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Microbial spatiotemporal succession and metabolic difference in multidimensional pit mud used for the production of Luzhou-flavor baijiu

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ABSTRACT

Pit mud (PM) prokaryotic community is essential for the solid-fermentation of Luzhou-flavor *baijiu*. In this study, the multidimensional (four vertical positions) microbial communities of different PMs were investigated. Microbial communities were significantly different in multidimensional PMs ($p = 0.001$). Horizontally speaking, the relative abundance of *Longilinea* at the position 1 (bottom of the cellar) was highest in 30-year PMs (16.19%) and decreased gradually in 100- (11.95%) and 300-year (0.27%) PMs. *Methanobrevibacter* had the highest relative abundance at the position 3 (up the huangshui fluid) in 100-year PMs (18.98%), and decreased in 300-year PMs (8.73%), at the position 4 (top site of the cellar), *Clostridium* had the highest abundance in 100-year PMs (8.24%). Vertically speaking, *Petrimonas* had the highest abundance (17.4%) at the position 3 in 100-year PMs, while for the 300-year PMs, *Lactobacillus* and *Caproiciproducens* had the highest abundance at the position 1. RDA analysis indicated that lactic, caproic, butyric, and acetic acids were the four key physicochemical factors. Amino acids, peptides, fatty acids, and conjugates were the main metabolites of PMs, and S-Acetyldihydrolipoamide-E, succinic acid, aspartic acid, asparagine and valine were key differential metabolites. Based on OPLS-DA analysis, 20 differential flavor compounds were identified, mainly including esters such as hexanoic acid methyl ester, ethyl acetate, hexanoic acid ethyl ester, butanoic acid hexyl ester and acids such as hexanoic acid, butanoic acid and heptanoic acid. Moreover, hexanoic, octanoic acid and their corresponding esters were concentrated on 300-year PMs. This study provides theoretical support for improving the quality of PM.

1. Introduction

Chinese Strong-flavor *baijiu* (CSFB), also called 'Luzhou-flavor *baijiu*', is famous for its distinctive flavor and unique *baijiu* culture and accounts for more than 70% of Chinese *baijiu* production (Liu & Sun, 2018). The materials of CSFB are cereals, mostly sorghum, rice wheat and corn, and the fermentation procedure is a recycling process and usually carried out in a mud pit lined with pit mud (PM), and the anaerobic fermentation in cellars (Fig. S1) lasts for 60–90 days. Microorganisms involved in CSFB fermentation mainly originate from *daqu*, *jiupei*, PM and fermentation environments. Synergistic cooperation between *jiupei* and PM microbiota drives *baijiu*'s typical flavor formation, such as acetate and lactate accumulated in *jiupei* were further converted to butyrate and hexanoate by pit mud microbiota (Qian et al., 2021). Meanwhile, as a small-scale micro-ecosystem, with the degradation of starch and other macromolecules during fermentation, microbiota inhabiting in the PM could produce various flavor compounds such as alcohols, fatty acids and esters, which are the key flavor substances of CSFB (He et al., 2020, 2022; Wang et al., 2014). Clostridia (mainly included the genera *Caproiciproducens*, *Hydrogenispora*, *Sedimentibacter*,

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Syntrophomonas and *Clostridium*), *Lactobacillus*, Bacteroidetes and methanogens were the most abundant microbiota in pit mud (Chai et al., 2019; Hu et al., 2016; Lu et al., 2021; Zhang et al., 2021). Stability and robustness of PM microbial structure is closely related to age and maintenance (Hu et al., 2016). In general, it takes at least 25 years for the microbial community of PM to mature naturally. Tao et al. revealed that PM diversity increased with the cellar age, while there was no significant difference between 25-year and 50-year PMs (Tao et al., 2014). Microbiota diversity of wall-PM is relatively lower than bottom-PM (Ding et al., 2014), and bottom-PM microbial communities differentiate in multidimensional (Zhang et al., 2020).

All of the above studies have only focused on the microbiota diversity in the whole cell or single position without considering the spatial locations. The assembly process of the microbial community is influenced by several environmental factors, such as moisture, pH and substrates (Kim et al., 2015; Lee et al., 2013; Tan et al., 2019; Wang et al., 2018), high temperature and acidity as key factors for bacterial community succession during *jiupei* fermentation (Shen et al., 2021). However, the factors that drive the microbiota succession in PM with hundreds of year intervals are not clear. Concurrently, production practice has proven that there are differences in the quality of raw liquor in different layers of the cellar (Rhee et al., 2011), and little is known about the contribution of microbiota metabolic potential and flavor profiles of PM in different spatial locations to raw liquor. The multidimensional exploration of microorganisms in old PMs can not only help guide and accelerate the maturation of new PMs, but also improve the raw liquor quality of different layers of *jiupei*.

To elucidate the microbial succession pattern in mature PMs, this study took 36 p.m. samples from different years and spatial positions, the microbiota composition and physicochemical factors differences among 30-, 100- and 300-year PMs were investigated, meanwhile, the differential metabolites and flavor compounds produced by microorganisms were elaborated. To date, this is the first specialized study to investigate the spatial and temporal distributions of microbial communities, physicochemical factors, metabolites and flavor compounds in PMs over 30-, 100- and 300-year. This study systematically described the relationship among microbes, metabolites, and flavor compounds in PM.

2. Materials and methods

2.1. Sample collection

PM samples were obtained from a CSFB-producing enterprise in Luzhou, the south-eastern part of Sichuan Province, China. The cellar was approximately 2.8 m in length, 1.8 m in width, and 2.0 m in height (Fig. S1). PM samples were shelled from the bottom of the cellar, under and up the middle site (80–100 cm below the upper edge), and the top site (0–20 cm below the upper edge), namely positions 1–4, respectively. The PM sample for each position was collected at approximately 100 g, three samples were collected as parallels for physicochemical analysis and sequencing, therefore, a total of 36 samples from four spatial locations were collected from cellars of different fermentation years. After collection, the samples were sufficiently mixed and placed in a sterilizing bag, placed in a foam box filled with dry ice, and returned to the laboratory for the further analysis.

2.2. Detection of PM physicochemical properties and organic acids

The moisture content of PM was measured using a dry/wet weight measurement method after drying at 105 °C for 6 h. The pH of fresh PM was determined in a 1:10 (w/v) ratio in deionised water using a pH meter (PB10; Sartorius, Gottingen, Germany). The concentration of available phosphorus (AP) was detected by extracting with ammonium fluoride and hydrochloric acid (Liu et al., 2014). Ammonia nitrogen was extracted with Seignette salt and ammonium chloride and its concentration was measured using UV–visible spectrophotometry. Organic acids such as caproic acid and butyric acid in the PM were detected by GC-MS (Gao et al., 2019). Lactic acid and acetic acid of the PM were quantified using HPLC as described previously (Gong et al., 2020).

2.3. DNA extraction, and 16S rRNA gene amplicon sequencing

The genomic DNA of PM samples was extracted using a Fast DNA spin kit for soil, according to the manufacturer's instructions. For bacteria, the 16S rRNA gene was amplified using the universal primers with the barcode. The SMRT Bell library was built as required and then purified by AM Pure PB beads (Pacific Biosciences, Menlo Park, CA, USA). Furthermore, an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) was used to detect the size of the library fragments. Sequencing was performed using the PacBio Sequel II instrument (Pacbio Technologies, USA). The original FASTQ file was processed using QIIME2 platform (version 2021.02, https://qiime2.org/), and the low-quality sequences were filtered out. For bacteria, UCLUST was used to cluster high-quality sequences into several Operational Taxonomic Units (OTUs) according to 97% similarity. SILVA release 138 database was used to compare the 16S rRNA sequences to determine the taxonomic status of the corresponding microbes. The Shannon and Chao1 diversity index were calculated by QIIME 2 platform. Finally, the NMDS analysis was determined to analyze the heterogeneity of community composition between samples.

2.4. Metabolomic analysis of PM

The PM samples from position 1 and 2 were mixed equally to form a new sample termed position under the huangshui fluid', and the PM samples from position 3 and 4 were mixed equally to form a new sample termed 'position above the huangshui fluid'. Untargeted metabolomic analysis was used to identify the differential metabolites between the different PM samples using GC-MS and LC-MS. Sample preparation was carried out as described method previously (Cai et al., 2015). The raw data were converted to the mzXML format using ProteoWizard and processed with an in-house program, which was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration (Smith et al., 2006). Then an in-house MS2 database (BiotreeDB) was applied to metabolite annotation. The cutoff for annotation was set at 0.3.

2.5. Analysis of volatile flavor compounds in PM

Headspace solid phase micro-extraction (HS-SPME) was performed for the extracted trace flavor compounds from PM. PM (2 g) was took in a headspace bottle, adding 8 mL of salt solution (0.85% NaCl and 1% $CaCl_2$) and 2.5 g NaCl. Finally, 10 μL of the internal standard substance 2- octanol (0.1018 g/mL in ethanol) was added.

The gas chromatography and mass spectrometry (GC-MS, Thermo Fisher Scientific, Massachusetts, USA) according to the previously described method (Xu et al., 2019). The concentration was calculated by the percentage of the peak area.

2.6. Statistical analysis

Origin 2017 (64 Bit, OriginLab Corporation, Northampton, MA, USA), and SPSS software (V. 21.0, IBM Corp, NY, USA) were used for data processing and analysis. RDA analysis was performed using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY). Principal component analysis was calculated by SIMCA (version. 13.0.0.0, UMETRICS, Sweden). To identify the microbial biomarkers in different PMs, Galaxy Web application (https://huttenhower.sph.harvard.edu/galaxy/) was used to calculate the linear discriminant analysis (LDA) effect size. The statistical significance of the difference between

the mean of samples was tested by one-way analysis of variance with the Tukey post hoc test. The cluster analysis and correlation coefficient between microbes and chemical properties were visualized using R software (V 3.6.3 and V 4.0.5).

3. Results and discussion

3.1. The physicochemical attributes

This study compared the levels of moisture content, ammonia N, AP, pH, as well as the acetic, caproic, butyric, and lactic acid content from different PM samples (Table 1, Fig. 1). pH is an important factor that has a cumulative effect on the properties of PM. However, it showed no differences ($p > 0.05$) at different spatial locations among the 30-, 100-, and 300-year PMs. The moisture content showed significant differences ($p < 0.05$) in the 100- and 300-year PMs. The moisture content at position 4 (36.05% ± 5.06%) was significantly lower ($p < 0.05$) than that at position 1 (45.79% ± 1.31%) and 2 (45.08% ± 1.13%) in the 300-year PMs. For the same spatial position, the moisture content in the 300-year PMs was slightly higher than that in the 100- and 30-year PMs, but the difference was not significant ($p > 0.05$). The moisture content produced by microbiota metabolism gradually accumulated and sank to the bottom of the cellar during fermentation, resulting in higher moisture content at the bottom of the cellar compared with that in other spatial positions (Qian et al., 2021). Nutrients are also important contributors to the biochemical properties of PM. The concentration of Ammonia N at position 4 was significantly lower than that of other spatial positions in the 30-, 100-, and 300-year PMs ($p < 0.05$). Notably, the concentration of Ammonia N at position 1 in the 300-year PMs was significantly lower than that in the 30- and 100-year PMs. The concentration of AP at position 1 in the 100- and 300-year PMs was significantly higher than other positions ($p < 0.05$). It is believed that physicochemical factors are the key determinants influencing the succession of microbial communities in the PM, pH and the content of NH_4^+ were the most important factors influencing the microbial communities of artificial PM (APM), were lower in aging APM (Sun et al., 2017). Therefore, the content of Ammonium N and AP could reflect the maturity of PM to a certain extent (Hu et al., 2016).

Organic acids are important precursors of esters in CSFL and inevitably affect the quality of CSFL. The acetic acid content (Fig. 1A) was significantly ($p < 0.05$) higher at position 3 than other positions in the 30- year PMs, while in the 300-year PMs, the acetic acid content at position 1 was significantly higher than other positions ($p < 0.05$). The same trend was also observed in the content of caproic acid, butyric acid and lactic acid in the 300-year PMs. The reason may be the organic acids formed during fermentation, along with huangshui gradually sinking to the bottom of the cellar (Qian et al., 2021). The butyric and caproic acid content (Fig. 1B and C) showed no significant spatial difference in the 30-year PMs ($p > 0.05$), while in 100- and 300-year PMs, butyric and caproic acid content at position 1 was significantly higher than other positions ($p < 0.05$). As for lactic acid content (Fig. 1D), position 4 showed significantly higher levels than other spatial locations in the 30-year PMs ($p < 0.05$), while in the 100-year PMs, the lactic acid content at position 1 and 2 was significantly higher than other spatial positions ($p < 0.05$). In 30-year PMs, the values of moisture, pH, lactic acid, acetic acid, caproic acid and butyric acid showed similar trends as in the old PMs, it indicates the aging trend occurring in 30-year PMs. As the major product during *jiupei* fermentation, the increase of lactic acid could lead to the decrease of pH, on the other hand, microorganisms can utilize lactic acid as a precursor substance to produce caproic acid. The lower acetic acid content in PMs at position 4 also indicated the weak microbiota metabolism, due to acetic acid is the end metabolite of many microbiota metabolism (Hamdi et al., 2015; Imachi et al., 2016).

Table 1
The spatiotemporal physicochemical properties difference of PM during CSFL fermentation.

	Position-1	Position-2	Position-3	Position-4
Moisture-30-year	38.21 ± 4.7a/A	39.1 ± 5.95a/A	37.95 ± 2.97a/A	38.27 ± 4.06a/A
100-year	43.48 ± 2.47a/A	41.97 ± 1.48a/A	39.91 ± 2.45 ab/A	38.11 ± 0.28 b/A
300-year	45.79 ± 1.31a/A	45.08 ± 1.13a/A	43.12 ± 3.32a/A	36.05 ± 5.06 b/A
pH-30-year	6.69 ± 0.19a/A	6.02 ± 0.57a/A	6.72 ± 0.22a/A	4.53 ± 0.53 b/A
100-year	6.38 ± 0.38a/A	6.37 ± 0.31a/A	5.66 ± 0.83a/A	6.26 ± 0.07a/B
300-year	6.33 ± 0.12a/A	6.5 ± 0.4a/A	6.52 ± 0.51a/A	6.63 ± 0.41a/B
AN (g/kg)-30-year	3.92 ± 0.34a/B	3.45 ± 1.15a/A	4.15 ± 0.19a/A	2.67 ± 0.66 b/A
100-year	3.74 ± 0.15 b/B	2.7 ± 0.39a/A	3.48 ± 0.24 b/A	1.94 ± 0.03c/A
300-year	2.82 ± 0.49a/A	2.9 ± 0.38a/A	2.97 ± 1.43a/A	2.22 ± 0.62 b/A
AP (g/kg)-30-year	4.57 ± 1.74a/A	3.25 ± 0.33a/A	4.19 ± 2.36a/A	1.87 ± 0.01a/A
100-year	5.38 ± 1.36a/A	5.69 ± 0.27a/B	3.47 ± 0.92 b/A	1.94 ± 0.03 b/B
300-year	4.07 ± 1.32 b/A	3.34 ± 0.77 ab/A	1.93 ± 0.59a/A	1.96 ± 0.23a/A

All data are presented as mean ± standard deviations (n = 3), Lowercase letters (a,b,c,d) represent a horizontal comparison of different spatial positions, upper case letters (A,B,C,D) represent a vertical comparison of pits in different years.
AP: Available phosphorus.
AN: Ammonia Nigroten

3.2. Microbial diversity in different layers of the cellar

3.2.1. Spatiotemporal differences of microbiota diversity in PM

Regarding 16S rRNA gene sequencing data, a total of 395,133 high-quality reads were obtained from 36 p.m. samples (with a range from 4369 to 19407 reads) and clustered into 2670 OTUs based on 97% similarity. Based on the sequencing results, the total reads were classified as bacterial and archaeal phyla. Fig. S2 showed the bacterial Shannon and the Simpson index with a range of 7 ± 0.82 to 8.35 ± 1.34 and 0.93 ± 0.03 to 0.96 ± 0.01 in all PM samples respectively. Vertically speaking, in 100- and 300-year PMs, the average diversity (Shannon) in the top-site of PMs was obviously smaller than that of bottom of the pit PMs without significance ($p > 0.05$). Horizontally speaking, the Shannon and Simpson index showed no significant difference ($p > 0.05$) between each other, expect for the position 4 in 100-year PMs. A previous study did not reveal significant difference in Shannon index of 40- and 400-year PM (Liu et al., 2017), which was in consistence with our results.

For microbial composition, 13 bacterial phyla and 4 archaea were detected in 36 p.m. samples., Firmicutes, Bacteroidetes and Euryarchaeota were the top-3 predominant phyla in 30-, 100-, and 300-year PMs. The genera with relative abundance of >1.0% in half of the PM samples were defined as core microbiota. The core microbiota genera were *Lactobacillus*, *Caproiciproducens*, *Clostridium*, *Proteiniphilum*, *Prevotella*, *Bellilinea*, *Longilinea*, *Petrimonas*, *Methanobacterium,* and *Methanobrevibacter* and accounted for 70.19%, 66.47%, and 70.2% in the 30-, 100- and 300-year PMs, respectively (Fig. 2A, B and C). LEFSe analysis was used to detect microbial biomarkers in the 30-, 100- and 300-year PM samples. Vertically speaking, with the biomarkers as follows: 100-year = *Petrimonas* (Fig. 3B), 300-year = *Longilinea*, *Bellilinea*, *Caproiciproducens* and *Lactobacillus* (Fig. 3C). Tao et al. deduced that the caproic acid production in the PM via chain elongation pathway with lactate or ethanol as electron donor respectively (Tao, Wang, et al., 2017), Tao, Zhu, et al., 2017t the bottom of the pit, lactic acid produced by *Lactobacillus* can be further utilized by *Caproiciproducens* to produce caproic acid. Horizontally speaking, for position 1 (Fig. 3D), the 30-, 100- and 300-year PM showed significant differences, with the biomarkers are as

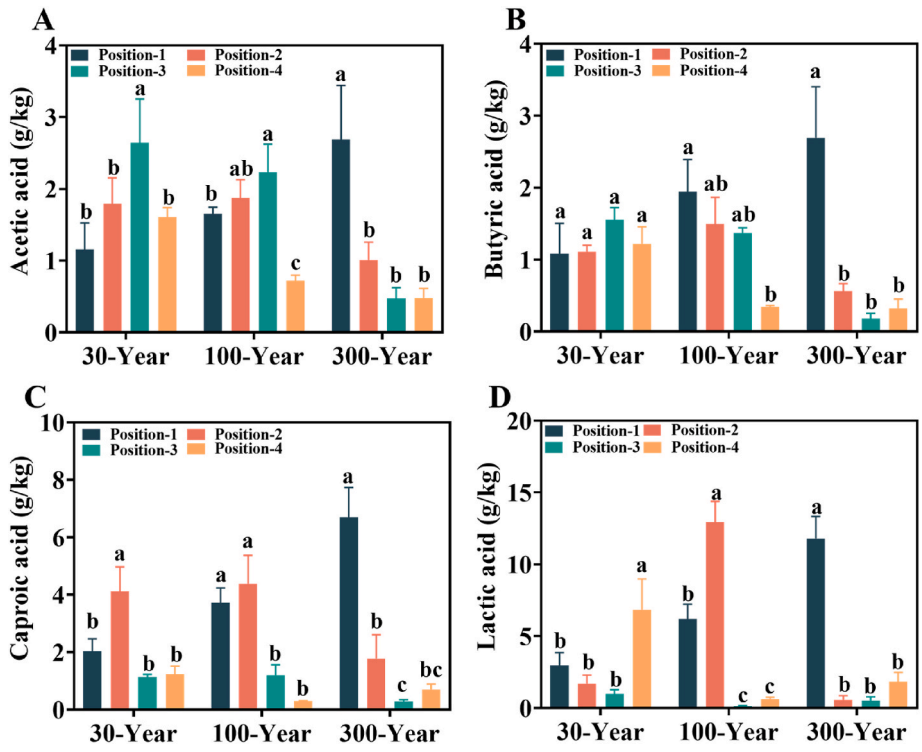

Fig. 1. Difference in organic acid contents in the spatiotemporal niche (the significance analysis refers to the comparison of different spatial locations of the same organic acid in the same year). The content of (A) acetic acid, (B) butyric acid, (C) caproic acid, and (D) lactic acid.

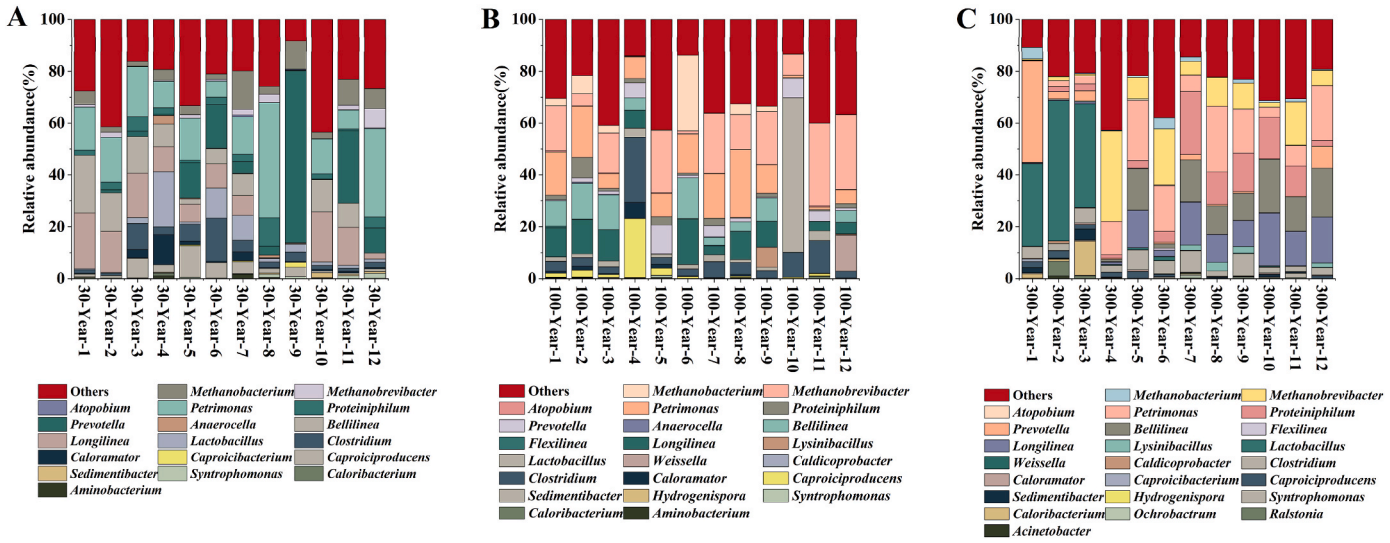

Fig. 2. Microbial community composition of PM in different cellars: (A) 30-year PMs, (B) 100-year PMs, (C) 300-year PMs.

follows: 30-year = *Longilinea*, 100-year = *Denitrobacterium*, *Flexilinea*, 300-year = *Pelotomaculum*. Compared with 300-year PMs (0.02%), *Longilinea* showed a big abundance in 30-year PMs (16.19%). The microbial communities showed no significant differences at position 2. While for position 3 (Fig. 3E), the biomarkers are as follows: 30-year = *Lactobacillus*, *Atopobium*, 100-year = *Methanobrevibacter*, 300-year = *Pelotomaculum*. Compared with 30-year (4.57%) and 100-year (1.65%) PMs, *Lactobacillus* showed the lowest abundance in 300-year PMs (0.07%). The relative abundance of *Methanobrevibacter* was 1.81%, 18.98% and 8.73% in the 30-, 100- and 300-year PMs with the relative respectively. For position 4 (Fig. 3F), the biomarkers are as follows: 30-year = *Methanobacterium*, 100-year = *Clostridium*. The *Methanobacterium* were relatively more abundant in 30-year PMs (6.77%), and the relative abundance of *Clostridium* was relatively more abundant in 100-year PMs (8.24%). According to previous reports, *Clostridium, Caproiciproducens, Syntrophomonas, Aminobacterium*, *Sedimentibacter, Prevotella*, *Petrimonas Methanoculleus* and *Methanobrevibacter* were the predominant microorganisms in mature PM (Liang et al., 2015; Liu et al., 2017). Notably, that the relative abundance of *Methanobacterium* and *Methanobrevibacter* was 0.7%–9.83% and 0.9%–22.82% in our PM samples, respectively, while the average abundance of *Methanobrevibacter* was less than 0.05% in new PM samples (Liu et al., 2020). In addition to the above microorganisms, *Proteinphilum*, *Bellilinea* and *Longilinea* were also detected as the key microorganisms in our

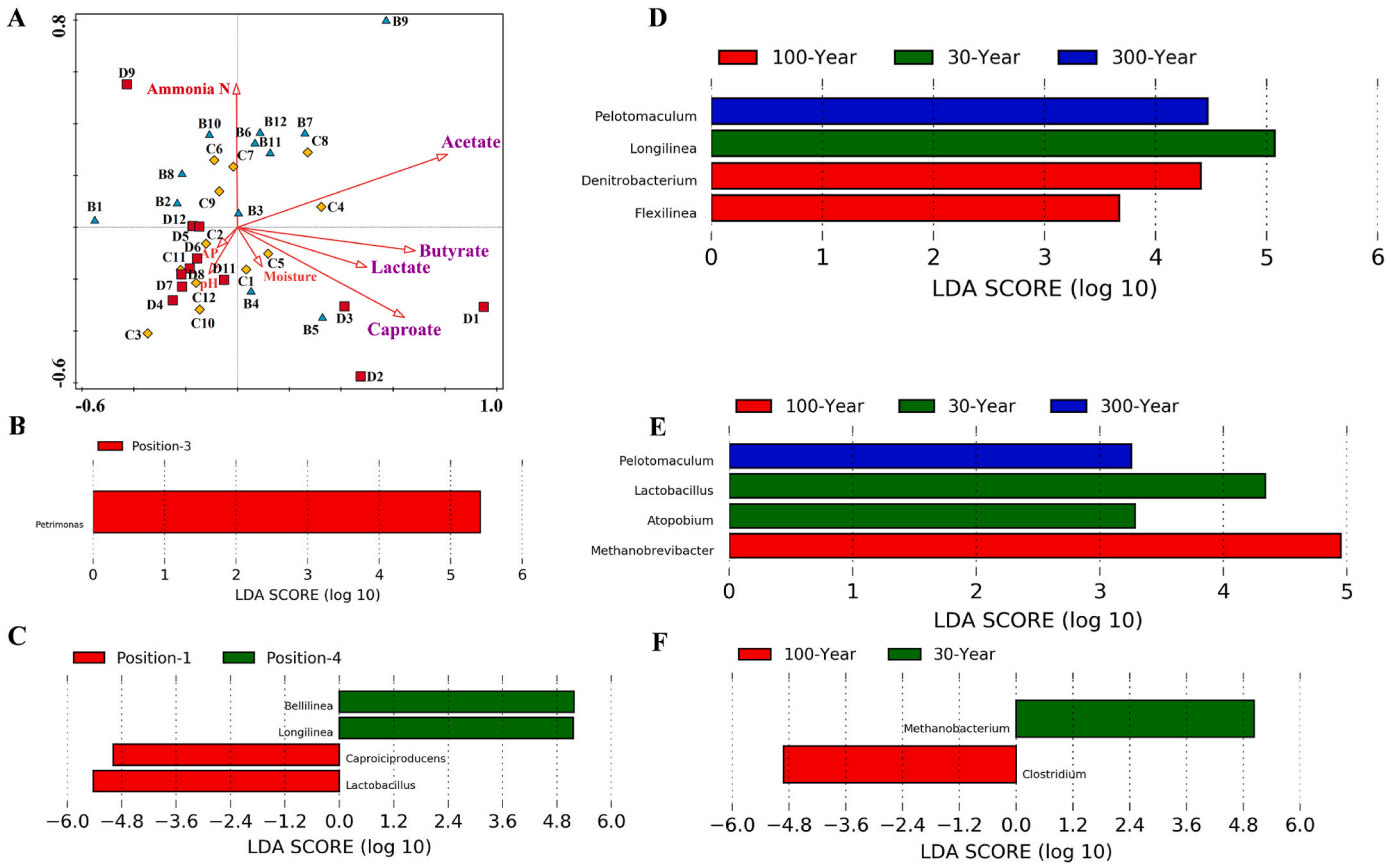

Fig. 3. (A) RDA between microbial community structure and environmental parameters. Linear discriminant effect size (LEfSe) analysis of the bacterial composition in the 30-, 100-, and 300-year PM samples. (B) 100-year (C) 300-year (D) Position-1, (E) Position-3, (F) Position-4 (LDA> 2 and $p < 0.05$).

study, indicating the complex microbial communities in PM.

3.2.2. *The β-diversity*

The similarities and differences in the microbiota using a Bray-Curtis approach. Combined non-metric multi-dimensional scaling and permanova analysis revealed that the microbial communities of 30-, 100-, and 300-year PMs were significantly difference ($p = 0.001$) (Fig. S3A), while there was no difference ($p = 0.2$) in 100- and 300-year PMs, suggesting 100- and 300-year PMs had more similar microbial communities. Previous studies indicated that microbial communities in 1-year PM were different from those in the 25-year and 50-year PM, while kept constant in the 25-year and 50-year PM (Tao et al., 2014).

3.3. *Relationships between microbial communities and physicochemical variables*

Redundancy analysis (RDA) was performed to investigate the possible relationship between microbial communities and physicochemical factors (Fig. 3A). The results showed lactate ($p = 0.046$), butyrate ($p = 0.004$), acetate ($p = 0.002$), and caproate ($p = 0.004$) significantly affected microbial communities. Previous studies revealed that lactic acid and pH were the two important factors influencing microbial composition in 1-, 10-, 25-, and 50-year PM samples (Tao et al., 2014). Lactic acid, pH, and soluble Ca^{2+} were considered as the important physicochemical factors for driving the microbial succession in bottom PMs (Zhang et al., 2020), at the same time, lactic acid also had a significant impact on the fungal community of *jiupei* (Mu et al., 2023). In our study, the pH and moisture content remained stable in different spatial locations of 30-,100- and 300-year PM. The organic acid content changes in the PM prokaryotic communities supported that environmental factors determine the shift in the structure of a prokaryotic community in the PM. The relationship between specific genera and driving factors was further investigated using spearman's coefficient ($p < 0.05$), about 6 genera were positively correlated with caproic, butyric and acetic acid, and 2 genera were positively correlated with lactic acid. Specifically, *Caproiciproducens* positively correlated with levels of lactic, butyric, acetic and caproic acids. *Lactobacillus* was positively correlated with levels of acetic, caproic and butyric acids. Yet *Methanobrevibacter* was negatively correlated with caproic acid and lactic acid. It is mainly because lactic acid and other substances produced by lactic acid bacteria could inhibit the growth and metabolism of some microorganisms (Hu et al., 2016). *Prevotella* was positively correlated with acetic acid and butyric acid. *Methanobacterium* was positively correlated with acetic acid, butyric acid and Ammonia N (Fig. S3B). Simultaneously, Ammonia N could provide a source of nitrogen for microbiota growth and has an important influence on the abundance of PM microbiota (Hu et al., 2016).

3.4. *Metabolites analysis in different layers of the PM*

3.4.1. *Classification of total metabolites*

Metabolites from PM also contribute to the flavor of *baijiu*. Fatty acids, amino acids, and peptides are decisive compounds because they could produce flavor compounds as precursors in CSFB production. In addition, lipids and lipid-like molecules, organoheterocyclic compounds, organic acids and derivatives were the most prevalent metabolites in artificial PM (Liu et al., 2020). In this study, 5991 metabolites were identified, of which 714 were identified by gas chromatography (GC-MS), and 5277 were identified by liquid chromatography (LC-MS). After filtering and quality control (deletion of unknown compounds), 982 metabolites were left for further analysis. The results showed that amino acids, peptides, and analogues, fatty acids, and conjugates were

the main metabolites and accounted for approximately half of the total metabolites (Fig. S4A). OPLS-DA score plot was applied to obtain better discrimination among three types of PM groups (Fig. S4B). Based on PCoA and anosim analysis, the metabolites among the 30-, 100- and 300-year PMs were significantly difference ($p < 0.05$) (Fig. S4C), while there was no difference in 100- and 300-year PMs ($p > 0.05$), indicating the similar metabolic potential in 100- and 300-year PMs (Fig. S4D).

3.4.2. Analysis of differential metabolites and pathways between different PMs

According to the standard of $P < 0.1$, VIP >1 and FC (fold change) > 1.5 were to obtain the differential metabolites in different PMs.

There were 85 differential metabolites in the 30-year PMs (Fig. 4A), of which 58 were up-regulated and 27 were down-regulated. There were 263 differential metabolites in the 100-year PMs (Fig. 4B), of which 195 were up-regulated and 68 were down-regulated. Of 189 differential metabolites found in the 300-year PMs (Figs. 4C), 119 were up-regulated and 70 were down-regulated. Based on this, there were 28, 55 and 39 KEGG pathways were annotated by differential metabolites at 30-, 100- and 300-year PMs, respectively.

According to the differential metabolites and KEGG pathways, important pathways were further obtained based on influence factors and p values in topological relationships (Xia et al., 2015). Compared to position 1 and position 2, there were 14, 40 and 26 differential pathways in 30-, 100- and 300-year PMs (Fig. 4D–F), respectively. The differential metabolic pathways mainly include β-Alanine metabolism, Vitamin B6 metabolism, Pyruvate metabolism, TCA cycle, Glycerolipid metabolism and Alanine aspartate and glutamate metabolism, Nicotinate and nicotinamide metabolism, etc. The differential metabolites related to the differential pathways mainly included β-alanine and L-aspartic acid (β-Alanine metabolism), S-Acetyldihydrolipoamide-E (Pyruvate metabolism/TCA cycle), Pyridoxine and Pyridoxamine (Vitamin B6 metabolism), aspartic acid, asparagine (Alanine, aspartate and glutamate metabolism), Glycerol (Glycerolipid metabolism) and L-valine (Pantothenate and CoA biosynthesis). S-Acetyldihydrolipoamide-E and succinic acid were key compounds in pyruvate metabolism, they can be converted into Acetyl-CoA and further converted to acetic acid. The relative abundance of S-Acetyldihydrolipoamide-E and succinic acid at position 1 was higher than position 2, indicating the metabolic activity at the bottom is higher than at the middle and upper of the cellar (position 2). Amino acids are also crucial compounds in microbiota growth and metabolism, *Sedimentibacter* (Imachi et al., 2016) and *Aminobacterium* (Hamdi et al., 2015) could ferment a variety of amino acids (such as valine, leucine, isoleucine, methionine, glycine, phenylalanine, tryptophan, lysine and arginine) to acetic and butyric acid. Alanine, aspartic acid and valine as differential metabolites showed high relative abundance at spatial position 1, which may result in higher flavor content at the bottom of the PM.

3.5. Analysis of flavor compounds in different layers of the PM

3.5.1. Spatiotemporal differences of flavor compounds in PM

The CSFL brewing process mainly relies on the metabolism of microbiota from pit mud and *jiupei* (He et al., 2019). To track the flavor profiles of the PM samples, HS-SPME-GC-MS analysis was conducted, and a total of 41 flavor compounds were detected in the PM samples, mainly including 31 esters, 6 fatty acids and 2 alcohols (Fig. S5A). The relative content changes of flavor compounds at different spatial locations were further compared, most of the flavor compounds such as butanoic acid, hexanoic acid, nonanoic acid and octanoic acid and their corresponding esters were concentrated at the bottom of the pit, especially in 300-year PMs. The butanoic acid heptyl ester, propanoic acid, 2-methyl-, pentanoic acid, 4-methyl- and nonanoic acid ethyl ester showed higher relative content at the position 2. In contrast, for the top of the pit (position 4), butanoic acid, 3-methylbutyl ester, butyl caprylate, pentanoic acid, ethyl ester showed higher relative content. Most of the microbes inhabited in PM are strictly anaerobic, the oxygen content at the bottom of the cellar is lower than in other spatial positions, which is conducive to microbial metabolism. Previous research has shown that flavor compounds in the bottom of FG were significantly higher than in

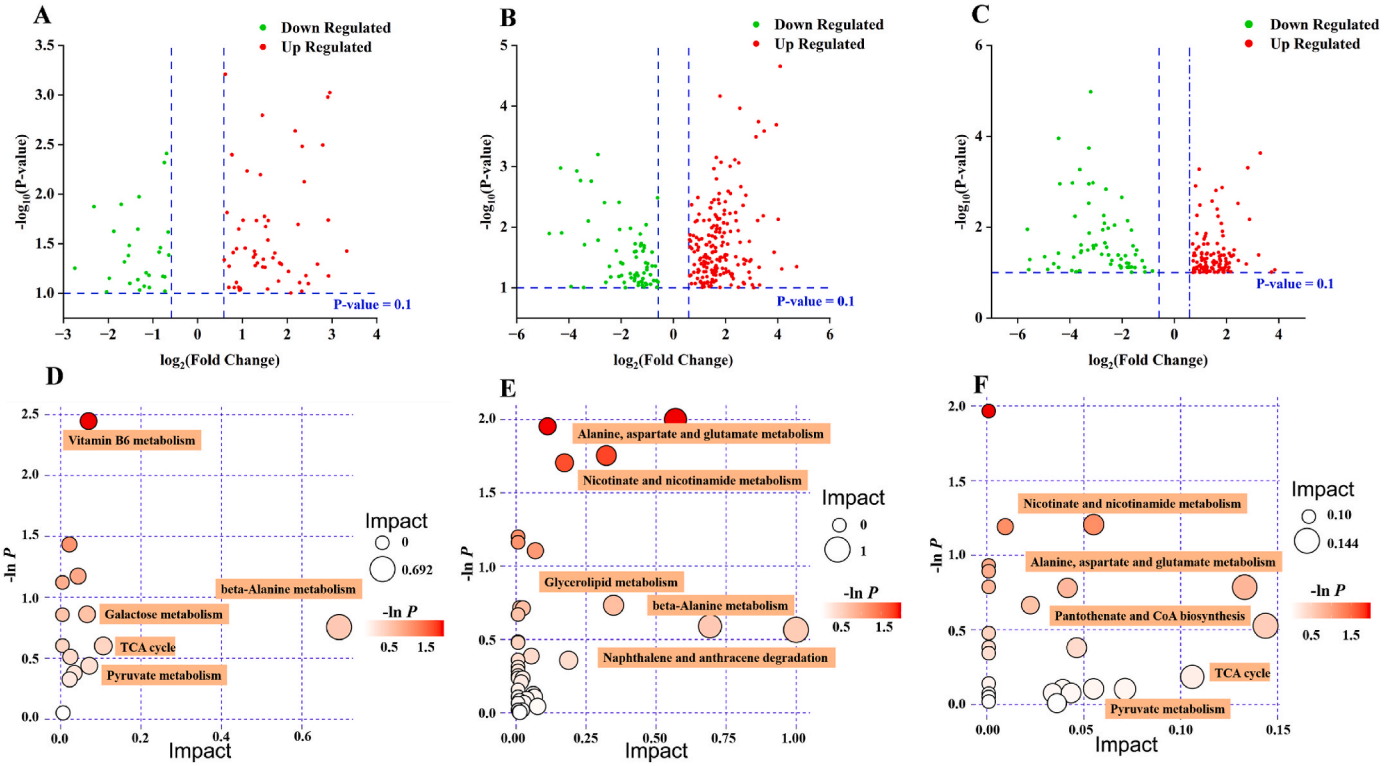

Fig. 4. Differential metabolites and KEGG pathways in different cellars (A and D, B and E, and C, F refer to the 30-, 100- and 300-year PMs, respectively).

the middle layer of FG (Jiao et al., 2022), at the same time, flavor compounds such as hexanoic acid ethyl ester, decanoic acid ethyl ester, octanoic acid and heptanoic acid were also detected in FG (fermented grains) (Hu et al., 2021) indicating potential substance exchange between FG and PM. Based on our results, the hexanoic and butyric showed higher relative content than other acids, indicating the microorganisms in PM were the main contributors of butyric and hexanoic acid, which were consistent with the previous results that butyric acid- and hexanoic acid-producers mainly inhabited the pit mud in CSFL (Tao, Zhu, et al., 2017; Zou et al., 2018). To obtain better discrimination among three types of PM groups by magnifying the covariance between the detected flavor compounds and different PM groups, an OPLS-DA score plot was applied. A clear separation among three types PM groups was observed (Fig. 5A). The model displayed goodness of fit ($R^2Y = 0.961$) and predictability ($Q^2 = 0.943$) for discrimination among the three groups. When the R^2 and Q^2 are >0.5, the model is considered successful (Wang & Xu, 2015). PMs showed distinct differences not only in microbiota distribution and metabolic profiles but also in their flavor compounds. The variables that significantly contributed to the discrimination among three groups were selected based on a VIP >1.0, and $p < 0.05$ (Fig. S5B), differential flavor compounds mainly include phenylethyl alcohol, hexanoic acid methyl ester, ethyl acetate, octanoic acid, hexanoic acid ethyl ester, butanoic acid hexyl ester, etc.

3.5.2. Relationships between microbial communities and physicochemical variables

The relationship between specific genera and flavor compounds were further analyzed using spearman's coefficient ($p < 0.05$). The results showed that *Lactobacillus* and *Clostridium* were positively correlated with hexanoic acid, heptanoic acid, butanoic acid, and esters such as butanoic acid hexyl ester, acetic acid hexyl ester, decanoic acid ethyl ester and ethyl acetate ($p < 0.05$), the *Methanobrevibacter* was positively correlated with octanoic acid, butanoic acid heptyl ester and benzenepropanoic acid ethyl ester ($p < 0.05$), while the *Petrimonas*, *Proteinphilum*, *Bellilinea* and *Longilinea* was negatively with esters such as pentanoic acid ethyl ester, benzeneacetic acid ethyl ester, ethyl acetate, and hexanoic acid and butyric acid ($p < 0.05$). Fig. 5B showed that one substance was positively correlated with several different genera, which was consistent with the result that some flavor compounds (such as ethyl lactate and ethyl isobutyrate) could be generated by co-fermentation of multi microbes (Kong et al., 2014). It is mainly due to the synthetic pathway of certain compounds requiring the enzymes produced by a variety of microorganisms (Hu et al., 2020).

4. Conclusion

This study comprehensively investigated the microbial succession, metabolites and flavor compounds composition of 30-,100- and 300-year PMs. *Longilinea*, *Lactobacillus*, *Clostridium*, *Caproiciproducens* and *Methanobacterium* were key differential microorganisms with spatial differences in 30-,100- and 300-year PMs. Lactic, acetic, butyric and caproic acid were considered as the key physicochemical factors for driving the microbiota succession. Meanwhile, Succinic acid, phenylalanine and S-Acetyldihydrolipoamide-E were key differential metabolites and relatively abundant at the bottom of the pit. Hexanoic acid methyl ester, ethyl acetate, hexanoic acid ethyl ester, butanoic acid hexyl ester and hexanoic acid, butanoic acid and heptanoic acid were key differential flavor compounds in 30-,100- and 300-year PMs. Moreover, hexanoic, octanoic acid and their corresponding esters were concentrated on 300-year PMs. The results of this study provided a reference for the precise quality improvement of young PM in the future.

Authors' contributions

Original draft, D.L.R.; Writing - review & editing, S.P.L.; Data curation, writing -review & editing, X. H.; Funding acquisition, J.M. The authors read and approved the final manuscript.

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CRediT authorship contribution statement

Dongliang Ren: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Shuangping Liu:** Writing – review & editing. **Hui Qin:** Investigation, Visualization. **Suyi Zhang:** Resources, Supervision. **Mengyang Huang:** Software,

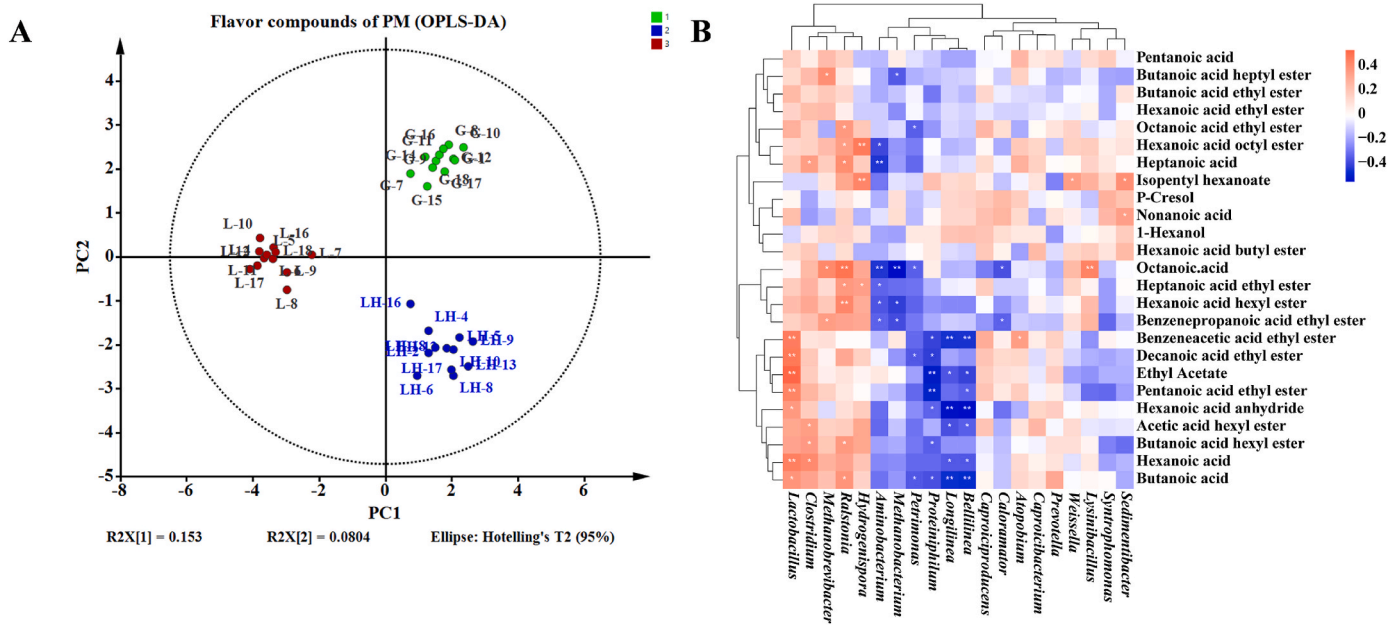

Fig. 5. (A) OPLS-DA analysis of flavour compounds in PM. (B) Spearman analysis between the microbiota and flavor compounds.

Validation. **Xiao Han:** Data curation, Writing – review & editing. **Jian Mao:** Funding acquisition, Resources, 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

Data will be made available on request.

Abbreviations

PM	Pit Mud
LC-MS	liquid chromatograph-mass spectrometer
GC-MS	Gas-chromatography-mass spectrometry
LEfSe	LDA Effect Size
RDA	redundancy analysis
NMDS	Non-metric multidimensional scaling
PCA	Principal Components Analysis
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
VIP	Variable importance in the projection

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.103413.

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