vs DWPM50 vs BPM50 analysis, the differential metabolites were enriched in the biosynthesis of unsaturated fatty acids, valine, leucine, and isoleucine degradation, degradation of aromatic compounds, and other pathways (Fig. 5c). Therefore, the primary metabolic pathways in 7-year and 50-year PM were significantly different.

3.6. Multivariate analysis of microorganisms, physicochemical factors, and differential metabolites

Redundancy analysis was used to determine the influence of physicochemical factors on dominant microbes (Fig. S6a). The microbial communities in the PM were significantly affected by pH ($p = 0.031$) and the HS ($p = 0.042$), LA ($p = 0.016$), and AA ($p = 0.020$) contents. According to Zhang, Meng, et al. (2020), pH and LA are the key physicochemical variables affecting microbial community succession in the BPM, and LA has a major influence on the fungal communities in *jiupei* (Mu et al., 2023). However, in this study, microbial community succession was driven by changes in pH, HS, LA, and AA ($p < 0.05$). In particular, the pH significantly increased and then stabilised at a near-neutral pH as the cellar age increased, whereas the relative abundances of *Lactobacillus* and *Ruminococcus* decreased.

Spearman's correlation was used to explore the connections between 9 physicochemical factors and 14 dominant microbes (Fig. S6b). *Lactobacillus* was positively correlated with HS, and *Ascodesmis* was positively correlated with LA, whereas *Lactobacillus* and *Ascodesmis* were significantly negatively correlated with AP and pH. *Pseudomonas*, *Monascus*, and *Hydrogenophaga* significantly positively correlated with AA, HS, and LA, whereas *Monascus* and *Hydrogenophaga* significantly negatively correlated with AP and pH. *Pseudeurotium* and *Methanobacterium* were negatively correlated with LA, HS, and AA, whereas they were positively correlated with pH. *Dekkera* was negatively correlated with the LA and HS. Furthermore, *Petrimonas* and *Prevotella* were negatively correlated with HS and AA, and *Petrimonas* was positively correlated with pH and AP. *Caloramator* and *Ruminococcus* were positively correlated with AP and AN. Additionally, moisture and CA contents were not significantly associated with the dominant microbes, which may be due to the low relative abundances of dominant microbes.

Unique flavour profiles form owing to the co-occurrence of microbiota in PM, and metabolites can influence the structure of the microflora (Wei, Shen, Wei, & Zhang, 2023). Notably, 294 pairwise correlations were observed between 9 bacterial biomarkers and 5 fungal biomarkers, with 21 differential metabolites (Fig. S7). The positive relationships between *Ruminococcus*, *Hydrogenophaga*, *Caloramator*, and *Monascus* and 18 metabolites indicated that they were the primary sources of important metabolites in PM. *Lactobacillus* was negatively correlated with diethyl(decyloxy)borane, azelaic acid, 3-hydroxy-benzoic acid, and ethylbenzene. *Pseudomonas* and *Ascodesmis* were positively correlated with 6-benzylquinoline, D-mannitol, neophytadiene, D-galactose 2, and 1-tetracosanol and negatively correlated with azelaic acid, L-isoleucine, valine, leucine, ethylbenzene, and hydroxyurea. *Clostridium* was positively correlated with azelaic acid, L-isoleucine, valine, and leucine. *Dekkera*, *Pseudeurotium*, *Prevotella*, and *Petrimonas* were negatively correlated with 6-benzylquinoline, D-mannitol, and neophytadiene. *Prevotella* and *Petrimonas* were positively correlated with L-isoleucine, valine, leucine, ethylbenzene, hydroxyurea, and diethanolamine levels. Additionally, one differential metabolite was positively correlated with several dominant microbes, possibly because of the production of metabolites that require enzymes produced by various microbes (Hu et al., 2020).

The correlation between the physicochemical indices, microbes, and differential metabolites in PM was explored using a structural equation model (SEM). Physicochemical properties significantly influenced the microbial community ($p < 0.001$) and differential metabolites ($p < 0.001$) (Fig. 6). The microbial community significantly influenced the differential metabolites ($p < 0.001$). Eight physicochemical factors exhibited $p < 0.05$, accounting for 88.89 % of all physicochemical properties. Physicochemical properties were significantly positively correlated with CA ($p < 0.01$), BA ($p < 0.05$), AA ($p < 0.01$), AN ($p < 0.05$), HS ($p < 0.01$), and LA ($p < 0.001$), whereas pH ($p < 0.01$) and AP ($p < 0.01$) were significantly negatively correlated with physicochemical properties. Six biomarkers exhibited $p < 0.05$, accounting for 43 % of all the biomarkers. Biomarkers were significantly positively associated with *Methanobacterium* ($p < 0.01$), *Clostridium* ($p < 0.05$), and *Ruminococcus* ($p < 0.01$), whereas they were significantly negatively associated with *Pseudomonas* ($p < 0.01$), *Monascus* ($p < 0.01$), and *Ascodesmis* ($p < 0.05$). Seven differential metabolites exhibited $p < 0.05$, accounting for 35 % of all key differential metabolites. Methyl (2*R*,3*R*,4*S*)-2,4-dimethyl-3-hydroxyhexanoate 2 ($p < 0.001$), D-galactose 1 ($p < 0.01$), 1-tetracosanol 2 ($p < 0.05$), 3-methyl-2,6-diphenylpyridine ($p < 0.01$), and neophytadiene ($p < 0.05$) were significantly positively associated with the differential metabolites, whereas diethanolamine ($p < 0.05$) and ethylbenzene 2 ($p < 0.05$) were significantly negatively associated with the differential metabolites.

4. Discussion

4.1. Physicochemical factors drive microbial community succession in Jinhui PM

The formation of flavour compounds is facilitated by microbial community succession, which is fuelled by complex material-energy metabolic pathways and environmental factors within the PM (Wang, Du, & Xu, 2017). In this study, LA, pH, HS, and AA were key factors influencing microbial community succession. The pH significantly increased and stabilised at near-neutral pH as the cellar age increased, confirming earlier results (Zheng et al., 2020). The increase in pH was primarily due to the relative abundance of *Lactobacillus,* which decreased as the cellar age increased, reducing the LA content. Higher pH levels can promote the growth of microbes that are sensitive to acids, such as *Clostridium* and *Sedimentibacter*, and inhibit the growth of microbes that inhabit low-pH environments, such as *Lactobacillus* (Imachi et al., 2016; Kobayashi et al., 2017). Generally, LA (pKa = 3.86) is approximately 2–5 times stronger than AA (pKa = 4.75) and BA (pKa = 4.81), which is responsible for lowering the pH of PM. In this study, HS and AP mitigated changes in pH. Therefore, the combined variation in the HS and AP contents influenced pH changes (Fig. S8).

4.2. Key microbiota succession patterns in PM

The α-diversity analysis indicated that fungal diversity and richness were lower than bacterial diversity and richness (Fig. S2). Microbial community analysis identified 5 dominant bacterial phyla and 13 dominant bacterial genera in PM, and their relative abundances significantly differed between cellar age and spatial position. Firmicutes, Euryarchaeota, Actinobacteria, Proteobacteria, and Bacteroidetes were the predominant bacterial phyla, consistent with previous results (Tao et al., 2014). As the cellar age increased, the relative abundance of Firmicutes increased, suggesting that these microorganisms can adapt to unique PM environments. This adaptation may benefit from their special stress-resistant structure, such as thick cell walls, and may also advantage from their diverse metabolic patterns. Additionally, the relative abundance of Bacteroidetes increased as the cellar age increased, which is consistent with earlier findings (Liu et al., 2022). Furthermore, the

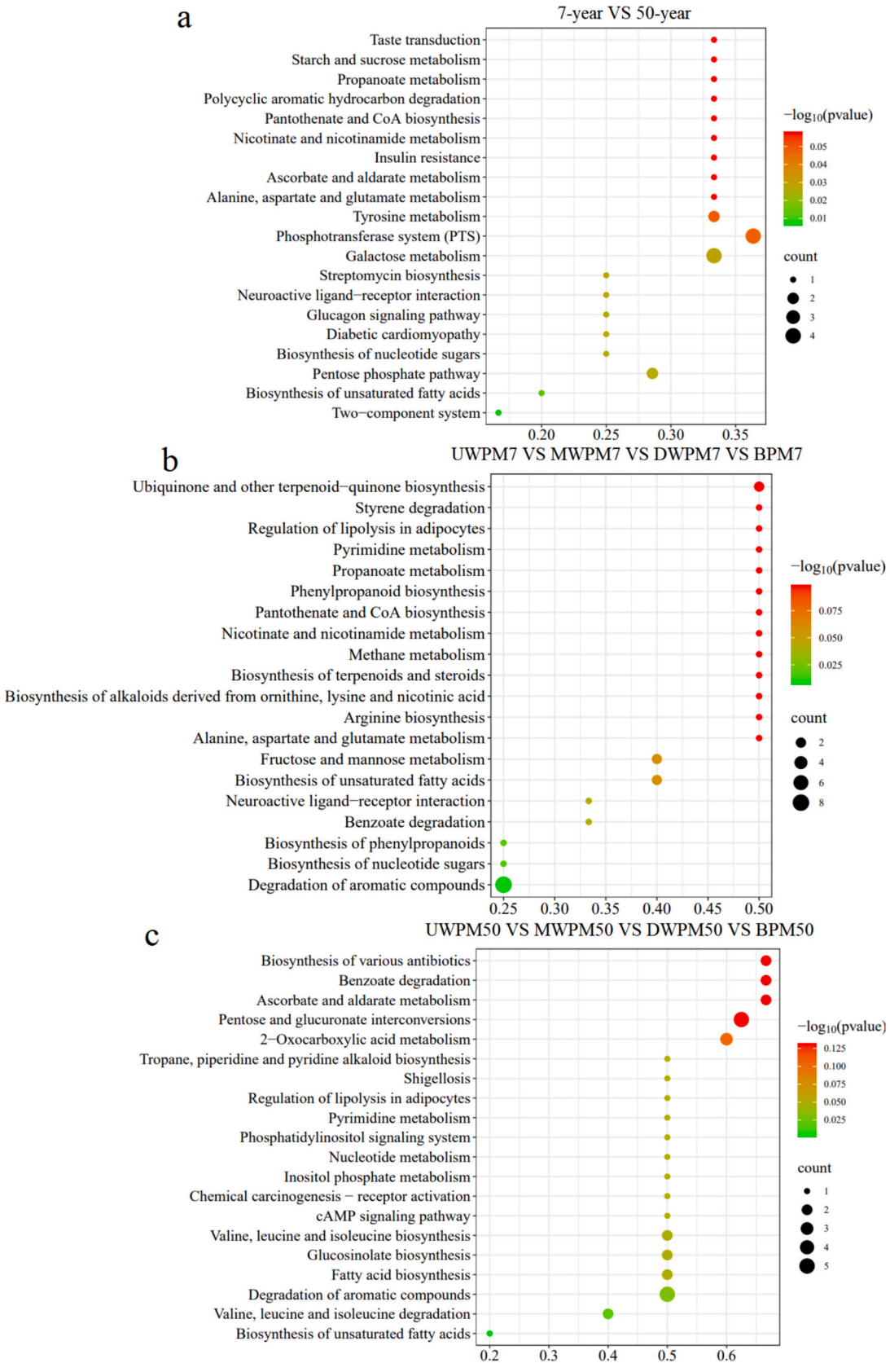
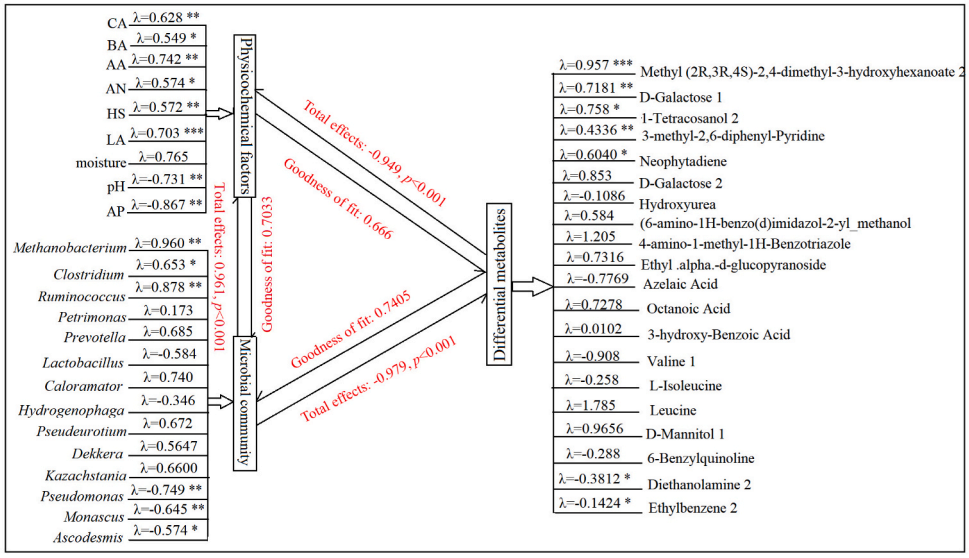


Fig. 5. KEGG annotations and enrichment results of the differentially expressed metabolites different PM.



Note: The path coefficient is shown by the value above the SEM line, and a significant difference is indicated by the symbol * (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$). The non-significant path coefficient is represented by the gray line or pane, the positive path coefficient by the red line or pane, and the negative path coefficient by the blue line.

Fig. 6. Structural equation modeling for physicochemical factors, differential microbes and key differential metabolites in PM. Note: The path coefficient is shown by the value above the SEM line, and a significant difference is indicated by the symbol * (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$). The non-significant path coefficient is represented by the gray line or pane, the positive path coefficient by the red line or pane, and the negative path coefficient by the blue line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relative abundance of Actinobacteria in the young PM was significantly higher than that in the old PM, which is consistent with our results (Fig. S3a) (Zhang et al., 2020).

The dominant genera in the 7-year PM in this study were *Lactobacillus* and *Pseudomonas*, which are essential for regulating the PM quality. As the cellar age increased, the consumption of nutrients and accumulation of products inhibited the development and procreation of *Lactobacillus* and *Pseudomonas*, leading to decreased relative abundances of *Lactobacillus* and *Pseudomonas*. *Lactobacillus* is a crucial microbe during *SFB* production, which can synthesise oligosaccharides and exopolysaccharides via the Maillard reaction, generate significant amounts of LA, and is crucial for controlling the flavour and quality of liquor (Xia et al., 2024). *Ruminococcus*, *Petrimonas*, *Clostridium*, *Syntrophomonas*, *Sedimentibacter*, and *Methanobacterium* were significantly enriched in 50-year PM. *Petrimonas* performs anaerobic fermentation by converting monosaccharides into organic acids, CO_2, and H_2. Moreover, *Petrimonas* and *Methanogens* work synchronously to regulate the microbial community in PM (Hu, Du, Ren, & Xu, 2016). *Clostridium* influences microbial community dynamics through interspecies hydrogen transfer and metabolite production and metabolises sugar, starch, and cellulose to synthesise CA (Cavalcante, Leitao, Gehring, Angenent, & Santaella, 2017). *Sedimentibacter* can generate AN and serve as a nitrogen source for other microbes (Lu et al., 2021). Through co-cultivation with *Methanogens*, *Syntrophomonas* can break down long-chain fatty acids into AA and H_2 (Zhang, Liu, & Dong, 2004). During fermentation of baijiu, ethyl acetate is metabolised by *Methanobacteria* (Xiao, Guo, Maspolim, Zhou, & Ng, 2016). Overall, the bacterial community constantly adjusts to form a balanced microbial ecosystem during fermentation, which contributes to the flavour and quality of liquor.

For fungi, the relative abundances of the dominant phyla and genera varied significantly as the cellar age and spatial position changed. *Aspergillus* can catalyse aromatic lipid synthesis, break down starch and cellulose macromolecules, and metabolise flavour-related substances to enhance the quality and flavour of liquor (Ali et al., 2019). Additionally, the esterifying, saccharifying, and liquefying abilities of *Daqu* are essential for solid-state fermentation of *SFB,* which is regulated by *Aspergillus* metabolites (Yang et al., 2017). *Monascus* can produce enzymes with strong esterification, fermentation, and saccharification capabilities, further enhancing the flavour and quality of the liquor (Chen, Liu, Zhen, & Fang, 2011). In this study, *Aspergillus* and *Monascus* were the dominant genera in the 7-year PM. As the cellar age increased, the relative abundances of *Aspergillus* and *Monascus* decreased. *Thermomyces*, *Pseudeurotium*, *Dekkera*, and *Kazachstania* were significantly enriched in the 50-year PM and were vital for PM quality. Among these, *Thermomyces* can produce key enzymes that break down proteins and macromolecular polysaccharides, providing raw materials for the reproduction and growth of microbes to further the formation of alcohols and aroma (Ren et al., 2024). Only a few species of *Kazachstania* have been characterised, and this genus has not been previously reported in SFB. *Kazachstania* has favourable aromatic attributes, and the fermentation capacity of *Saccharomyces cerevisiae* is higher than that of *Kazachstania* (Jood, Hoff, & Setati, 2017).

In summary, the relative abundances of *Petrimonas, Clostridium*, *Ruminococcus*, *Syntrophomonas*, *Sedimentibacter*, *Methanobacterium, Thermomyces*, *Kazachstania*, *Dekkera*, and *Pseudeurotium* increased as the cellar age increased. However, the relative abundances of *Aspergillus*, *Lactobacillus*, *Monascus*, and *Pseudomonas* decreased as the cellar age increased. These results suggest that the Jinhui PM shaped a stable microbial community using interaction and sustained collaboration among various microorganisms. Therefore, the progressive growth of adaptable microorganisms and the appearance of dominant species form a stable microbial community as the cellar age increases (Wang et al., 2020).

4.3. Relationship among dominant microbes and key differential metabolites

In Jinhui PM, the primary metabolites identified were acids, carbohydrates, heterocyclic compounds, and alcohols. In contrast, the primary metabolites in Luzhou PM are conjugates, peptides, fatty acids, and amino acids (Ren, Cai, et al., 2023). These differences may be due to differences in geographical location, brewing techniques, raw materials, or other factors. Furthermore, amino acids were also the important differential metabolites in *Jinhui* PM and essential for the development and metabolism of microbiota. Most amino acids (including valine, leucine, and isoleucine) are fermented by *Sedimentibacter* to produce AA and BA (Imachi et al., 2016). Correlation analysis indicated that *Hydrogenophaga* and D-mannitol 1, neophytadiene, D-galactose 2, and 1-tetracosanol 2 were significantly positively correlated (Fig. S7). In addition, *Monascus* was significantly positively correlated with D-mannitol 1. Moreover, *Caloramator*, *Ruminococcus*, and *Clostridium* were positively correlated with amino acids, such as L-isoleucine, valine 1, and leucine. In conclusion, the dominant microbes and differential metabolites were correlated.

4.4. Interaction among physicochemical factors, microbes, and metabolites

Changes in the physicochemical indices of PM can affect the microbial community, and differences in the microbial community can further affect the metabolites in PM (Ren et al., 2024). *Monascus* was significantly negatively correlated with the pH. As the cellar age increased, the relative abundance of *Monascus* decreased, possibly owing to a pH-limiting factor that inhibited the growth of *Monascus*. *Monascus* exhibited a significant positive correlation with octanoic acid, and octanoic acid content decreased as the pH increased. The HS, LA, and AA contents exhibited a significant positive correlation with *Hydrogenophaga*, and the relative abundance of *Hydrogenophaga* decreased as the cellar age increased. *Hydrogenophaga* was significantly positively correlated with D-mannitol 1, neophytadiene, D-galactose 2, and 1-tetracosanol 2, whereas the HS, LA, and AA contents decreased as the relative abundance of *Hydrogenophaga* decreased. These results were consistent with the SEM results (Fig. 6), suggesting a significant correlation between physicochemical factors, microbes, and differential metabolites.

5. Conclusion

This study comprehensively investigated the differences in physicochemical indices, microbes, and metabolites among different Jinhui PM samples. The physicochemical properties, microbes, and metabolites exhibited significant spatiotemporal differences, although the temporal heterogeneity was more significant than the spatial heterogeneity. The pH, AN, and AP contents increased as the cellar age increased, whereas the HS, LA, and AA contents decreased. Moreover, pH, HS, LA, and AA are crucial factors that drive microbial community succession. The metabolic activities of key microbes produce many metabolites that provide precursors for the flavour substances of liquor. Therefore, 50-year PM is better for balancing and enriching microbes and producing high-quality baijiu. Additionally, physicochemical indices significantly influenced the microbial community and metabolite content, and the microbial community significantly affected metabolite contents. This study provides important insights into regulating PM quality and enhancing the flavour and quality of *SFB*.

CRediT authorship contribution statement

Haiwei Ren: Writing – review & editing, Supervision, Methodology, Conceptualization. **Zhijuan Li:** Writing – original draft, Validation, Software, Methodology. **Qin Zhou:** Formal analysis. **Hongyuan Zhao:** Writing – review & editing. **Donglin Ma:** Writing – review & editing. **Xiaopeng Guo:** Writing – review & editing. **Zaoning Cai:** Software, Methodology, Data curation. **Yantao Li:** Resources. **Zhiliang Zhang:** Resources. **Yi Zheng:** Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary material

Supplementary data of this article can be found in the supplementary material. Supplementary data to this article can be found online at [https://doi.org/10.1016/j.fochx.2025.102287].

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102287.

Data availability

Data will be made available on request.

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