Multi-Omics Reveals Microbial Roles and Metabolic Functions at the Spatiotemporal Niche in Pit Mud

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Abstract

Background

Popular distilled Chinese strong-flavoured liquor (CSFL) is produced by solid fermentation in ground pits. Pit mud (PM), as a habitat for microbes, plays an important role in the production of CSFL. However, understanding the taxonomic composition, metabolic potential, and functional diversity of core microbes of PM in spatiotemporal niche remains a major challenge.

Results

Using a multi-omic approach of high-throughput full-length 16S rRNA, ITS sequencing, and metagenomics, we identified bacteria such as *Caproiciproducens*, *Clostridium*, *Lactobacillus*, *Bellilinea*, *Petrimonas*, *Proteiniphilum*, and *Prevotella* and the archaea *Methanobrevibacter* and *Methanobacterium* as the core microbiota in 30-, 100-, and 300-year-old cellars. Significant correlations between microbial communities and environmental factors showed that lactic, caproic, butyric, and acetic acids were the core driving forces for microbiota succession in different spatial locations and were mainly correlated with *Caproiciproducens*, *Clostridium*, *Lactobacillus*, *Methanobrevibacter*, and *Methanobacterium*. A total of 982 metabolites were detected using GC/MS and LC/MS, mainly including amino acids, peptides, and fatty acids, and correlations were shown between seven microorganisms and 12 amino acids and fatty acid metabolites. The crucial genes of flavour-relevant and substrate degradation pathways mainly included nine microorganism orders, with the most important being *Clostridiales, Bacteroidales*, and *Methanobacteriales*. However, only *Clostridiales* and *Bacillales* were potentially involved in alcohol–aldehyde–carboxylate–ester metabolism.

Conclusions

The 30-, 100-, and 300-year-old cellars provided an ideal opportunity to understand the effect of cellar age on the microbial composition and functional diversity of the microorganisms in PM. Our detailed metagenomic analyses across the continuously used cells extend the known diversity of microorganisms involved in flavour generation and substrate degradation over a wide range of environmental conditions. The results indicate that *Clostridiales* and *Bacteroidales* are major microbiota orders for flavour generation and substrate degradation pathways, while archaea also play an important role in cooperation with bacteria in various flavour generation pathways. This study helps elucidate the core microbiota composition, different metabolic roles of microorganisms, and the formation mechanism of PM partial functions, thus providing a basic theory to support the regulation of Baijiu production.

Background

Numerous fermented foods, such as cheese, wine, bread, and yoghurt, have been consumed for years. These fermented foods are produced with complex microbiota from raw materials and the environment[1]. Chinese liquors are traditional fermented distilled spirits and are classified into four basic aroma types: strong, light, soy sauce, and rice aroma types. Chinese strong-flavoured liquor (CSFL), also called 'Luzhou-flavour liquor', is famous for its distinctive flavour and unique liquor culture and accounts for more than 70% of Chinese liquor production[2, 3]. CSFL production is a typical recycling process that uses solid-state fermentation (Fig. 1). In brief, raw material mixtures are anaerobically fermented for 45–90 days or longer in a specialised rectangular soil pit (Fig. 1) lined with pit mud (PM)[4]. Fermentation involves numerous microbes originating from the PM and Daqu[1]. PM, a fermented clay, is considered a small-scale micro-ecosystem that contains various microbes, contributing to the simultaneous saccharification and alcoholic fermentation of raw materials. Microbes in the PM can produce complex and diverse metabolites such as small-molecule organic acids (e.g., caproic, butyric, acetic, and lactic acids), which are the key precursors of flavour esters, to largely determine the style and quality of CSFL[2, 5–8]. Many studies have elaborated that the class *Clostridia* such as *Clostridial cluster* IV and *Clostridium* are crucial microbes in PM and are responsible for the formation of various aromatic compounds (e.g., fatty acids, alcohols, and phenols) in CSFL[9–11]. *Clostridial* microbes can utilise ethanol, lactic acid, and acetic acid as substrates to synthesise hexanoic and butyric acids [12, 13]. *Lactobacillus* is the dominant genus responsible for ethanol oxidation and lactate production[14]. With the degradation of starch and other macromolecules during fermentation, Huangshui, a leaching solution of fermented grains, accumulates with CSFL fermentation as by-product, which contains a variety of microbial metabolites such as lactic acid, ethanol, and acetic acid[15], which can be further metabolised by PM microbiota. In general, CSFL quality also improves with increasing cellar age; high-quality liquor is produced only in old cellars, which are maintained for at least 20 years by continuous use[16]. In particular, some cellars have been used for several hundred years without interruption: for example Wuliangye, Jiannanchun, and Luzhou Laojiao are brewed in such long-aged cellars[17, 18], and PM with high quality has a stable and robust microbial structure[19].

Recently, great progress has been made in investigating the microbial composition of PM. Taxonomic studies using culture- and molecular-based (such as sequencing and profiling of 16S rRNA genes and metagenomics) methods have shown that bacteria and archaea are core populations in the PM microbial community. The dominant prokaryotic phyla mainly consists of bacterial phyla such as *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, *Synergistetes*, *Proteobacteria*, and other unclassified bacteria, which include *Clostridium*, *Caproiciproducens*, *Ruminococcus*, *Caloramator*, *Sedimentibacter*, *Syntrophomonas*, *Lactobacillus*, and *Petrimonas* genera, and archeal phylum *Euryarchaeota* that includes *Methanobrevibacter*, *Methanobacterium*, and *Methanoculleus* genera[14, 20]. It is noteworthy that *Firmicutes* predominate in many PM microbial communities[21, 22]. The genome approach is a reference-free assembly that provides a powerful tool for understanding the metabolic diversity of microbiomes and improves the taxonomic classification of microbiome populations[23, 24]. Subtle relationships between the environment and microbial species can affect the microbial community structure[25], and the

moisture content in the pit can directly affect the pH, humus, and growth of the microbial flora. The proper pH of PM not only promotes CSFL fermentation but also facilitates the formation of aroma precursor substances and improves the quality of the original liquor. Microorganisms are continuously enriched, and additional populations are derived during the long-term reciprocating fermentation process. In fact, the interaction between these microorganisms and physicochemical indices has formed a complex microbial ecosystem[26].

Although many studies have elaborated the microbiota composition and the factors driving the microbiota succession in PM, previous studies have only focused on the microbiota composition in the whole cell without considering the spatial locations. Moreover, only limited knowledge of the microbial composition in different spatial locations of PM is available, and the factors that drive the microbiota succession in PM with hundreds of year intervals need to be clarified. Concurrently, little is known about the metabolic potential and functional properties of the PM microbiota owing to the lack of knowledge of complex microbiota interactions. Therefore, understanding the relationship among microbes, metabolites, and functional enzymes in PM remains a major challenge.

To address some of these problems, using a multi-omic approach, this study aimed to (1) characterise the microbiota community composition, analyse the driving factors for microbial succession, and reveal the relationships between chemical properties and microbial community in different PMs; and (2) investigate the differential metabolites and functional enzymes produced by microorganisms related to flavour generation and substrate degradation pathways.

Results

Analysis of PM physicochemical factors

The levels of PM moisture, TN, AP, pH, and acetic, caproic, butyric, and lactic acid content from different cellar samples changed significantly with increasing age of the cellar (Table 1, Fig. 2). pH is an important factor that has a cumulative effect on the properties of PM; however, it did not show differences in different spatial locations in the 30-, 100-, and 300-year-old cellars ($p>0.05$). The relative moisture content showed a significant difference in the 100- and 300-year-old cellars. The moisture content in position 4 was significantly lower than that in positions 1 and 2 ($p<0.05$). For the same positions, the moisture content in the 300-year-old cellars was slightly higher than that in the 100- and 30-year-old cellars, but the difference was not significant ($p>0.05$). The moisture content produced by microbial 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 at other spatial positions. Nutrients are also important contributors to the biochemical properties of PM. The concentration of TN at position 4 was significantly lower than that at other positions in the 30-, 100-, and 300-year-old cellars ($p<0.05$). Notably, the concentration of TN at position 1 in the 300-year-old cellar was significantly lower than that in the 30- and 100-year-old cellars. The concentration of AP at position 1 in the 100- and 300-year-old

cellars was significantly higher than that at positions 2, 3, and 4 ($p<0.05$) and at positions 2 and 4, respectively.

Organic acids are important precursors of esters in CSFL and inevitably affect the quality of CSFL. We compared the organic acid contents spatiotemporally in the PM. The acetic acid content (Fig. 2A) was significantly higher at position 3 ($p<0.05$) and position 1 ($p<0.05$) than that at other positions in the 30- and 100-year-old cellars and 300-year cellars, respectively. The butyric acid content (Fig. 2B) showed no significant spatial difference in the 30-year-old cells ($p> 0.05$) but was significantly higher at position 1 than that at other spatial locations ($p<0.05$) in the 100- and 300-year-old cellars. The caproic acid (CA) content (Fig. 2C) showed different trends in the different cellar types. The CA content at positions 2 and 1 was significantly higher than that at other positions ($p <0.05$) in the 30- and 300-year-old cellars, respectively. As for lactic acid content (Fig. 2D), positions 4 and 1 showed significantly higher levels than other spatial locations in the 30- ($p>0.05$) and in the 100- and 300-year-old cellars ($p<0.05$), respectively. In summary, organic acids accumulated at the bottom of the pit with continuous CSFL fermentation, leading to a high content of organic acid at the bottom of the pit, when compared with other spatial locations. In addition, the total organic acid content increased with the continuous use of pits.

Microbial succession in different cellars

Spatiotemporal differences of microbial diversity in PM

Succession patterns of PM microbial communities in 30-, 100-, and 300-year-old cellars were investigated. High-throughput sequencing was applied to obtain information on the active microbial community in the three cellar types at four positions, and sequence similarity thresholds were used to define the OTUs. Based on the sequencing results, the total reads were correlated with bacterial and archaeal phyla. The *Good's* coverage estimator revealed that 91.72–94.05% of bacterial OTUs were obtained in all PM samples, which indicates that the generated data were reasonable reflection of the microbiota community. In addition, the bacterial Shannon index with a range of 5.41±0.96 to 5.48±0.86 and the bacterial Simpson index with a range of 0.9±0.07 to 0.95±0.02 at different PM samples were obtained, and there were significant differences ($p<0.05$) among the different PM samples (Table 1), suggesting similar overall species diversity.

For bacterial community composition, only prokaryotic abundance differences were found, without prokaryotic structural differences. At the phylum level, *Firmicutes*, *Bacteroidetes*, *Euryarchaeota*, *Actinobacterium*, *Chloroflexi*, and *Proteobacteria* were predominant in the 30-, 100-, and 300-year-old PM samples. At the genus level, 221 genera were detected. Bacterial genera such as *Caproiciproducens*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Weissella*, *Sedimentibacter*, *Atopobium*, *Brachybacterium*, *Ralstonia*, *Acinetobacter*, *Proteiniphilum*, *Anaerocella*, *Prevotella*, *Bellilinea*, *Petrimonas*, *Staphylococcus*, *Caloramator*, *Lysinibacillus*, *Caldicoprobacter*, *Caloribacterium* and archeal genera such as *Methanobacterium* and *Methanobrevibacter* were detected with relative abundances higher than 0.1% in the 30-, 100-, and 300-year-old PM samples (Fig. 3). In this study, we defined core microbiota genera as

those detected in all PM samples and with relative abundance higher than 1.0% in all samples. Based on this, the core microbiota genera were *Lactobacillus*, *Caproiciproducens*, *Clostridium*, *Proteiniphilum*, *Prevotella*, *Bellilinea*, *Petrimonas*, *Methanobacterium,* and *Methanobrevibacter* and accounted for 60.28%, 84.39%, and 80.65% in the 30-, 100- and 300-year-old PM samples, respectively. Notably, *Prevotella*, *Bellilinea*, *Petrimonas*, *Methanobacterium*, and *Methanobrevibacter* were increased in the 100- and 300-year-old PM samples when compared with those in the 30-year-old PM samples, especially for *Methanobacterium* and *Methanobrevibacter*. We further investigated the differential microbes among the three PM samples. The results (Fig. 3D) indicated that *Proteiniphilum*, *Caproiciproducens*, *Methanobacterium*, and *Methanobrevibacter* were the main differential microorganisms among the 30-, 100-, and 300-year-old samples. With the continuous use of the cellars, microbes will inevitably undergo succession progress. We calculated the beta nearest taxonomic index (βNTI) value to compare the influence of the two (deterministic and stochastic) assembly processes of the 30-, 100-, and 300-year-old cellars (Fig. 3E) to compare and analyse the differences in the construction of microbial communities in the different PM cellars. The results showed that both deterministic and stochastic processes promoted the assembly of the microbiome in the PM cellars, with the pit used for a long time, especially for 300-year-old cellars, the microbial composition follows a deterministic process.

Microbial diversity in different spatial locations

The cellar environment changed with the CSFL fermentation process, such as the Huangshui generation. It gradually changed from a micro-anaerobic to an anaerobic environment, leading to a diversity of microbial communities in different spatial locations. At position 1 (Additional file: Figure S1-A), *Lactobacillus, Bellilinea, Methanobrevibacter, Petrimonas,* and *Prevotella* were found in the 30-, 100-, and 300-year-old cellars. *Petrimonas* (13.18%) showed a higher relative abundance in the 100-year-old PM samples than that in the 30- and 300-year-old PM samples. Moreover, *Lactobacillus* (41.82%) and *Prevotella* (1.78%) were higher in the 300-year-old PM samples than in the 30- and 100-year-old PM samples, but the relative abundance of *Bellilinea* (0.7%) for the 300-year cellar was lower than that for the 30- and 100-year samples. In contrast, *Bacillus*, *Weissella*, *Brachybacterium*, *Staphylococcus*, *Anaerocella*, *Caloramator*, *Caldicoprobacter*, and *Caloribacterium* were only detected in the 30-year, 100-year, and 300-year-old PM samples, respectively.

At position 2 (Additional file: Figure S1-B), the dominant microbes were *Lactobacillus*, *Bellilinea*, *Methanobrevibacter*, *Methanobacterium*, *Petrimonas*, *Caproiciproducens*, and *Prevotella*. The *Prevotella* (12.38%) and *Lactobacillus* (12.34%) in the 30-year-old samples were higher than in the 100- and 300-year-old PM samples. *Bellilinea* (18.04%), *Petrimonas* (21.18%), and *Methanobrevibacter* (30.16%) in the 300-year-old samples were higher than in the 30- and 100-year-old samples, and *Methanobacterium* (9.96%) and *Caproiciproducens* (15.63%) in the 100-year-old samples were higher than in the 30- and 300-year-old samples. *Anaerocella* and *Lysinbacillus* were only detected in the 30- and 300-year-old samples, respectively.

At position 3 (Additional file: Figure S1-C), the core microbes were *Bellilinea*, *Methanobrevibacter*, *Methanobacterium*, *Petrimonas*, *Proteiniphilum*, *Clostridium*, and *Prevotella*. *Prevotella* (26.45%) and *Methanobacterium* (9.28%) in the 30-year-old samples were higher than in the 100- and 300-year-old samples, whereas *Proteiniphilum* (14.27%), *Bellilinea* (29.09%), and *Clostridium* (6.68%) in the 300-year-old samples were higher than in the 30- and 100-year-old samples. *Methanobrevibacter* (41.79%) in the 100-year-old PM samples was higher than in the 30- and 300-year-old PM samples.

At position 4 (Additional file: Figure S1-D), the core microbes were *Bellilinea*, *Methanobrevibacter*, *Petrimonas*, *Lactobacillus*, and *Prevotella*. *Petrimonas* (19.27%) and *Prevotella* (14.53%) in the 30-year-old PM samples were higher than in the 100- and 300-year-old PM samples, and *Methanobrevibacter* (52.33%) and *Lactobacillus* (21.78%) in the 100-year-old samples were higher than in the 30- and 300-year-old samples. Moreover, *Bellilinea* in the 300-year-old PM samples was higher than in the 30-and 100-year-old PM samples.

Differences in the physicochemical properties of different spatial positions in the pit, such as oxygen, moisture content, nutrients, and the contact between the *jiupei* (fermented grains) and PM, will inevitably lead to differences in microbial composition and abundance. Notably, the relative abundance of the same microorganism is also different in the use of the same year pit. In summary, the microbiota composition among the four spatial locations were different; for example, *Caproiciproducens* and *Lactobacillus* were concentrated at the bottom of the cellar, while *Proteiniphilum* and *Clostridium* were highly abundant at positions 3 and 4. In addition, some genera were only detected in specific spatial locations, indicating the complex interactions between the microorganisms. Moreover, the microbial composition also correlated with the physicochemical environment in the cellars, and the diversity of microbes could influence the flavour substances in CSFL.

After studying the microbial communities of different PM samples, we further evaluated the similarities and differences in the microbiota using a Bray-Curtis approach. A non-metric multi-dimensional scaling analysis revealed that all samples in bacteria were clustered into two parts and showed year-dependent clustering (Fig. 4A). In summary, samples from the 30-, 100-, and 300-year-old samples were centralised and formed clusters, especially for 30- and 100-year-old samples, suggesting that samples from different years are possibly in different transitional states and bacterial communities in the 30- and 100-year-old cellars had similar microbial compositions. The microbial community continues to evolve over time, stabilises, and gradually forms a stable microbial environment. Therefore, the age of the cellar is the main factor driving microbial succession.

Relationships of microbial community with physicochemical properties

Environmental factors in fermentation can affect the growth and metabolism of microorganisms. Hence, physicochemical factors (pH, moisture content, TN, AP, acetate, caproate, butyrate, and lactate) were selected and analysed to reveal their connection with the microbiota community (Fig. 4B). The results indicate that the contents of lactate, butyrate, acetate, and caproate significantly affected microbial succession change ($p<0.05$) and that lactate and caproate

contents were positively correlated with prokaryotic communities in the 30-, 100-, and 300-year-old samples at positions 1 and 2. Moreover, butyrate and acetate contents were positively correlated with prokaryotic communities in the 30- and 100-year-old PM samples at position 2.

To clarify the relationships between specific genera and driving factors, we analysed their correlation using Spearman's coefficient ($p<0.05$). Organic acids significantly affected the core microbiota *Lactobacillus*, *Caproiciproducens*, *Clostridium*, *Proteiniphilum*, *Prevotella*, *Bellilinea*, *Petrimonas*, *Methanobacterium*, and *Methanobrevibacter*, while TN, AP, and moisture content affected a few families. Specifically, at position 1 (Additional file: Figure S2-A), lactic acid was positively correlated with levels of *Caproiciproducens*. In addition, butyric acid and acetic acid were positively correlated with *Methanobacterium* and *Caproiciproducens*, Caproic acid was negatively correlated with *Petrimonas*, and TN was negatively correlated with *Prevotella* and *Lactobacillus*. At position 2 (Additional file: Figure S2-B), lactic acid was negatively correlated with *Clostridium* yet positively correlated with *Lactobacillus*, butyric acid was negatively correlated with *Petrimonas* yet positively correlated with *Prevotella*, acetic acid was positively correlated with *Lactobacillus* and *Caproiciproducens*, CA was negatively correlated with *Petrimonas* yet positively correlated with *Lactobacillus*, and moisture content was negatively correlated with *Prevotella*. At position 3 (Additional file: Figure S2-C), lactic acid and AP were the two important factors influencing microbial community composition. For example, lactic acid was positively correlated with *Methanobrevibacter*, but AP was negatively correlated with *Ralstonia*, *Bellilinea*, and *Proteiniphilum* and negatively correlated with *Clostridium*. At position 4 (Additional file: Figure S2-D), CA and butyric acid were negatively correlated with *Methanobrevibacter*, while butyric acid and lactic acid were positively correlated with *Methanobacterium*.

In summary, physicochemical factors such as TN, AP, and organic acids played an important role in determining the microbiota composition, and microbes in different spatial locations were affected by different factors, because the microbial composition differed spatially.

Metabolomic analysis of PM

In the untargeted metabolomics analysis, we identified 5991 metabolites, of which 714 were identified by gas chromatography (GC-MS), and 5277 were identified by liquid chromatography (LC-MS) from PM samples collected over different years that are continuously used. After filtering and quality control (deletion of unknown compounds), 982 metabolites were left for further analysis.

Classification of total metabolites

We first classified the metabolites detected by GC-MS and LC-MS. The results showed that amino acids, peptides, and analogues, fatty acids, and conjugates were the main metabolites and accounted for approximately half of the metabolites (Additional file: Figure S3). Metabolites from PM also contribute to the liquor flavour. Fatty acids, amino acids, and peptides are decisive compounds in CSFL because they can produce flavour compounds as precursors in CSFL production.

Analysis of differential metabolites

We selected the metabolites according to the standard of P value <0.1 and VIP value> 1 to obtain the differential metabolites in different cell types.

In the position under the Huangshui fluid, there were 129 differential metabolites in the 30- and 100-year-old cellars (Additional file: Figure S4-A), of which 105 were up-regulated and 24 were down-regulated. There were 86 differential metabolites in the 30- and 300-year-old cellars (Additional file: Figure S4-B), of which 60 were up-regulated and 26 were down-regulated. Of 100 differential metabolites found in the 100- and 300-year-old cellars (Additional file: Figure S4-C), 41 were up-regulated and 59 were down-regulated.

In the position of the Huangshui fluid, 68 differential metabolites were found in the 30- and 100-year-old PM samples (Additional file: Figure S4-D), of which 29 were up-regulated and 39 were down-regulated, and of the 247 differential metabolites in the 30- and 300-year-old cellars (Additional file: Figure S4-E), 95 were up-regulated and 152 were down-regulated. There were 178 differential metabolites in the 100- and 300-year-old cellars (Additional file: Figure S4-F), of which 77 were up-regulated and 101 were down-regulated.

Analysis of differential metabolic pathways between different cellars

The main flavour compounds in CSFL are esters, alcohols, aldehydes, ketones, phenol, and pyrazine compounds. According to our results, differential metabolites were related to a variety of metabolic pathways. We selected differential metabolites for their potential participation in the substrate degradation and flavour development of PM. Dodecanoic, caprylic, myristic, arachidonic, γ-linolenic, and eicosapentaenoic acids were related to the biosynthesis of fatty acids and unsaturated fatty acid pathways, while threonine, asparagine, phenylalanine, isoleucine, aspartic acid, valine, proline, and phenylpyruvic acid were related to the biosynthesis of amino acids. Malic acid, succinic acid, and γ-aminobutyric acid were related to the butanoate and pyruvate metabolism pathways. The relative concentrations of the metabolites were also compared. Under the Huangshui fluid (Table S1), compared to the 30-year-old samples, the compounds γ-aminobutyric acid, threonine, proline, phenylalanine, dodecanoic acid, succinic acid, and valine were increased in the 100-year-old PM samples and decreased in the 300-year-old PM samples. The concentrations of arachidonic acid, eicosapentaenoic acid, caprylic acid, malic acid, myristic acid, isoleucine, glyceric acid, and β-hydroxypyruvate in the 30-year-old PM samples were higher than in the 100- and 300-year-old PM samples. The concentrations of phenylpyruvic acid and γ-linolenic acid in the 300-year-old samples were higher than in the 30- and 100-year-old PM samples. At the position of the Huangshui fluid (Table S2), compared to the 30-year-old samples, the compounds dodecanoic acid and α-linolenic acid were increased in the 100-year-old PM samples and decreased in the 300-year-old samples. The concentrations of homoserine, saccharopine, threonine, succinic acid, and aminoadipic acid in the 30-year-old PM samples were higher than in the 100- and 300-year-old PM samples. The concentrations of γ-linolenic acid, aspartic acid, caprylic acid, glucuronic acid, myristic acid, asparagine, and arachidonic acid in the 300-year-old PM samples were higher than in the

30- and 100-year-old PM samples. Figure 6A shows the reciprocal connections between the differential metabolites and KEGG pathways.

We further analysed the relationship between core microbiota and PM metabolites (Additional file: Figure S5). The correlation coefficients indicated a strong relation (0.6<|Spearman's|<1). Seven of the nine main genera were involved in the distribution of certain metabolites. Positive correlations were observed between *Caproiciproducens* and isoleucine, gluconic lactone, and glyceric acid, *Lactobacillus* and CA, *Methanobrevibacter* and lactic acid, and *Bellilinea* and *Proteiniphilum* and caprylic acid.

Metagenomic analysis

Metagenomic microbial community composition

Among all sequences that passed the quality control, 62.15–64.1%, 21.81–27.92%, and 0.01% were identified as fragments originating from bacteria, archaea, and eukaryotes, respectively. The dominant bacterial phyla were *Firmicutes* (19.84–35.71%) and *Bacteroidetes* (8.11–19.57%). The archaeal reads were mainly affiliated with *Euryarchaeota* (21.75–27.8%). A small proportion of eukaryotic reads (less than 0.1%) were detected in the PM samples. This indicated that the PM microbiota was dominated by prokaryotes (Additional file: Figure S6).

Functional gene profiles of the PM metagenome

To investigate the metabolic potential of the PM microbiome, microbial genes aligned against the KEGG database among all PM samples were categorised into three levels of pathways based on metagenomic sequencing data. The functional gene profile at level 2 was mainly composed of carbohydrate metabolism, amino acid metabolism, energy metabolism, and translation metabolism in PM samples (Additional file: Figure S6-C).

We selected 135 enzymes from the KEGG database for their potential participation in the substrate degradation and flavour development of PM, and they were then grouped into 20 functional assemblies. Starch and sucrose metabolism are the primary sources of all compounds in the process of fermentation. According to starch and sucrose metabolism, the key enzymes alpha-amylase (EC 3.2.1.1) and glucan 1,4-alpha-maltohydrolase (EC 3.2.1.133) can catalyse starch hydrolysis to dextrin and maltose. The major producers of these two enzymes were *Clostridiales* (mainly *Clostridium*), *Anaerolineales* (mainly *Bellilinea*), *Methanomicrobiales* (mainly *Methanoculleus*), and *Methanosarcinales* (mainly *Methanosarcina*). Glucose is generally generated from starch during fermentation and is converted to pyruvate through the activities of aldose 1-epimerase (EC 5.1.3.3) and glucokinase (EC 2.7.1.2), while aldose 1-epimerase catalyses the interconversion of α- and β-terminal isomers of hexose, which are mainly produced by *Petrimonas*, *Prevotella*, and *Clostridium*. Moreover, glucokinase is related to the degradation of glucose and can catalyse glucose phosphorylation, which is mainly produced by *Petrimonas*, *Clostridium*, *Prevotella*, *Methanoculleus*, and *Syntrophomonas*. The above-mentioned

microbiota genera were considered the major users of polysaccharides, and the generated glucose could be utilised by other taxa.

As for the flavour-generation metabolism pathways, CA is generally produced by anaerobic bacteria via the chain elongation pathway. The oxidation of ethanol can provide energy (acetyl-CoA) to sequentially elongate the carbon chain of carboxylic acids (acetic acid to n-butyric acid to CA). *Petrimonas*, *Methanoculleus*, *Syntrophomonas*, and *Methanobacterium* were the most prevalent genera that produced the enzymes (EC 2.3.1.9, 1.3.1.9, 2.3.1.16, and 4.2.1.55) involved in CA synthesis. Moreover, *Lactobacillus*, *Clostridium*, and *Methanoculleus* were the predominant genera that produced lactic acid from pyruvate, mainly catalysed by L-lactate dehydrogenase (EC 1.1.1.27) and D-lactate dehydrogenase (EC 1.1.1.28). In addition, lactate dehydrogenase (EC 1.1.2.3, EC 1.1.2.4), which can consume lactic acid to produce pyruvate, reduces the production of lactic acid. Conversely, *Sedimentibacter* and *Clostridium* were the main potential groups converting lactate back to pyruvate via D-lactate dehydrogenase (EC 1.1.2.4), while only a few species were annotated to produce lactate dehydrogenase (EC 1.1.2.3). *Petrimonas* was the predominant microbiota using pyruvate dehydrogenase (EC 1.2.4.1 and EC 2.3.1.12), which oxidised pyruvate to generate acetyl-CoA. This is a critical link in multiple metabolic pathways, contributing to aromas in CSFL, and can be oxidised to alcohol via acetyl-CoA synthetases (EC 6.2.1.13), aldehyde dehydrogenase (EC 1.2.1.3), and alcohol dehydrogenase (EC 1.1.1.1, EC 1.1.1.2). Among these, alcohol dehydrogenase is one of the most important enzymes involved in ester substance generation according to the alcohol–aldehyde–carboxylate–ester metabolism pathway. The gene coding for alcohol dehydrogenase was mainly enriched in microbes belonging to *Clostridium*. Butyrate is another basic aroma in CSFL and is produced from butyryl-CoA by the catalytic activities of acetate CoA transferase (EC 2.8.3.8) or butyrate kinase (EC 2.7.2.7). The genus source of butyrate kinase (EC 2.7.2.7) was *Caloramator*, *Petrimonas*, and *Sedimentibacter*, while the genus source of acetate CoA-transferase (EC 2.8.3.8) was *Petrimonas*, *Sedimentibacter*, and *Proteiniphilum*. Genes of various pathways to form acetate were detected in the microbiota, with acetyl-CoA and acetyl-P as intermediates. The key enzymes aldehyde dehydrogenase (EC 1.2.1.3), acetate kinase (EC 2.7.2.1), acetate-CoA ligase (EC 6.2.1.13), and pyruvate dehydrogenase (EC 1.2.5.1) involved in acetate metabolism were mainly produced by *Clostridiales*, *Bacteroidales*, and *Anaerolineales*, while acetyl-CoA synthetase (EC 6.2.1.1) was mainly produced by *Methanobacteriales*, *Methanosarcinales*, and *Methanomicrobiales*. The critical enzymes acetate CoA-transferase (EC 2.8.3.18) and acetyl-CoA synthetases (EC 6.2.1.13) helped acetyl-CoA change into acetate. Archaea were also the dominant microbes in the 30-, 100-, and 300-year-old PM samples, while cdhA (EC 1.2.7.4), acetyl-CoA synthetase (EC 6.2.1.1), and formate dehydrogenases (EC 1.17.1.9 and EC 1.17.1.10) were the most important enzymes related to methane metabolism. Acetyl-CoA synthetase (EC 6.2.1.1) catalyses the conversion of acetic acid to acetyl-CoA, and formate dehydrogenase (EC 1.17.1.9) catalyses the conversion of formate to CO_2, while EC 1.17.1.10 catalyses the conversion of CO_2 to formate.

Metabolic network related to substrate–flavour metabolism in PM microbiota

To better explain the link among the 20 functional assemblies summarised in Fig. 5, a specific metabolic network in PM microbiota among the nine orders and 30 microbial genera is presented schematically in Fig. 6, showing the pattern of enzyme-coding genes involved in the substrate–flavour metabolic pathway.

Discussion

CSFL, a fermented and traditional distilled alcoholic beverage, is produced by spontaneous solid-state fermentation containing various microbes and their complex interactions. The standard production of Chinese liquor involves four steps, including cooking, saccharification, alcohol fermentation, and distillation, using grain as the main raw material. In CSFL production, the core microorganisms in PM play important roles in the characteristics of flavour metabolites and determine the quality and safety of CSFL to a large degree.

PM cellar is a microbial ecosystem that provides a natural model for a broader understanding of microbiota composition and succession. Combining 16S rRNA, ITS full-length sequencing, metabonomics, and metagenomic analysis may be valuable for elucidating the microbial community composition, differential metabolites, and the relationship between the microorganisms and the metabolites in PM samples. In our study, sequencing data revealed that PM microbiomes are commonly dominated by bacteria such as *Firmicutes*, *Euryarchaeota*, *Bacteroidetes*, *Chloroflexi*, *Petrimonas*, *Proteiniphilum*, *Prevotella*, *Bellilinea*, *Caproiciproducens*, *Clostridium*, and *Lactobacillus* and by archaea such as *Methanobacterium* and *Methanobrevibacter* (Fig. 3). Previous studies have shown that prokaryotic communities of 30-, 300-, 40-, and 400-year-old PMs were only found to have prokaryotic abundance differences, without prokaryotic structural differences between them[27], *and Clostridium, Caproiciproducens, Syntrophomonas, Sedimentibacter,* and *Methanobrevibacter* were clearly the predominant microorganisms in mature PM[28, 29]. The difference in the PM bacterial groups in different studies could be explained by a combination of methodological and environmental factors[30]. Along with the relatively steady eco-environment and microbiota structure formed through long-term fermentation progress, *Caproiciproducens*, *Prevotella*, and *Lactobacillus* were identified as the most important bacteria at the bottom of the pit, while *Clostridium*, *Bellilinea*, and *Proteiniphilum* showed a higher relative abundance in the Huangshui fluid of the pit. The accumulation of *Lactobacillus*, *Clostridium*, and other acid-producing microorganisms at the bottom of the pit leads to an increase in organic acid content (Fig. 2). It is notable that the relative abundance of *Methanobacterium* and *Methanobrevibacter* was 1.44% to 4.58% and 2.54% to 34.42% in our PM samples, respectively, but the average abundance of *Methanobrevibacter* was less than 0.05% in new PM samples in a previous study [31]. Low pH inhibits methanogenic activity in anaerobic biological processes[32]. The archaeal genera in PM can utilise H_2, acetate, methanol, and methylamine[33]. In addition to fermentative bacteria, methanogens can enhance organic acid production through syntrophic interactions[34].

For fungal microbiota, we did not detect fungi in the 30-, 100-, and 300-year-old PM samples. Previous studies have shown that fungi were not detected in the older PM and were only detected in the new cellars that were used for 2 years[18, 35]. There are several possible reasons for this: (1) the long fermentation environment is not suitable for the growth of fungi, and oxygen deficiency in the inner compartment partially leads to a low survival rate of fungi, especially *Aspergillus*[36]. (2) The presence of fungi in PM can also be attributed to the addition of the Daqu starter. It is known that the Daqu starter contains abundant microbes such as *Lactobacillus*, *Pseudomonas*, *Aspergillus*, *Saccharomyces*, *Wickerhamomyces*, and *Pichia*, and enzymes such as α-amylase, β-amylase, glucoamylase, and proteases[37, 38]. During long-term use of the pit, microbes may undergo exchanges between the PM and fermented grains during the fermentation process of the raw material. Therefore, bacteria are more dominant than fungi in older PM samples[9].

Function enzymes related to substrate degradation pathways

Glycolysis is a central pathway in carbohydrate metabolism and is regarded as a feeder that prepares glucose for further catabolism and energy conservation[39] and has many steps that lead to the catabolism of glucose into pyruvate[40], which diverges according to the availability of oxygen. Thus, the most dominant raw material synthesising the four dominant acids in PM is starch. In our study, the nine orders were all related to starch and sucrose metabolism and glucose utilisation pathway. Moreover, the absolute abundance of *Clostridiales*, *Bacillales*, and *Lactobacillales* were highest in the 300-year-old PM, suggesting that the amount of starch in the 300-year-old PM samples, which turned into the four dominant acids, was much greater than in the 30- and 100-year-old PM samples. The potential capacity for the comprehensive utilisation of different substrates may contribute to their dominance in the entire microbial community.

Function enzymes related to flavour generation pathways

Like beer and wine, the synergistic effect of microorganisms is thought to be responsible for the production of various flavour compounds, such as CA, butyric acid, acetic acid, lactic acid, and alcohols[3, 41], in CSFL production[42]. Previous studies have shown that pyruvate, ethanol, glutamate, and 4-aminobutanoate are related to the four dominant acid syntheses, especially pyruvate and ethanol[14, 43]. Thus, investigating the ethanol and organic acid metabolism pathways and the core enzymes is vital for improving CSFL quality. Ethanol is the main component of CSFL and is mainly generated through the conversion of pyruvate to acetaldehyde and reduction to ethanol. Some genera such as *Bacteroides*, *Porphyromonas*, and *Sedimentibacter* have been reported to produce succinic acid, propionic acid, and alcohols by using starch and glucose[44]. Increasing the content of ethyl CA is an effective method for improving the quality of CSFL. The common synthesis method of CA can occur via carboxylic acid chain elongation using ethanol as an electron donor[45]. Acyl-CoA hydrolase is the final enzyme in CA generation and has been previously observed to be enriched within *Clostridium spp.*, indicating that the *Clostridium* genus was the most important microbiota participating in CA production. Generally, some

of the most CA-producing *Clostridium* species include *C. kluyveri*[6] and *Clostridium* sp. BS-1[46]. In addition, the CA production is a process of H_2 and CO_2 production[47], and *C. ljungdahlii* could synthesize acetic acid and ethanol from H_2 and CO_2 produced by *C. clarkii*[48]. Concurrently, the presence of hydromethanogens and CA-producing bacteria can lead to interspecific hydrogen transfer, which is beneficial to increase CA production [14]. Thus, the co-occurrence of the high abundance of *Clostridium*, *Methanobacterium*, and *Methanobrevibacter* may indicate efficient CA production.

Lactic acid not only can inhibit some bacteria but also is an ethyl lactate precursor[49], and it contributes to the formation of other flavour substances[27]. *Clostridiales* (mainly *Clostridium*) and *Lactobacillales* (mainly *Lactobacillus*) were the important potential orders for producing lactic acid in our study. *Lactobacillus* produces lactic acids from sugar by homofermentative metabolism or produces alcohols in addition to lactic acid via heterofermentative metabolism[50]. In addition, ldh (EC 1.1.2.3, EC 1.1.2.4) could catalyse too much lactic acid to pyruvate and then generate acetic acid, butyric acid, and CA[51], which could contribute to the recovery of pH of PM and enhance the production of organic acids. Moreover, the decrease in lactic acid is due to its catalysis not only to pyruvate, but also to acetic acid through EC 1.13.12.4. Excess lactate can lead to high levels of ethyl lactate, which can deteriorate CSFL quality[8]. Key genes (e.g., alcohol dehydrogenase and L-lactate dehydrogenase) involved in reverse β-oxidation with both ethanol and lactate as substrates were found in PM samples, indicating that the PM microbiome possesses metabolic potential for CA production from ethanol and lactate. Our results verified the importance of lactic acid in CA production. *Lactobacillus* was positively correlated with CA (Additional file: Figure S5), because lactic acid is mainly produced by *Lactobacillus* and converted into CA by *Caproiciproducens*[52].

Butyric acid, one of the crucial flavour substances in CSFL, is synthesised through two alternative pathways, namely butyrate kinase (EC 2.7.2.7) and butyryl-CoA:acetate CoA-transferase (EC 2.8.3.8) [53]. A previous study showed that *Clostridium* was most likely involved in butyrate production through the buk pathway in PM[14]. In our study, besides *Clostridiales* and *Bacteroidales*, we also found that *Synergistales* (mainly *Aminobacterium*) have the ability to produce enzymes of butyrate kinase (EC 2.7.2.7) and butyryl-CoA: acetate CoA-transferase (EC 2.8.3.8). In addition, butyric acid can provide more precursors for CA synthesis[14]. For example, *Sedimentibacter* and *Aminobacterium* can ferment amino acids to acetic and butyric acids [54, 55]. Butanoate metabolism and pyruvate metabolism pathways were related to the production of CA, butyric acid, and lactic acid, and two metabolites, malic acid and succinic acid, were enriched, indicating their importance for the production of organic acids.

Ethyl acetate produced by acetic acid was also important for the formation of flavour in CSFL, and in addition to L-lactic acid, more acetic acid was produced by acetyl-CoA through the catalytic action of pct (EC 2.8.3.1) and acdA (EC 6.2.1.13). The major genus sources of butyrate kinase (EC 2.8.3.1) were *Clostridium* and *Lactobacillus*, which had a high absolute abundance in the 300-year-old cell, while the major genus sources of acdA (EC 6.2.1.13) were *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, *Bellilinea*, and *Syntrophomonas*. *Syntrophomonas* can degrade long-chain fatty

acids (C_4–C_8) into acetic acid, propionic acid, and H_2[56]. Furthermore, specific members of the *Clostridium* species were also identified with acetate biosynthesis from various substrates, such as sugar, starch, and cellulose.

Methane metabolism is also an important pathway during pit fermentation because it can enhance the production of organic acids during fermentation. *Methanosarcina* co-cultured with bacteria can metabolise carbon dioxide to methane[57]. *Methanobrevibacter* can produce CH_4 from H_2 and CO_2[58], and the interspecies hydrogen transfer between archaea and *Clostridiaceae* (mainly *Clostridium*) plays an important role in improving the quality of CSFL[59]. In our study, the *Methanomicrobiales* (mainly *Methanoculleus*) and *Methanobacteriales* (mainly *Methanobrevibacter* and *Methanobacterium*) were the most abundant microbiota orders participating in methane metabolism. Further, the genera *Caloramator* and *Clostridium*, belonging to *Clostridiaceae*, mainly produced the enzymes EC 5.4.2.12 and 1.17.1.9. In our study, *Methanobrevibacter* was positively correlated with lactic acid by metabonomics (Additional file: Figure S5), indicating that *Methanobrevibacter* played an important role in the production of lactic acid.

Environmental factors drive microbiota succession in PM

Microbial community succession is a complex long-term process. Physicochemical factors are believed to be the main determinants that influence the shift of the microbiota community and structure in the soil and PM[19]. This study revealed that organic acids such as acetic acid, CA, butyric acid, and lactic acid were the most important indicators influencing microbial community structure in the 30-, 100-, and 300-year-old PM samples (Fig. 4B). The organic acid content was highest at position 1 of the cellar, especially in the 300-year-old PM samples (Fig. 2). Previous studies revealed that lactic acid and pH were the two important factors influencing microbiota composition in 1-, 10-, 25-, and 50-year-old PM samples. Hartman et al.[60] also found that pH was the most essential environmental factor influencing the bacterial community structure in soil, and pH broadly altered the prokaryotic diversity and affected specific taxa, including *Acidobacteria* and *Actinobacteria*. In our study, pH and moisture were stable in the cellars and influenced a few bacteria. The moisture content can affect the microbiota composition, which may be related to the lack of free water available to PM microorganisms. In our study, the moisture content gradually increased with cellar use and was higher in positions 1 and 2 than in other spatial locations, especially for the 100- and 300-year-old cellars. This may be because PM was soaked in Huangshui for a long time during long-term CSFL fermentation, thus leading to a high moisture content. Simultaneously, ammonium nitrogen can provide a source of nitrogen for microbial growth and has an important influence on the abundance of PM microbiota[19]. The concentrations of TN and AP increased with the cellar age and showed certain spatial rules; the content of AP and TN in position 1 was higher than in other spatial locations. Therefore, the microbial community structure can be adjusted by adjusting environmental factors such as moisture and pH of the PM to achieve the purpose of increasing CA and decreasing lactic acid content[61]. Consequently, a challenge in Chinese liquor production is to achieve a reduction in ethyl lactate synthesis during CSFL production.

In our study, we investigated the core microbiota composition in PM using sequencing technologies and identified metabolites using GC-MS and LC-MS. Amplicon analysis revealed fundamental information about microbial succession, and RDA revealed positive correlations between the core microbiota and major endogenous factors. The metagenomic data illustrated the effectiveness of the analysis in increasing our understanding of the possible mechanisms responsible for the microbial community structure in PM, providing a reference for further utilisation of PM microbiota genetics and paving the way for the optimisation of CSFL production. However, a limitation of this study is that some microorganisms present throughout the process may have died and left their DNA in the samples. This process requires further exploration via isolation of microbes and functional genomic analysis.

Conclusions

These cellars have been used for several years without interruption, providing an ideal opportunity to uncover the effect of cellar age on the microbial succession and functional diversity of the microorganisms in PM. Although the different cellars harboured the taxonomically and functionally diverse and abundant microbial community, the functional microbiota related to flavour generation as a hotspot for microbial diversity, genes for flavour generation, and substrate degradation were identified throughout the 30-, 100-, and 300-year-old cellars. However, metagenomic datasets revealed that the pathways and taxa involved in flavour generation and substrate degradation were complex and diverse. *Clostridiales* (mainly *Clostridium*) and *Bacteroidales* (mainly *Petrimonas* and *Proteiniphilum*) are major microbiota orders for flavour generation and substrate degradation pathways, while archaea also play an important role, in cooperation with bacteria, in various flavour generation pathways. Our data help elucidate the core microbiota composition, different metabolic roles of microorganisms, and disclose the formation mechanism of PM partial functions, providing a basic theory to support the regulation of Baijiu production.

Methods

Sample collection

PM samples were obtained from an SFB-producing enterprise in Luzhou, the south-eastern part of Sichuan Province, China. The cellar was approximately 2.8 m length, 1.8 m width, and 2.0 m height (Fig. 1). We sampled the PM from different fermentation pit cellars that have been used continuously for 30, 100, and 300+ years. We shovelled the PM samples 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 samples were collected at approximately 400 g. After collection, the samples were sufficiently mixed and placed in a sterilising bag, placed in a foam box filled with dry ice, and finally returned to the laboratory for total DNA extraction.

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[62]. Total nitrogen was extracted with Seignette salt and ammonium chloride and its concentration measured using UV-visible spectrophotometry. Organic acids such as Caproic acid, butyric acid, and acetic acid in the PM were detected by GC-MS: 1 g PM samples and 2 mL 60% ethanol were placed in a 5-mL centrifuge tube with a vortex mixer. After 30 minutes of extraction, the mixture was centrifuged at 10000 rpm for 10 minutes. Afterwards, the supernatant was collected through the membrane for GC-MS analysis. The conditions were as follows: the inlet temperature was 230℃, the carrier gas was high-purity helium, and the flow rate was 1.0 mL/min. Moreover, the following gradient conditions were used: a temperature of 35℃ maintained for 10 min and then increased to 60℃ at 4℃/min and maintained for 4 min. The temperature was further increased to 195°C at 6°C/min for 20 min. Lactic acid in the PM was quantified using HPLC: 1 g PM samples and 2 mL ultra-pure water were placed in a 5-mL centrifuge tube, and after 30 min of extraction, the supernatant was collected through the membrane for HPLC analysis, as described previously [63].

DNA extraction, 16S rRNA gene, and ITS amplicon sequencing

Genomic DNA of PM samples was extracted using a FastDNA spin kit for soil, according to the manufacturer's instructions. The concentration and purity of DNA were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA) and 1% agarose gel electrophoresis, and the library was constructed and sequenced after passing the test. To analyse the microbial community, a target variable region of the small ribosomal subunit RNA gene was PCR-amplified with the KAPA HiFi Ready Mix (Kapa Biosystems, KK2602). The universal primers (16S: Forward Primers sequences AGRGTTYGATYMTGGCTCAG, Reverse primers sequences RGYTACCTTGTTACGACTT and ITS: Forward Primers sequences TCCGTAGGTGAACCTGCGG, Reverse primers sequences TCCTCCGCTTATTGATATGC) with the barcodes were used to amplify the bacterial 16S rRNA and ITS regions of fungal rRNA, respectively. The PCR product was then purified by 0.6x AMPure PB Beads, and the library was constructed using the SMRTbell Express Template Prep Kit 2.0 (PacBio) with damage repair, end repair, A-tailing, and ligation of sequencing adapters. The sequencing was performed on a PacBio Sequel II instrument with Sequencing Primer V4 and Sequel II Binding Kit 2.1 in Grandomics at Wuhan Biotechnology Co., Ltd (Wuhan, China).

16S rDNA and ITS amplicon data analyses

The original FASTQ file was processed using QIIME software[64], and the low-quality sequences were filtered out. After analysing the sequencing data, the SILVA database (v13.2) was used to compare the 16S rRNA and ITS gene sequences to determine the taxonomic status of the corresponding microbes. QIIME software was used to define sequence similarity >97% as an operational taxonomic unit (OTU)[65]. Only OTUs containing at least five reads were considered valid in this study, and we selected a

representative sequence of each OTU for taxonomic analysis. The alpha diversity index was calculated to analyse species richness and uniformity in the samples, after which the Shannon and Chao1 indices of the samples were determined to obtain the diversity of the community and the number of OTUs in the samples. The genus-level clustering results for each sample were used as inputs for LEfSe analysis. Finally, the beta diversity index was determined to analyse the heterogeneity of community composition between samples.

Metagenomic sample preparation, processing, and assembly of metagenomic sequencing data

Metagenomic shotgun sequencing libraries were constructed and sequenced by Beijing Novogene Bioinformatics Technology Co. Ltd. Briefly, 8 μg DNA per sample was used as the input material for DNA sample preparations. Sequencing libraries were generated using the Ligation Sequencing Kit (SQK-LSK109) following the manufacturer's recommendations. DNA was disrupted with a Megaruptor (Diagenode, NJ, USA), and fragments greater than 10 kb were screened using BluePippin. After end repair and addition of A tail, the barcodes were added, and then the fragment length was detected. Samples with different barcodes were mixed in equimolar amounts, and the linker and template were purified. DNA concentration was measured using Qubit to complete the preparation of the DNA library. The barcoded samples were clustered using the PromethION Flow Cell Priming Kit (EXPFLP001.PRO.6) according to the manufacturer's instructions. After sequencing, the clean data were assembled and analysed by Flye (version: 2.4.2)[66] after data pre-processing, and the assembled scaftigs were checked with the clean data of every sample. Additionally, all clean data samples were compared to each scaffold using Bowtie 2.2.4 software to acquire the PE reads not used. Filter fragments shorter than 500 bp in all scaftigs for statistical analysis were generated from a single assembly. Every unigene sequence of PM samples was aligned to bacterial, archaeal, fungal, and viral sequences of the NCBI-NT library using BLASTN (*E*-value<0.001).

Metabolomic analysis of PM

The PM samples from positions 1 and 2 were mixed equally to form a new sample termed 'positions under the Huangshui fluid'. The PM samples from positions 3 and 4 were mixed equally to form a new sample termed 'positions 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. GC-MS analysis was performed using a 7890A gas chromatograph (Agilent) coupled to a PEGASUS HT mass selective detector (LECO). LC-MS analysis was performed on an Acquity UPLC system (Thermo Fisher Scientific) coupled with a Q Exactive HFX (Thermo Fisher Scientific). Detailed sample preparation and MS analysis methods are described in Supplementary text S1.

Statistical analysis

Origin 2017, 64 Bit, and SPSS 21 software were used for data processing and analysis. A redundancy analysis (RDA) was performed using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY). The statistical significance of the difference between the mean of samples was tested by a one-way analysis

of variance with the Tukey post hoc test. The cluster analysis and correlation coefficient between microbes and chemical properties were visualised using R software (V 3.6.3 and V 4.0.5).

Abbreviations

PM
Pit Mud
LC-MS
liquid chromatograph-mass spectrometer
GC-MS
Gas-chromatography-mass spectrometry
RDA
redundancy analysis
NMDS
Non-metric multidimensional scaling
LEfSe
Linear discriminant analysis Effect Size. βNTI:beta nearest taxonomic index

Declarations

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Authors' contributions

Conceptualization, methodology, formal analysis, investigation, visualization, writing 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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Availability of data and materials

No obtained data required submission to a public repository. Raw data or further details related to the conducted experiments can be obtained upon request from the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

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±2.45ab/A	38.11±0.28b/A
300-year	45.79±1.31a/A	45.08±1.13a/A	43.12±3.32a/A	36.05±5.06b/A
pH-30-year	6.69±0.19a/A	6.02±0.57a/A	6.72±0.22a/A	4.53±0.53b/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
TN (g/Kg)-30-year	3.92±0.34a/B	3.45±1.15a/A	4.15±0.19a/A	2.67±0.66b/A
100-year	3.74±0.15b/B	2.7±0.39a/A	3.48±0.24b/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±0.62b/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±0.92b/A	1.94±0.03b/B
300-year	4.07±1.32b/A	3.34±0.77ab/A	1.93±0.59a/A	1.96±0.23a/A
Simpson	0.9±0.05a	0.95±0.02a	0.9±0.07a	0.92±0.05a
Shannon	5.41±0.96a	5.96±0.71a	5.12±0.93a	5.48±0.86a
Coverage (%)	92.35±3.88	93.65±1.86	94.05±1.84	91.72±4.96
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				
TN: Total Nigroten				

Figures

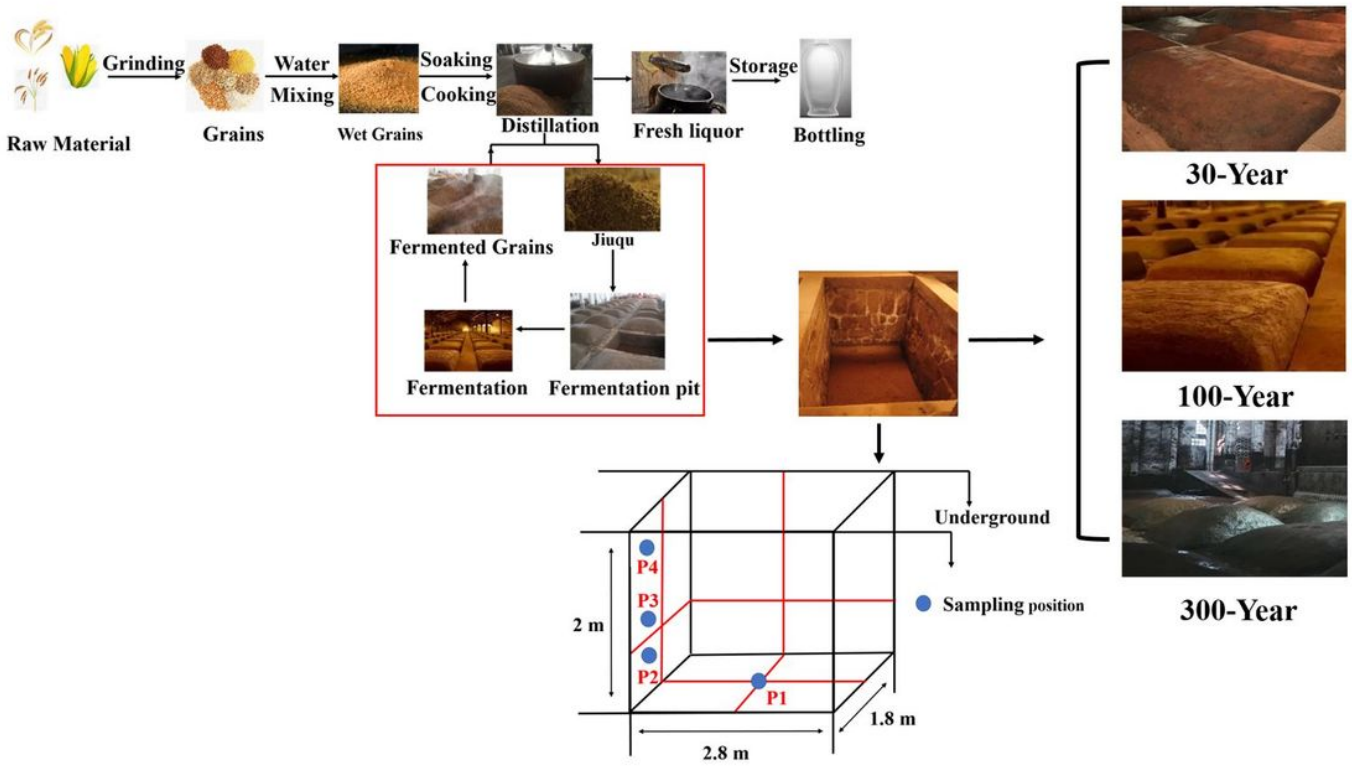

Figure 1

Traditional production processing of Chinese Strong-Flavoured Liquor (CSFL, the raw materials of CSFL are cereals, mostly sorghum, or a mixture of corn, rice, millet, and wheat; raw material mixtures are anaerobically fermented for 45–90 days or longer in a specialized rectangular soil pit, and we selected the pit cellar that was continuously used for the 30-, 100-, and 300-year cellars as a model to investigate microbial compositions, roles, and metabolic functions)

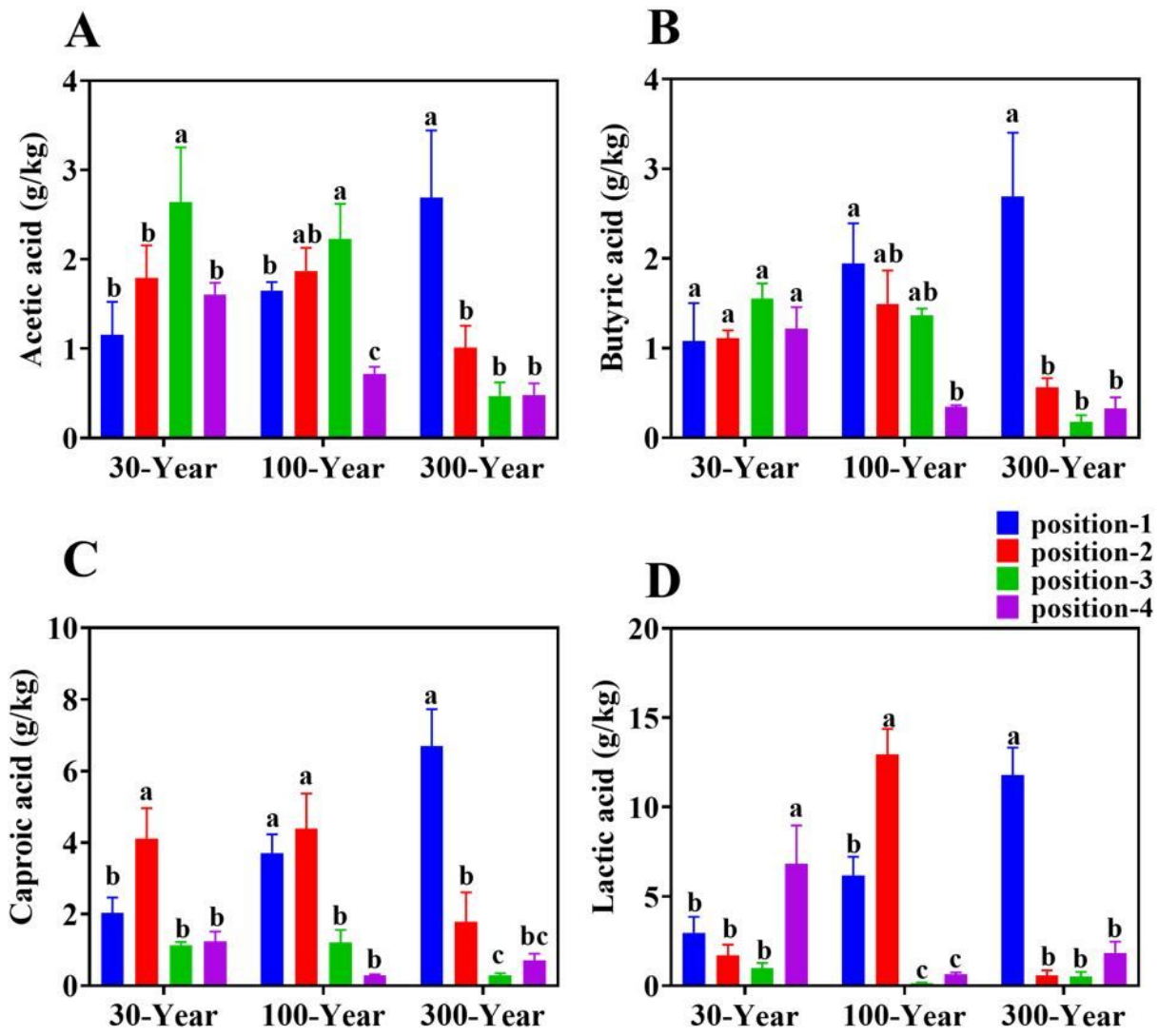

Figure 2

Difference in main 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.

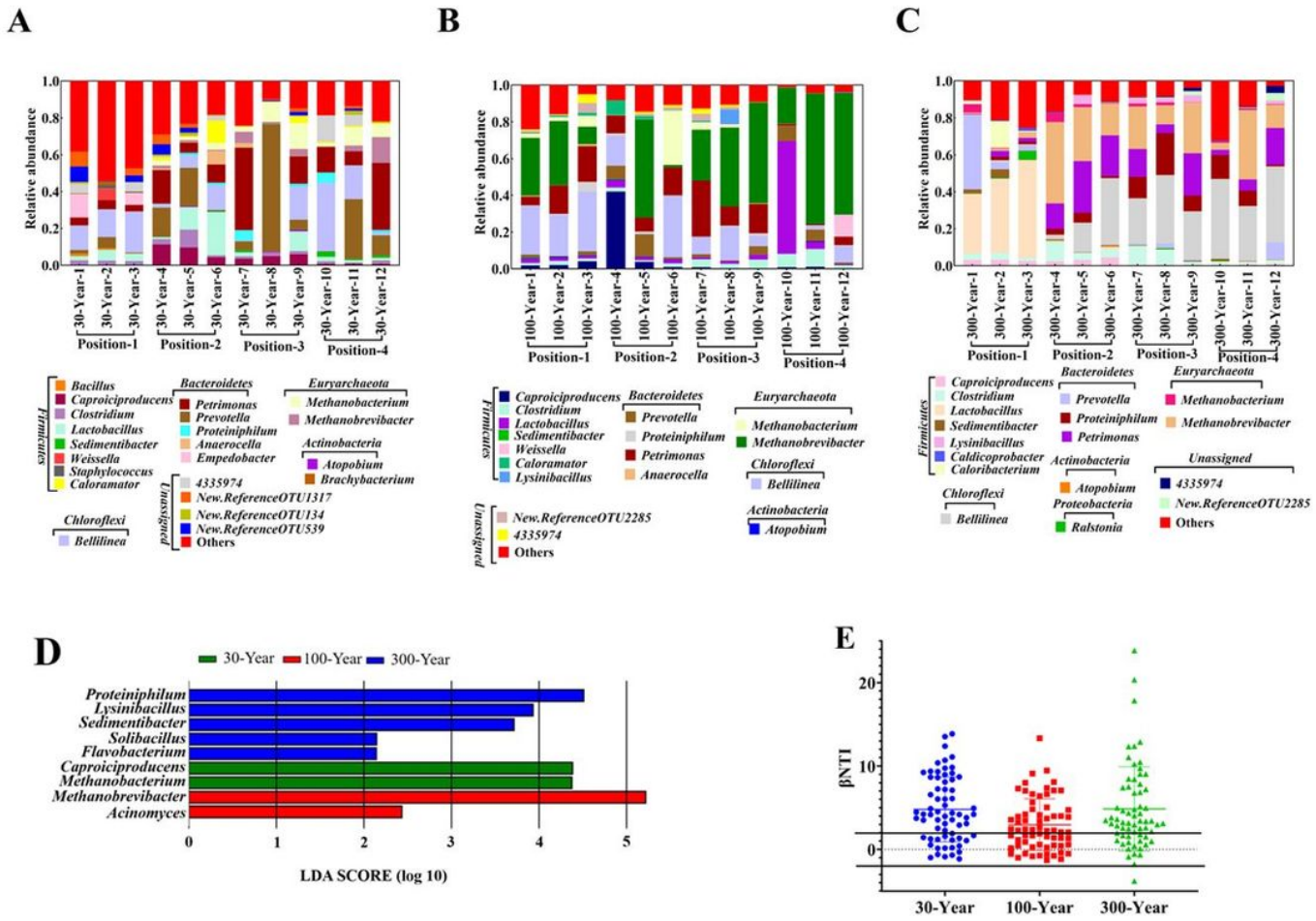

Figure 3

Microbial community composition of PM in different cellars: (A) 30-year-old cellar, (B) 100-year-old cellar, and (C) 300-year-old cellar (D) Linear discriminant effect size (LEfSe) analysis of the bacterial composition in the 30-, 100-, and 300-year-old PM samples (LDA> 2 and p<0.05) (E) Distribution box plots of βNTI of bacteria in the 30-, 100-, and 300-year-old PM samples. The horizontal red-dashed lines indicate the upper and lower significance limits of βNTI = +2 and -2, respectively.

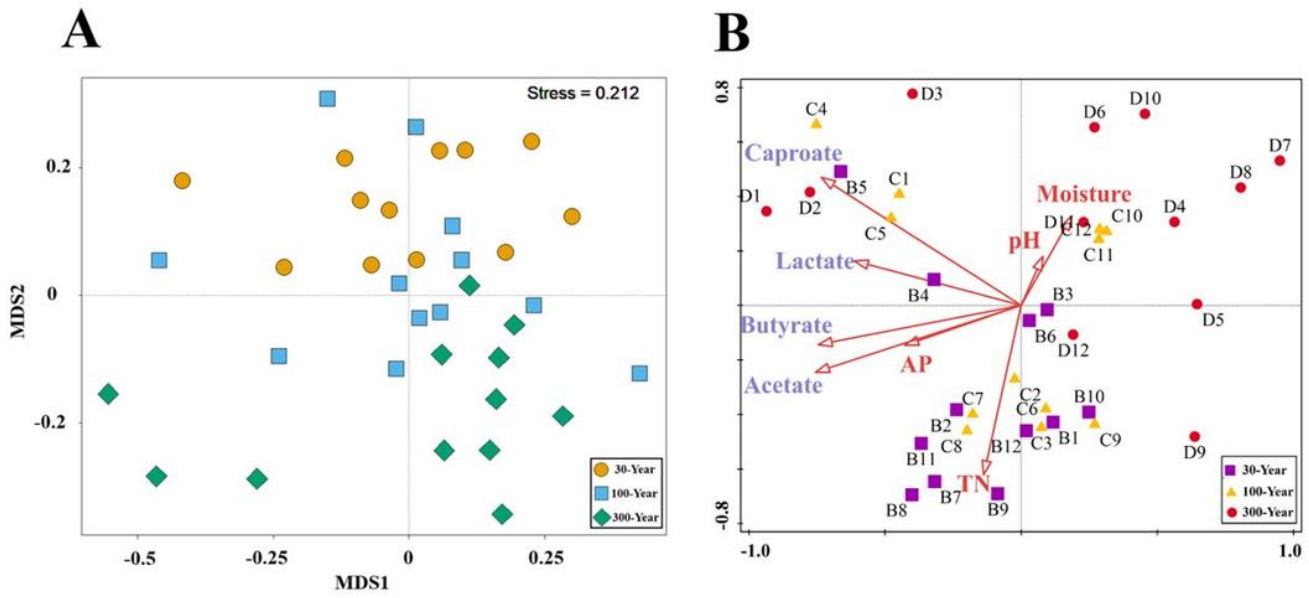

Figure 4

(A) NMDS analysis of bacterial microbiota community in the 30-, 100-, and 300-year-old PM samples. (B) RDA between prokaryotic community structure and environmental parameters in the 30-, 100-, and 300-year-old PM samples.

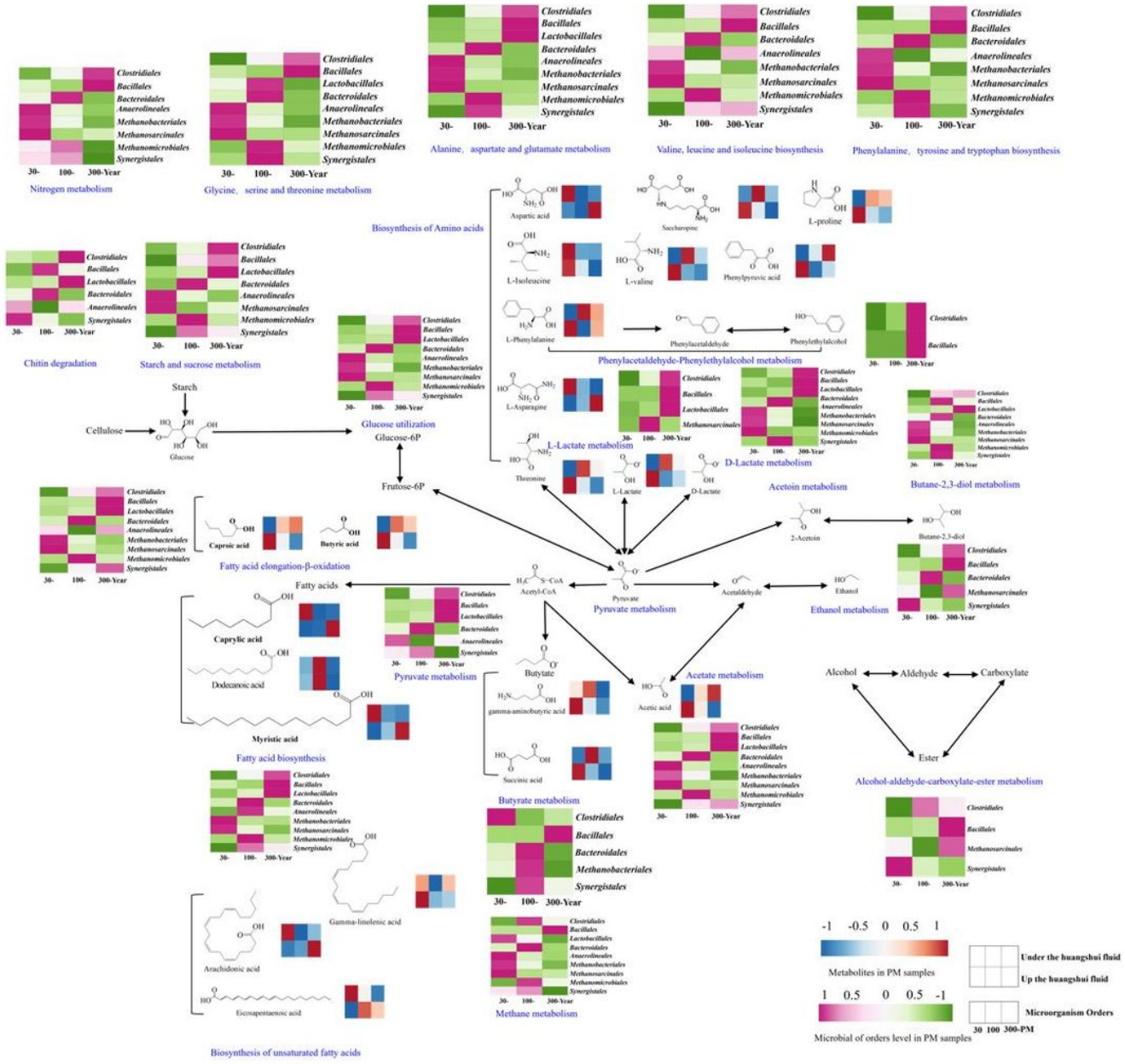

Figure 5

Schematic presentation of metabolites and microbiota orders related to flavour generation and substrate degradation pathways in the 30-, 100-, and 300-year-old PM samples. The heat maps of all genes annotated to the 135 KEGG enzymes in a predicted metabolic network for substrate degradation and flavour generation in PM, relating to the top 9 orders. The values were transformed with Z-score normalization. Blue tests represent key substrate and flavour metabolic pathways in PM. The blue–red heat map refers to the abundance of metabolites at positions under the huangshui fluid and above the huangshui fluid. The green–purple heat map refers to the abundance of the enzymes related to the substrate and flavour generation pathways.

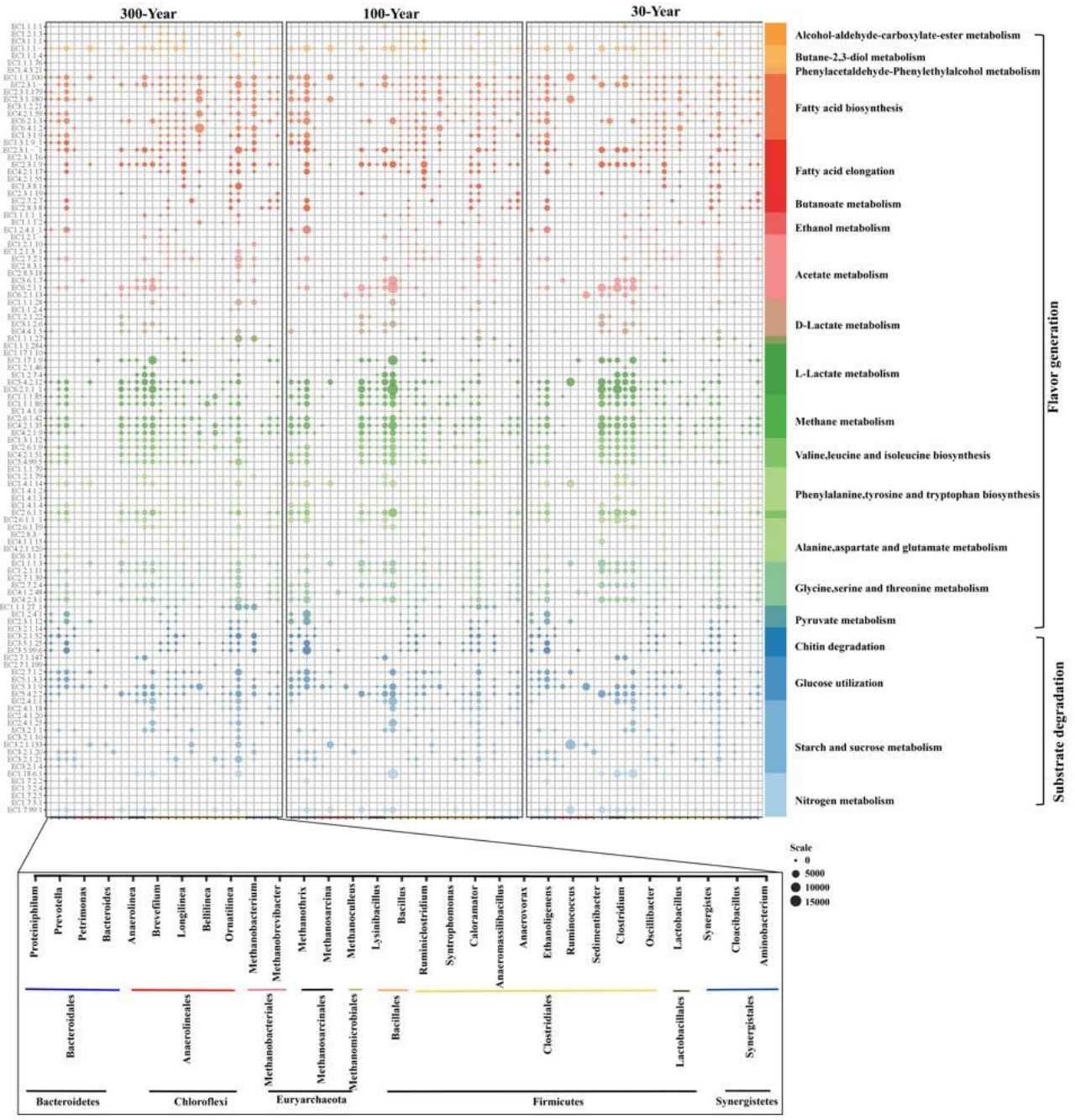

Figure 6

Taxonomic and functional distribution for substrate degradation and flavour generation, relating to the top 30 genera and 135 KEGG enzymes. The diagram is composed of three sub-plots, which correspond to the samples (30-, 100-, and 300-year-old samples). Vertical coordinates indicate the EC numbers of the functional enzymes. Horizontal coordinates, which indicate taxonomic classifications, are magnified at the bottom. Bubble size is proportional to the absolute abundance of the corresponding enzyme-coding genes. Highlighted colour bars help to rapidly locate the corresponding position of specific families (on X-axis) and functions (on Y-axis).

Supplementary Files

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