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Cooperation within the microbial consortia of fermented grains and pit mud drives organic acid synthesis in strong-flavor Baijiu production

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ABSTRACT

Mud cellars have long been used as anaerobic bioreactors for the fermentation of Chinese strong-flavor Baijiu, where starchy raw materials (mainly sorghum) are metabolized to ethanol and various flavor compounds by multi-species microorganisms. Jiupei (fermented grains) and pit mud are two spatially linked microbial habitats in the mud cellar, yet their metabolic division of labor remains unclear. Here, we investigated the changes in environmental variables (e.g., temperature, oxygen, pH), key metabolites (e.g., ethanol, organic acids) and microbial communities in jiupei and pit mud during fermentation. Jiupei (low pH, high ethanol) and pit mud (neutral pH) provided two habitats with distinctly different environmental conditions for microbial growth. Lactic acid accumulated in jiupei, while butyric and hexanoic acids were mainly produced by microbes inhabiting the pit mud. Biomass analysis using quantitative real-time PCR showed that bacteria dominated the microbial consortia during fermentation, moreover cluster and principal coordinate analysis (PCoA) analysis showed that the bacterial communities of jiupei and pit mud were significantly divergent. The bacterial community diversity of jiupei decreased significantly during the fermentation process, and was relatively stable in pit mud. *Lactobacillus* dominated the jiupei bacterial community, and its relative abundance reached 98.0% at the end of fermentation. Clostridia (relative abundance: 42.9–85.5%) was the most abundant bacteria in pit mud, mainly distributed in the genus *Hydrogenispora* (5.3–68.4%). Fungal communities of jiupei and pit mud showed a similar succession pattern, and *Kazachstania*, *Aspergillus* and *Thermoascus* were the predominant genera. PICRUSt analysis demonstrated that enzymes participating in the biosynthesis of acetic and lactic acid were mainly enriched in jiupei samples, while the bacterial community in the pit mud displayed greater potential for butyric and hexanoic acid synthesis. Assays from an in vitro simulated fermentation further validated the roles of jiupei microbiota in acetic and lactic acid production, and these acids were subsequently metabolized to butyric and hexanoic acid by the pit mud microbiota. This work has demonstrated the synergistic cooperation between the microbial communities of jiupei and pit mud for the representative flavor formation of strong-flavor Baijiu.

1. Introduction

Baijiu is a Chinese distilled alcoholic beverage, produced from the semi-solid/solid-state fermentation of cereals involving multiple microbial species. Although the main constituents of Baijiu are ethanol (38–65%, v/v) and water, a small amount (~2%) of volatile and non-volatile compounds can determine the flavor profiles of different types of Baijiu (Fang, Du, Jia, & Xu, 2019; Zheng & Han, 2016). Based on their concentration and aroma intensity, the characteristic flavor compounds of strong-flavor Baijiu include hexanoic, butyric, acetic and lactic acids, and their corresponding ethyl esters (Fang et al., 2019; Liu & Sun, 2018); ethyl hexanoate and hexanoic acid make the highest contribution to its typical flavor, and the concentration of ethyl hexanoate is an important quality index in the Chinese national standard (GB/T 10781.1-2006).

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The complex flavor of Baijiu can be affected by the raw materials, jiuqu (a fermentation starter), fermentation, distillation, blending, aging, etc. (Liu & Sun, 2018), and among these, microorganisms involved in the fermentation process are vital for the flavor formation and quality of Baijiu. Therefore, understanding the mechanisms of key compound production by microbial consortia during fermentation may facilitate the development of strategies to modulate the flavor and quality of Baijiu.

The most typical characteristic of strong-flavor Baijiu is that the fermentation process occurs in a mud cellar. Jiupei (fermented grains) and the pit mud provide the habitats for microbial growth. Bacilli were shown to be the most abundant microbiota present in jiupei and their relative abundance increased from ~65% to ~95% throughout fermentation, followed by Bacteroidia and Clostridia; and *Lactobacillus* dominated at the genus level, accounting for ~60% to ~90% of the total bacterial population (Chai, Lu, et al., 2019; Wang, Du, & Xu, 2017; Xiao et al., 2019). The predominant bacterial strains isolated from jiupei were distributed in genera *Bacillus*, *Lactobacillus* and *Acetobacter* (Zou et al., 2018). *Kazachstania* dominated the jiupei fungal community during fermentation with a relative abundance of ~60–90%; other abundant genera included *Aspergillus*, *Thermoyces*, *Thermoascus* and *Saccharomyces* (Xiao et al., 2018). Starch and other macromolecules are degraded and utilized during fermentation, resulting in the accumulation of water, ethanol, acids and other compounds in jiupei (Gao, Wu, & Zhang, 2020). This seeping solution of jiupei, referred to as huangshui, accumulates in the fermentation cellar, gradually sinking to the bottom under gravity and contacting the pit mud. Huangshui can supply a convenient channel for the exchange of compounds between jiupei and pit mud. However, whether the compounds produced by the jiupei microbiota are further metabolized by microbes inhabiting the pit mud remains unclear.

So far, studies of pit mud microbiota have mainly focused on samples taken from different aged cellars, and data from the analysis of pit mud microbiota throughout the fermentation process of strong-flavor Baijiu was lacking. Clostridia was the most abundant bacterial group in pit mud microbiota, and mainly included the genera *Caproiciproducens*, *Hydrogenispora*, *Sedimentibacter*, *Syntrophomonas* and *Clostridium* (Chai, Xu, et al., 2019; Hu, Du, Ren, & Xu, 2016; Liu, Tang, Guo, et al., 2017; Tao et al., 2017). Metagenomic analysis revealed that Clostridial cluster IV and *Clostridium* were the potential hexanoic acid-producers, possibly via chain elongation of butyryl-CoA with two carbons under the catalysis of acetyl-CoA acetyltransferase, hydroxy-acyl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, and acyl-CoA hydrolase (Spirito, Richter, Rabaey, Stams, & Angenent, 2014; Tao et al., 2017). The potential predominant butyric acid-producers in pit mud were also distributed in the class Clostridia, especially the genus *Clostridium* (Chai, Xu, et al., 2019). Liu, Tang, Zhao, et al. (2017) used denaturing gradient gel electrophoresis analysis to show that Ascomycota dominated the fungal community of pit mud, and the compositions of dominant genera from different aged samples varied significantly. Although it is widely acknowledged that the pit mud microbial community makes a major contribution to the formation of the key flavor compounds (i.e., hexanoic acid and butyric acid) of Baijiu, more definite evidence is still required.

Previous studies of species and community levels have provided the profiles of microbial consortia in jiupei and pit mud. Nevertheless, the underlying mechanisms explaining the division of labor between the microbial communities of jiupei and pit mud within a single fermentation cellar remains elusive. In this study, we focused on elucidating the contributions of the microbial communities in jiupei and pit mud within a single mud cellar to the formation of the key flavor compounds in Baijiu (i.e., hexanoic, butyric, acetic and lactic acids). Changes in environmental variables (e.g., temperature, oxygen, pH) and key metabolites (e.g., ethanol and organic acids) were measured in jiupei and pit mud. Using high-throughput sequencing, the succession and function of microbial communities (including bacteria and fungi) in jiupei and pit mud throughout fermentation were studied for the first time. The potential roles of the microbial communities in jiupei and pit mud in the synthesis of the key flavor organic acids were validated using an in vitro simulated fermentation process.

2. Materials and methods

2.1. Sample collection

Samples of jiupei and pit mud were collected throughout fermentation (0, 1, 3, 5, 7, 9, 11, 16, 21, 26, 31, 36, and 46 d) from Luzhou Laojiao Group Co., Ltd., a representative strong-flavor Baijiu producer located in Sichuan Province, China (105°29′50′′ E, 28°53′47′′ N) (Supplementary Fig. 1A). Three replicates were collected at each sampling time point using a sterile hollow cylindrical sampler (Puluody, China) (Supplementary Fig. 1B). Samples of jiupei and pit mud were collected from three different points near the center of the cellar (4.3 m length × 3.3 m width × 2.3 m height); replicate samples were combined and mixed fully on site in sterile plastic bags. Samples were immediately transferred to the laboratory, ground into a homogeneous powder under liquid nitrogen, and stored at −80 °C until required for analysis (Supplementary Fig. 1C).

2.2. Determination of environmental variables and metabolite contents

Changes of temperature and oxygen content in the jiupei and pit mud were monitored throughout fermentation using a thermometer and a microsensor monometer with an Unisense OX-50 microelectrode sensor (Aarhus, Denmark) (Lu et al., 2016), respectively. Moisture content was analyzed immediately post sampling by drying jiupei and pit mud samples to constant weight in an oven at 105 °C for 4 h.

Samples for pH measurement and chemical analysis (30 g) were reconstituted in sterile ultrapure water (150 mL), vortex mixed for 1 h at 100 rpm at room temperature, centrifuged for 15 min at 5000*g*, and the supernatant collected for analysis. pH was determined using a Mettler Toledo pH meter (Shanghai, China). Ethanol concentration and total acidity were determined by distillation and sodium hydroxide titration respectively according to methods of Chai, Lu, et al. (2019). The contents of acetic, lactic, butyric and hexanoic acids were determined by HPLC with diode array detection using a 1260 Infinity LC system (Agilent Technologies, USA) after separation on a SEPAX Carbomix H-NP column (5 μm, 300 × 7.8 mm) (Chai, Lu, et al., 2019).

2.3. DNA extraction

DNA was extracted from homogeneous frozen samples (0.5 g) using a PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. A NanoDrop 2000 UV spectrophotometer (Thermo Scientific, USA) was used to determine the DNA quality and quantity. All DNA samples were stored at −80 °C until required for analysis.

2.4. Quantitative real-time PCR (qPCR)

Bacterial and fungal cell numbers in the *jiupei* and pit mud samples were investigated by qPCR on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) using the primer sets Eub338/Eub518 and ITS1f/5.8S, respectively (Fierer, Jackson, Vilgalys, & Jackson, 2005)). qPCR reactions were carried out using the SYBR™ Select Master Mix (Applied Biosystems, USA) according to the previous work (Xiao et al., 2017). Bacteria and fungi in jiupei and pit mud were enumerated from standard curves obtained by plotting quantification cycle against the log of the *Escherichia coli* 16S rRNA gene and *Aspergillus niger* ITS1 PCR amplification fragment concentrations, respectively (Supplementary Fig. 2). The copy numbers of 16S rRNA gene and ITS sequence were calculated using the method described by Zhang et al. (2008). Three replicates were performed for each reaction.

2.5. Illumina MiSeq amplicon sequencing

Amplicon libraries for bacterial community analysis were prepared by amplifying the sequences targeting the 16S rRNA gene V3-V4 region using the universal primer set 338F/806R (Dennis et al., 2013). Primers ITS1F and ITS2R targeting the ITS1 region were used for fungal community analysis (Gardes & Bruns, 1993). Prior to the Illumina MiSeq sequencing (2 × 300 bp), the PCR products were purified and adjusted to an appropriate concentration for library construction using the SanPrep Column PCR Product Purification Kit (Sangon Biotech, China) and TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA).

Analysis of amplicon sequencing data was conducted using QIIME (V 1.9.1) pipeline for quality control; de-multiplexing and subsequent analysis followed the procedure of Caporaso et al. (2010). The high-quality data from all samples were classified into operational taxonomic units (OTUs) by USEARCH using 97% identity as a cutoff after subsampling to the same sequencing depth (Edgar & Flyvbjerg, 2015). Representative OTU sequences were annotated against the SILVA database and RDP classifier at 80% confidence (Wang, Garrity, Tiedje, & Cole, 2007). Calculation of the alpha diversity (α-diversity) indices was carried out using QIIME (V1.9.1). Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis, principal coordinate analysis (PCoA), mantel test and analysis of similarities (ANOSIM) were carried out in R (V 3.2.4) of the vegan package (V 2.3–4). Functional composition of bacteria in jiupei and pit mud throughout fermentation was analyzed by PICRUSt (Langille et al., 2013).

2.6. In vitro modular fermentation of jiupei and pit mud consortia

To validate the division of labor between the microbial communities of jiupei and pit mud during synthesis of the main organic acids during Baijiu fermentation, an in vitro sequential dual module fermentation processes was used. Briefly, the fermentation of jiupei was performed in a bioreactor without pit mud (module one), after which, the liquid residue was used to inoculate the pit mud in a second bioreactor and start the fermentation of module two. The procedure for module one was

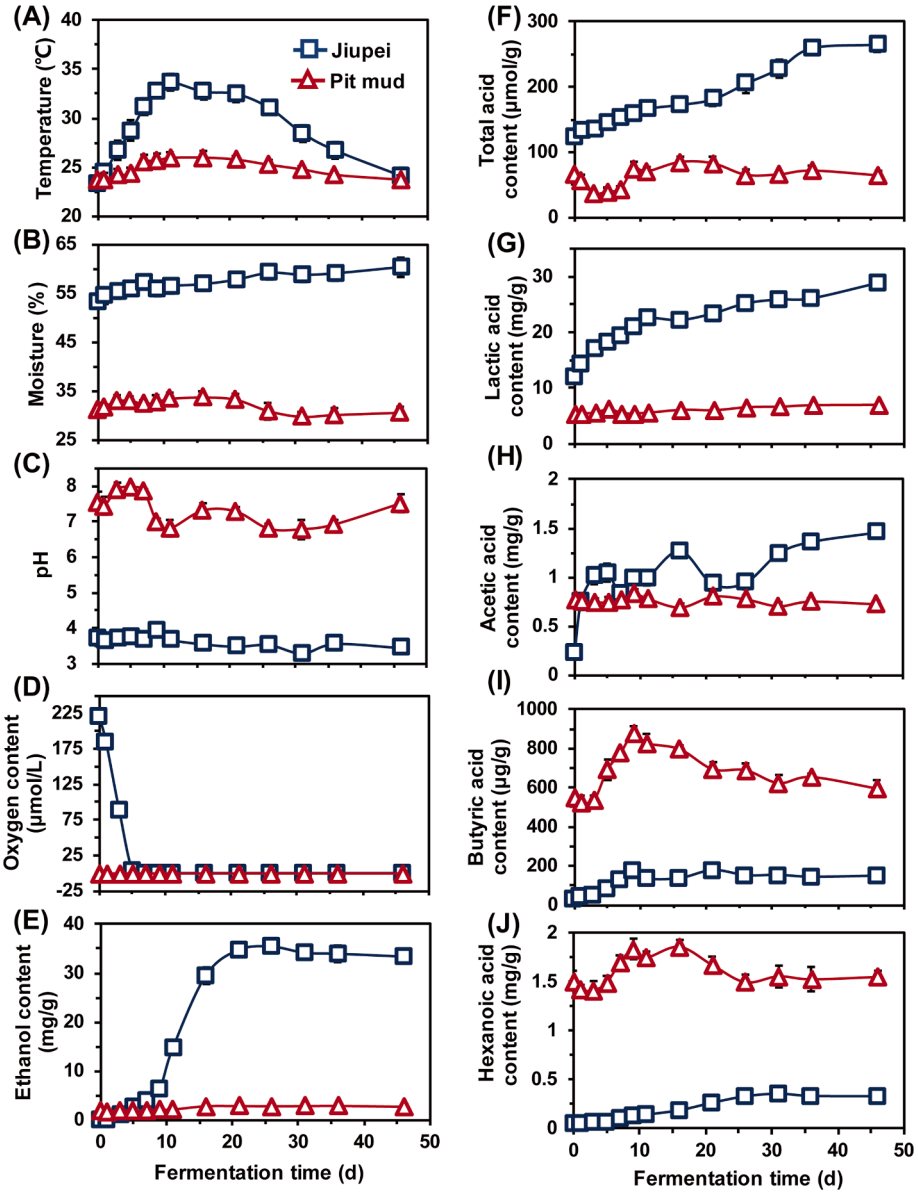

Fig. 1. Dynamics of jiupei and pit mud environmental and chemical properties during Chinese strong-flavor Baijiu fermentation. (A) Temperature, (B) moisture, (C) pH, (D) oxygen content, (E) ethanol content, (F) total titratable acidity, (G) lactic acid, (H) acetic acid, (I) butyric acid, and (J) hexanoic acid. Squares and triangles represent jiupei and pit mud samples, respectively. Each data point is the mean of three measurements ± SD.

as follow: 200 g of sorghum granules (~3 mm in diameter) was brewed in hot water for 15 min; when cool, the pretreated sorghum was inoculated with 4 g of fermentation starter (Xiao et al., 2017) and cultured for 16 d at 25 °C; the volume of the inoculum was adjusted to 2 L with water on day two. For module two: the fermentation broth was removed from module one, centrifuged for 15 min at 5000*g*, and the supernatant adjusted to 7.0 pH with NaOH (i.e., close to the pH of pit mud in the Baijiu process); pit mud (10%, w/v) was suspended in the supernatant (about 1.5 L), flushed with N_2 for 30 min to remove oxygen (anaerobic environment) and cultured for 16 d at 30 °C; samples of suspension (50 mL) were removed from each module at 0, 4, 8, 12, and 16 d, centrifuged for 15 min at 5000*g*, and the supernatant was retained for the analysis of environmental and chemical parameters. The bacterial community structure was determined by amplicon sequencing analysis at the end of fermentation in each module.

2.7. Sequence deposit

The raw amplicon sequencing data sets were deposited in the NCBI Sequence Read Archive with the accession number PRJNA558446.

3. Results

3.1. Temporal changes in the physical and chemical profiles of jiupei and pit mud throughout baijiu fermentation

Divergent changes in the physical and chemical environment of jiupei and pit mud were observed during the single cellar fermentation process (Fig. 1, Supplementary Table 1). At the beginning of fermentation, the temperatures of jiupei (23.3 ± 0.5 °C) and pit mud (23.7 ± 0.6 °C) were similar as Baijiu is produced in an open environment. As fermentation progressed, the temperature of the jiupei increased rapidly to 33.7 ± 0.9 °C on day 11 and gradually decreased to 24.1 ± 0.8 °C at the end. The temperature of the pit mud fluctuated within a narrow range (23.7–26.0 °C) (Fig. 1A). Overall, the average moisture content of jiupei (57.0 ± 2.3%) was significantly higher than that of pit mud (31.9 ± 1.7%; $P < 0.01$) (Fig. 1B). The moisture content of jiupei showed a slight upward trend throughout fermentation, while it remained relatively stable in the pit mud. During fermentation, jiupei exhibited an acidic environment (pH 3.3–3.9), however, the pH remained approximately neutral (pH 6.8–8.0) in pit mud (Fig. 1C). The whole

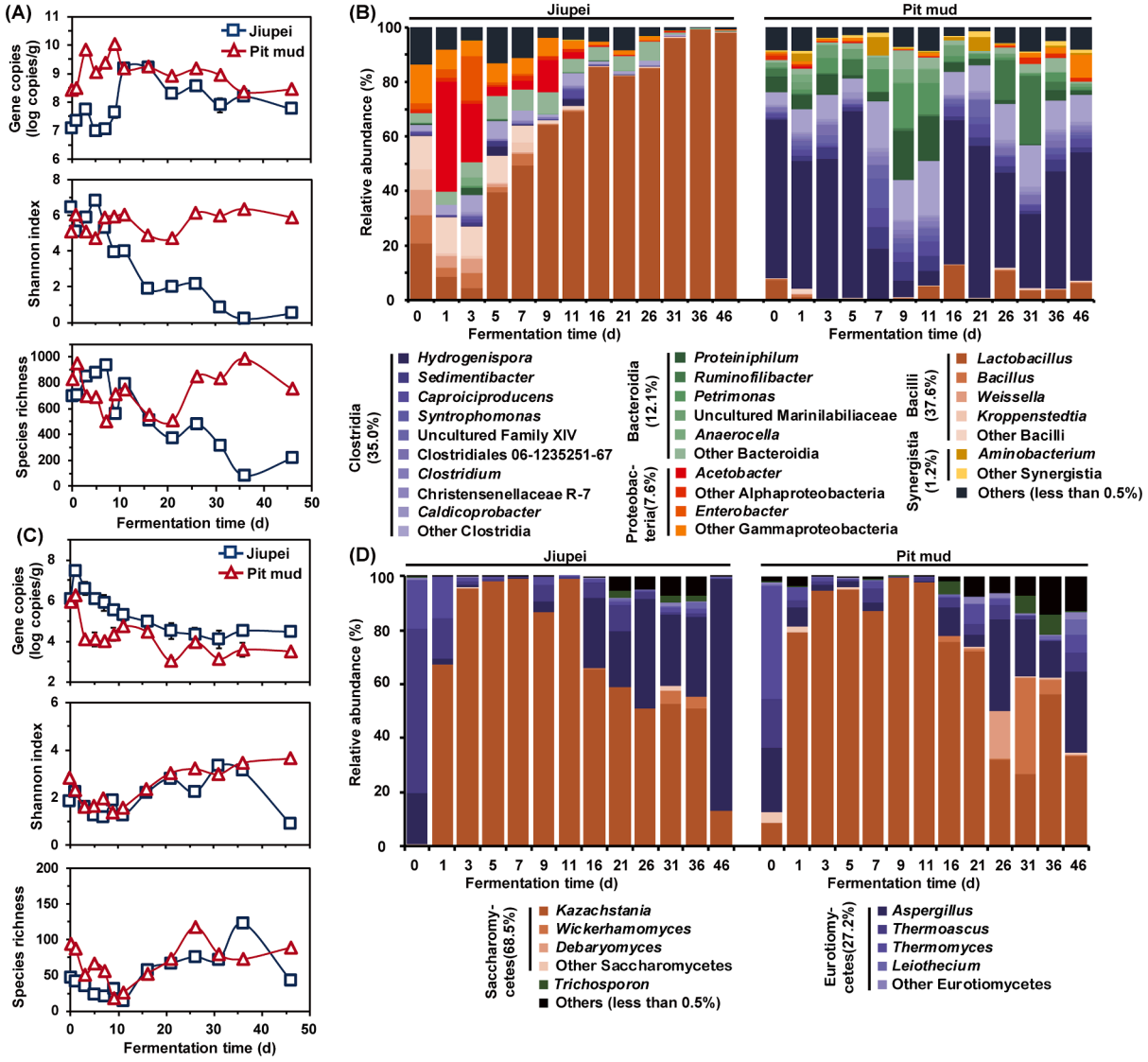

Fig. 2. Biomass, α-diversity and the composition of microbial communities in jiupei and pit mud during fermentation. Plot showing the biomass expressed as log (gene copy numbers), and changes in Shannon index and species richness of (A) bacterial and (C) fungal communities in jiupei and pit mud throughout the fermentation process. (B) Bacterial and (D) fungal community compositions based on Silva and Unite databases. Taxonomy is displayed at the level of genus. Genus compromising <0.5% of the total relative abundance across all samples are grouped as others.

fermentation system was anaerobic as the cellar was sealed with mud, except the first 5 days in jiupei (Fig. 1D). Ethanol content rocketed from 6.5 ± 0.6 mg/g on day 9 to 34.8 ± 1.5 mg/g on day 21 in jiupei; in contrast, trace amounts of ethanol were detected in the pit mud samples (Fig. 1E).

Total titratable acids accumulated in jiupei through fermentation reaching 264.8 ± 11.9 μmol/g at the end of the process; only low levels were found in the pit mud (Fig. 1F). HPLC analysis showed that lactic acid was the main organic acid in jiupei and pit mud, reaching 28.8 ± 1.1 mg/g and 7.0 ± 0.2 mg/g respectively at day 46 (Fig. 1G); acetic acid increased in the later stage of fermentation in jiupei, while it remained constant in the pit mud (Fig. 1H); butyric and hexanoic acids were mainly accumulated in the pit mud at the end of fermentation, and their concentrations were respectively ~3 and ~4 times higher than that of jiupei (Fig. 1I & J).

3.2. The succession of microbial communities in jiupei and pit mud

The dynamic features of the microbial populations in jiupei and pit mud were explored using qPCR and amplicon sequencing analysis (Fig. 2, Supplementary Table 2). Cell numbers of total bacteria increased rapidly to $5.58 \pm 2.09 \times 10^7$ 16S rRNA gene copies/g in jiupei and $6.57 \pm 0.74 \times 10^9$ copies/g in pit mud on day three, decreasing to $9.50 \pm 1.99 \times 10^6$ and $1.11 \pm 0.15 \times 10^9$ copies/g on day five, respectively. Further peaks of bacterial cell number appeared at day 16 in jiupei ($1.57 \pm 0.07 \times 10^9$ copies/g) and day nine in pit mud ($1.07 \pm 0.08 \times 10^{10}$ copies/g). At the end of fermentation, the bacterial cell numbers in jiupei and pit mud had decreased to $5.83 \pm 1.13 \times 10^7$ and $2.80 \pm 0.19 \times 10^8$ copies/g respectively (Fig. 2A). Enumeration of the fungi showed that cell numbers at day one were $2.98 \pm 0.69 \times 10^7$ and $1.84 \pm 0.58 \times 10^6$ ITS copies/g in jiupei and pit mud, respectively; at the end of fermentation these values had decreased to $2.97 \pm 0.41 \times 10^4$ copies/g (jiupei) and $3.29 \pm 0.60 \times 10^3$ copies/g (pit mud) (Fig. 2C).

High-throughput sequencing resulted in 882,533 qualified 16S rRNA gene reads and 865,567 ITS reads from all jiupei and pit mud samples; 23,990–46,119 clean bacterial reads and 22,671–40,154 fungal reads were obtained from each sample (Supplementary Table 3). In total, 2,719 bacterial OTUs and 321 fungal OTUs were clustered at 97% identity after subsampling. The Shannon diversity and species richness indices represented the α-diversity of the microbial consortia. For the bacteria, the diversity and richness of the community showed a decreasing trend in jiupei, while these indices fluctuated in pit mud. The Shannon diversity and species richness indices for the pit mud bacteria were 11.7 and 3.5 times that of jiupei on day 46 (Fig. 2A). These results demonstrated the different succession patterns of the bacterial consortia in jiupei and pit mud. The bacterial community of the pit mud maintained a dynamic balance, while the bacterial community of jiupei was gradually dominated by a few species. The Shannon diversity and species richness indices of the fungal community were much lower than those of the bacterial community. Both indices showed a downward trend from the beginning of the fermentation process in jiupei and pit mud, increasing thereafter; these increases occurred from days nine to 36 for jiupei and days nine to 46 for the pit mud (Fig. 2C).

The results from temporal profiling of the bacterial community structure of jiupei and pit mud during fermentation are shown in Fig. 2B. The most predominant bacterial classes in jiupei and pit mud were Bacilli and Clostridia, accounting for 70.7% (average relative abundance) and 65.9%, respectively. *Lactobacillus*, *Bacillus* and *Weissella* were the predominant genera in jiupei at the beginning of fermentation. *Lactobacillus* dominated the bacterial community after day five, and its relative abundance was 98.0% at the end of fermentation. However, the relative abundances of *Bacillus* and *Weissella* decreased to <1.0% from days nine and five, respectively, until the end of fermentation. The relative abundance of *Acetobacter* increased markedly to 40.4% by day one, reducing to <1.0% from day 16. *Enterobacter*, also belonging to the class Proteobacteria, showed a similar trend. The average abundance of all genera distributed in Clostridia and Bacteroidia was <1.0% in jiupei. With respect to pit mud, Clostridia and Bacteroidia dominated the bacterial community, and their total relative abundance was >85.0%. The top sixteen genera (relative abundance per sample > 0.1%) accounted for 65.1–86.6% of the total bacterial abundance throughout fermentation. Amongst these, the relative abundances of *Hydrogenispora* and *Proteiniphilum* ranged from 5.3–68.4% and 0.6–18.1%, respectively; the relative abundance of *Hydrogenispora* in pit mud decreased from 39.7% to 5.0% between days nine and 11; the relative abundance of *Proteiniphilum* reached ~16.3% between days nine and 11, maintaining at 3.3% in the rest samples.

Eurotiomycetes (98.8% relative abundance) dominated the fungal community at the beginning of fermentation in jiupei samples, comprising mainly *Thermoascus*, *Aspergillus* and *Thermomyces* (Fig. 2D). The relative abundance of *Kazachstania*, belonging to the class Saccharomycetes, proliferated rapidly to 67.2% by day one, and continued to increase until day 36. After day 16, the relative abundance of *Aspergillus* remained above 20% in jiupei. The relative abundance of *Thermoascus*, one of the predominant Eurotiomycetes, decreased sharply from 60.6% to 0.4% during the first 7 d, and then it fluctuated slightly at ~3.3%. The temporal dynamics of the fungal community in pit mud showed a similar pattern to those of jiupei (Fig. 2D).

3.3. Divergence of microbial communities in jiupei and pit mud

UPGMA cluster and PCoA analyses were performed using the Bray-Curtis algorithm to evaluate beta-diversity (β-diversity) in the microbial consortia of jiupei and pit mud samples throughout fermentation. The results demonstrated that the bacterial community profiles of the Baijiu fermentation cellar could be divided into a jiupei group and a pit mud group during fermentation (Fig. 3A & C). In addition, cluster analysis showed that the jiupei group could be further deconvoluted into two phases representing 0–5 d and 7–46 d, while in the pit mud group, samples from 7 to 11 d were clustered and distinct from all other samples (Fig. 3A). Principal coordinate axis one (PCo1) explained 48.6% discrepancy across all samples, and a significant difference ($P < 0.01$) was detected between the PCo1 scores for the jiupei and pit mud groups, which was also supported by ANOSIM ($R = 0.858$, $P = 0.001$) (Fig. 3C).

The fungal communities in jiupei and pit mud could not be distinguished by cluster and PCoA analyses (Fig. 3B & D), and this was also confirmed by ANOSIM ($R = -0.032$, $P = 0.845$). However, the mantel test showed that the dynamics of the fungal communities in jiupei and pit mud were significantly correlated with each other ($R = 0.768$, $P = 0.001$). Inspection of the biomass and community succession patterns suggested that the fungal community composition and succession of pit mud were affected by the fungal community in jiupei, mainly due to direct contact.

3.4. Bacterial flow in different layers of the Baijiu fermentation cellar

The bacterial communities in jiupei and pit mud layers were studied further. Compared with the fungi, bacteria were the more dominant group in the fermentation system, and their biomass was ~100 and 10,000 times that of the fungi in jiupei and pit mud, respectively (Fig. 2A & C). In addition, the remarkable divergence of the bacterial community structure and succession was mainly responsible for the differences in environmental and chemical properties of the different layers (Fig. 3).

Tracking individual OTUs in different samples revealed widespread species migration and colonization between the jiupei and pit mud bacterial communities (Fig. 4). During the first 11 d of fermentation, the number of shared OTUs increased to 149, decreasing to 25 at day 46 (Fig. 4B). The number of species entering jiupei from the pit mud was greater than those entering the pit mud from jiupei. The relative abundance of shared OTUs for jiupei increased throughout fermentation reaching 98.3% on day 46. The relative abundance of shared OTUs in the pit mud decreased from 82.0% to 28.8% after 11 d (Fig. 4A). Within the

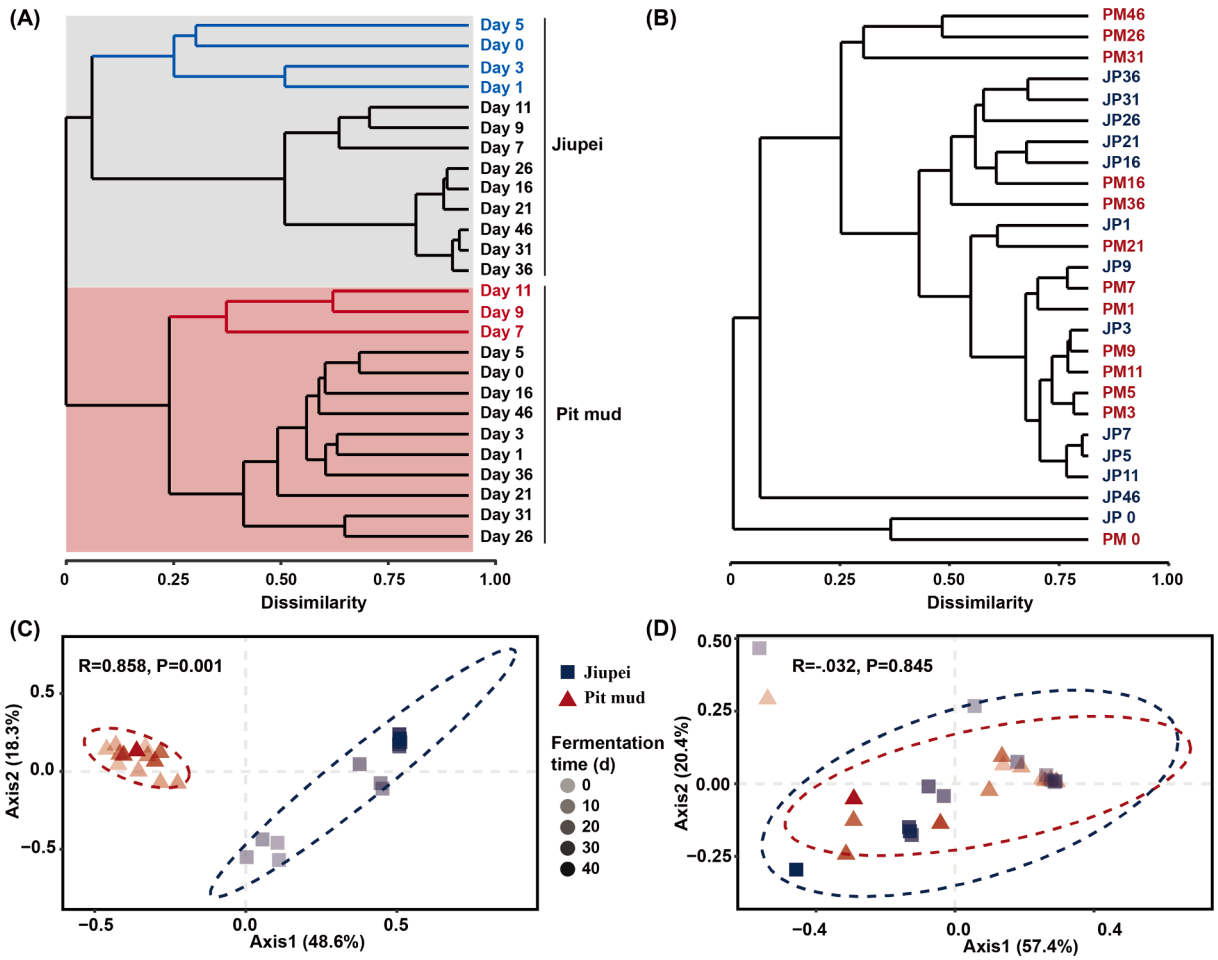

Fig. 3. β-diversity of the microbial communities in jiupei and pit mud. UPGMA cluster analysis and PCoA analysis of cellar consortia. (A, C) bacteria, (B, D) fungi. Each square and triangle represents averaged community composition data for each jiupei and pit mud sample.

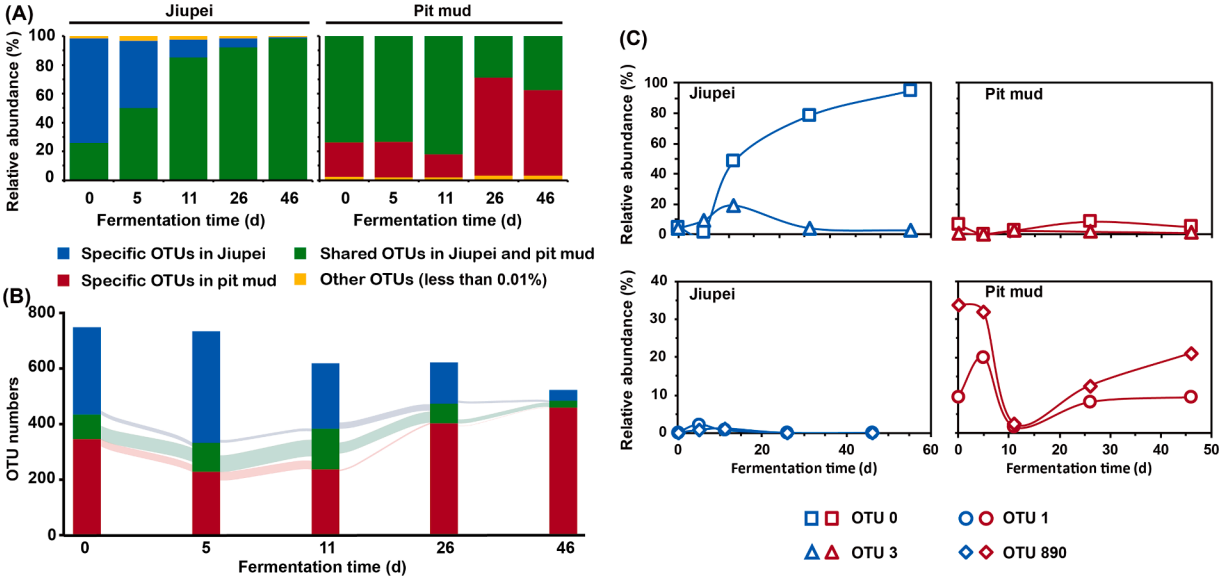

Fig. 4. Bacterial flow between the different microbial habitats in the Baijiu fermentation cellar. (A) Histogram showing the relative abundance of shared OTUs for jiupei and pit mud samples at different stages of fermentation. (B) Sankey plots tracking the shared OTUs for jiupei samples at different stages of fermentation (heights of the rectangles correlate with the number of OTUs). (C) Dynamics of the relative abundance of the main OTUs in jiupei and pit mud.

shared OTUs, OTU0 (24.9%), OTU3 (4.4%), OTU890 (10.4%) and OTU1 (5.2%) accounted for the highest average abundance (Fig. 4C). OTU0 and OTU3, assigned to *Lactobacillus*, were more abundant in jiupei samples; OTU890 and OTU1, assigned to *Hydrogenispora*, were more abundant in the pit mud samples. Shared OTUs of different relative abundances were identified in jiupei and pit mud, which could be attributed to species migration between the two different layers during fermentation.

3.5. Functional composition analysis of the jiupei and pit mud microbial consortia

The temporal functional profiles for the metabolism of glucose and organic acids by bacterial communities of jiupei and pit mud during fermentation were predicted using the PICRUSt tool and the 16S rRNA gene sequencing results (Fig. 5). We focused on the eighteen key enzymes involved in glucose utilization and the biosynthesis and catabolism of acetic, lactic and butyric acids. The results showed that, over the whole fermentation process, the jiupei- and pit mud-predicted metagenomes were statistically different (ANOSIM, $P < 0.001$). Enzymes participating in the biosynthesis of acetic acid (EC 1.2.1.3, aldehyde dehydrogenase (NAD^+)) and lactic acid (EC 1.1.1.28, D-lactate dehydrogenase) were mainly enriched in the jiupei group ($P < 0.05$) (Fig. 5A & C). This demonstrated that the bacteria in jiupei were the main contributors of acetic and lactic acids in the Baijiu fermentation cellar, which was consistent with results from the environmental and chemical analyses (Fig. 1G & H). In contrast, the bacterial consortia in pit mud displayed greater potential for the synthesis (EC 2.7.2.7, butyrate kinase and EC 2.8.3.9, butyryl-CoA:acetate CoA-transferase) and utilization (EC 6.2.1.2, medium-chain acyl-CoA synthetase) of butyric acid, and EC 6.2.1.2 was also reported as a key enzyme for the synthesis of hexanoic acid from butyric acid (Fig. 5A & D) (Seedorf et al., 2008; Zou et al., 2018). These results could help to explain the observation that the accumulation of butyric and hexanoic acids was greater in pit mud than in jiupei during fermentation (Fig. 1I & J). The enzyme involved in catalyzing the reversible conversion between pyruvate and L-lactate (EC 1.1.1.27, L-lactate dehydrogenase) was enriched in the jiupei and pit mud groups. Based on the lactic acid concentrations in the jiupei and pit mud samples (Fig. 1G), we inferred that L-lactate dehydrogenase played a more important role in lactate synthesis in jiupei. Compared with the jiupei group, the microbial community in the pit mud was predicted to have a higher utilization of glucose since glucokinase (EC 2.7.1.2) was more abundant. The significant variation ($P < 0.01$) of 16S rRNA gene copy numbers in each cell of the microbes from jiupei and pit mud was considered as an index of growth rate in response to favorable conditions (glucose as substrate in this study). Hence it could be inferred that the jiupei community overwhelms the microorganisms in pit mud and utilize glucose rapidly (Fig. 5B) (Green, Bohannan, & Whitaker, 2008; Roller, Stoddard, & Schmidt, 2016).

3.6. Evaluation of the contributions of microbial communities in jiupei and pit mud to the metabolism of organic acids

The distinct environmental and chemical properties, microbial

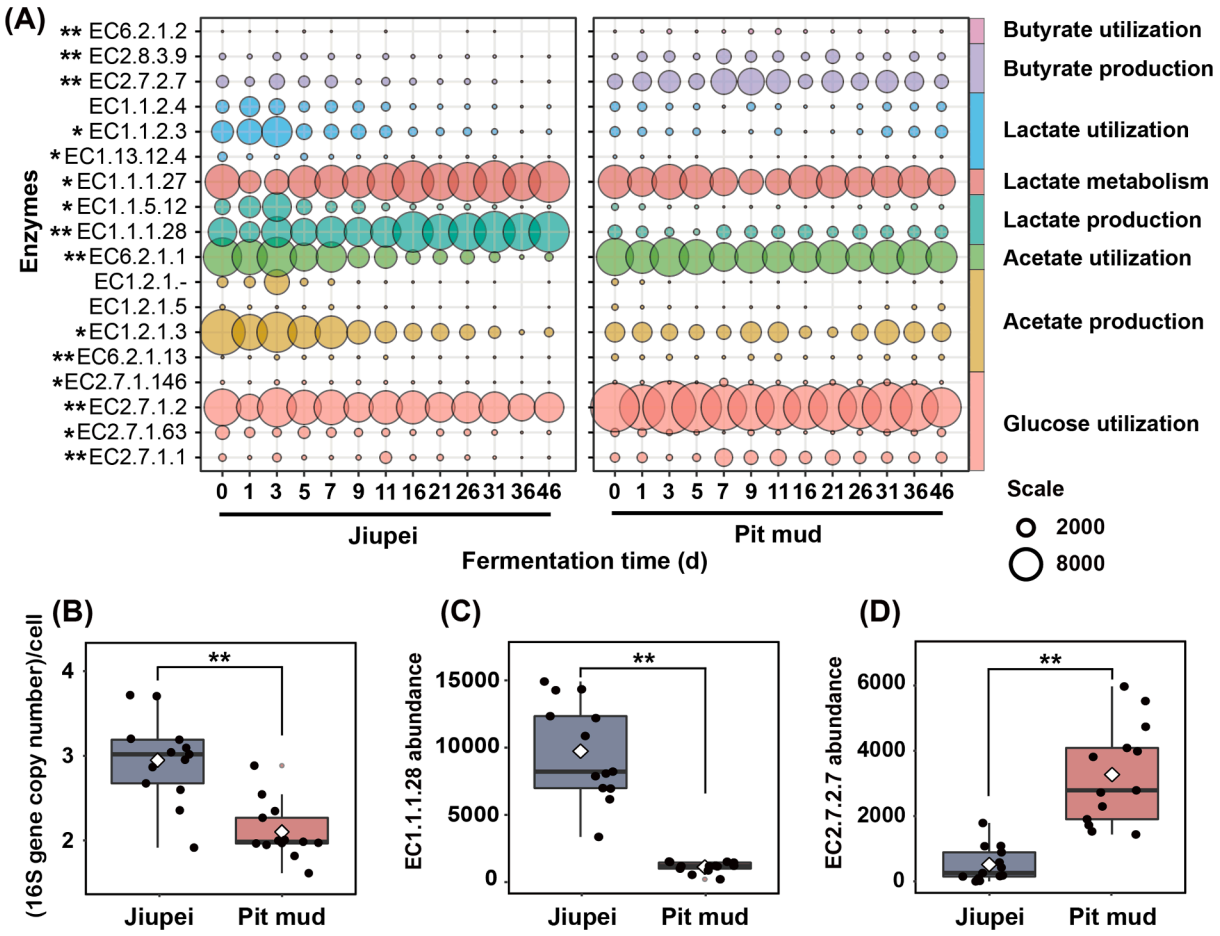

Fig. 5. Predicted functional composition of samples throughout fermentation involved in glucose and organic acid metabolism. (A) Temporal patterns of enzyme reads for substrate utilization and flavor formation in the microbial communities of jiupei and pit mud samples. The bubble diameter represents the number of enzyme reads. Glu, glucose; Ace, acetic acid; Lac, lactic acid; But, butyric acid; (B), (C) and (D) one-way ANOVA of the average 16S rRNA gene copy number per cell, EC 1.1.1.28 reads and EC 2.7.2.7 reads between jiupei and pit mud groups, respectively. *, $P < 0.05$; **, $P < 0.01$.

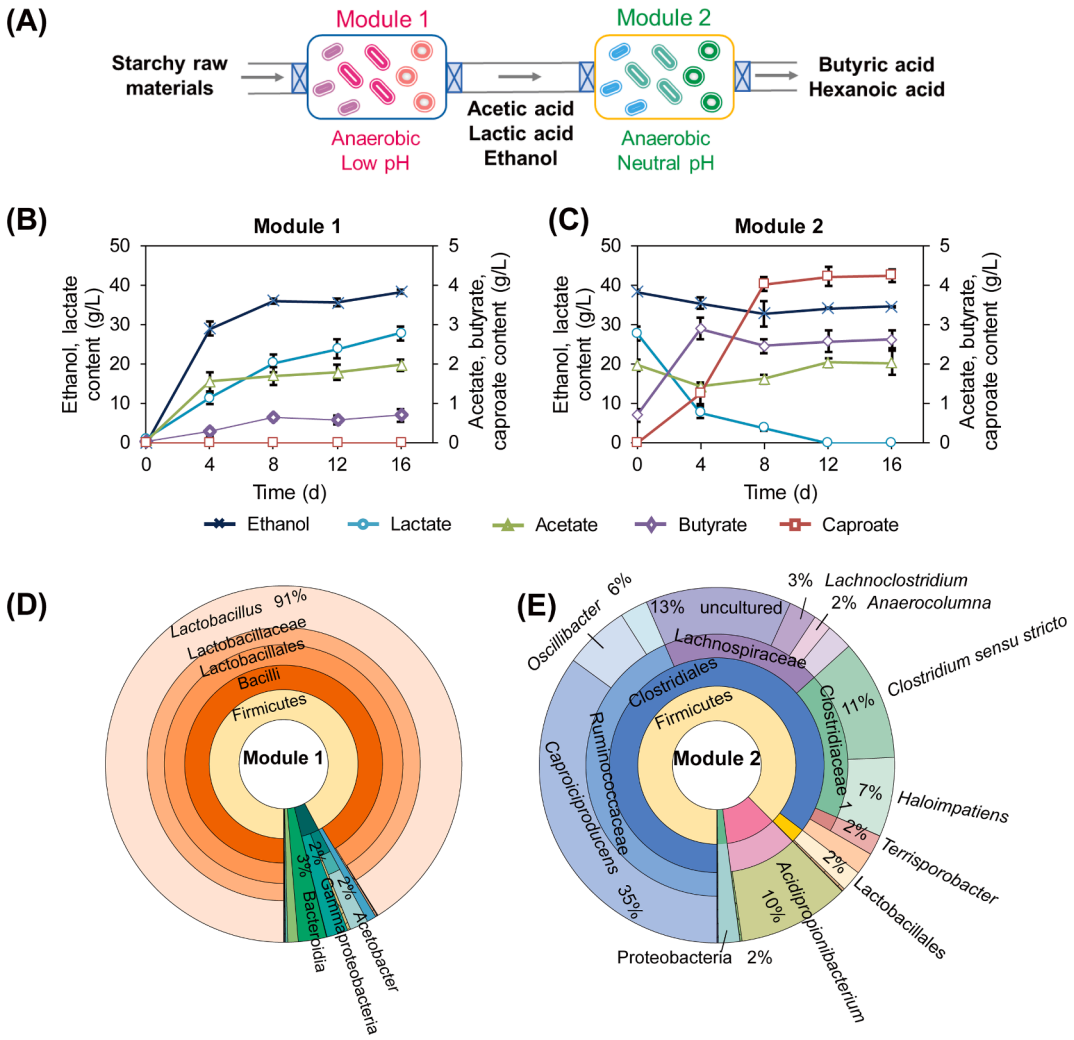

Fig. 6. Metabolite profiles and bacterial community patterns of each module during in vitro sequential fermentation. (A) Schematic of the sequential fermentation system. (B) and (C) Metabolite contents in fermentation broth of module one and two. (D) and (E) Bacterial community composition genus to phylum of each module.

consortia and potential metabolic functions provided a framework to support the concept of jiupei and pit mud functioning as distinct modules exhibiting a cooperative function for organic acid production (Fig. 6A). A modular reactor was constructed to verify this hypothesis. Module one contained the jiupei consortium and sorghum granules (substrate), and module two contained the pit mud consortium and fermentation residue (inoculum) from module one.

Fig. 6B shows the changes in organic acids and ethanol during the in vitro fermentation of jiupei in module one over 16 d. The rate of lactic acid formation was 1.7 g/L/d, reaching 27.9 ± 1.8 g/L on day 16; ethanol increased to 36.11 ± 0.73 g/L on day eight and remained constant until day 16; acetic acid and butyric acid were 2.0 ± 0.2 g/L and 0.7 ± 0.2 g/L at day 16, respectively; hexanoic acid was not detected across all samples.

The metabolic characteristics of the microbial consortia from the pit mud fermentation in module two were different from those in module one (Fig. 6C). Lactic acid was the favored carbon source in this module, and it was consumed at a rate of 2 g/L/d before being exhausted on day 12; the concentration of butyric acid reached a maximum value on day four (2.9 ± 0.3 g/L), while the content of hexanoic acid increased at 0.5 g/L/d until day eight. The concentration of acetic acid remained relatively stable, and we considered that its production and utilization by the pit mud microbiota was in the homeostatic range, which was also consistent with the in situ results from the Baijiu cellar (Fig. 1H).

Community structure analysis indicated that the population of module one was dominated by *Lactobacillus* in late fermentation (Fig. 6D), like the microbial community composition of jiupei in cellar production. From 295 OTUs, 21 (91.4%) were assigned to *Lactobacillus*. *Lactobacillus* is known for its ability to convert carbohydrates into lactic acid, which was consistent with measured concentrations of the acid (Fig. 1G & Fig. 6B). In module two, the main phyla were Firmicutes, Actinobacteria and Proteobacteria, accounting for 87.5%, 10.1%, and 1.9% of total reads respectively (Fig. 6E). Clostridiales was the most abundant order within Firmicutes, comprising three families, i.e., Ruminococcaceae (43.5%), Lachnospiraceae (19.8%), and Clostridiaceae (18.2%). The community pattern also differed from that of the pit mud community at the genus level. The most prevalent genus in the module two consortium was *Caproiciproducens* (35.0%), which was 10 times more abundant than that in the pit mud.

4. Discussion

After undergoing long-term (several decades/centuries) directional selection and artificial domestication, the mud fermentation cellar for Chinese strong-flavor Baijiu production remains an anaerobic bioreactor converting starchy biomass into ethanol and various flavor compounds. Jiupei, which mainly comprises sorghum, is the fermentation substrate in Baijiu production, and pit mud provides a vessel for its fermentation. Previous studies of flavor formation in strong-flavor Baijiu have focused on the microbiome of either jiupei or pit mud (Chai, Xu, et al., 2019; He,

Huang, Zhou, Wu, & Jin, 2019), resulting in an incomplete understanding of the roles of the various microorganisms. In this study, we explored the division of ecological functions of the microbial communities in jiupei and pit mud during fermentation by following the metabolism of four key organic acids (hexanoic, butyric, acetic and lactic acids). The microbial community structures and metabolic functions in jiupei and pit mud were systematically investigated within a single mud cellar for the first time. The results showed that the microbes in jiupei and pit mud occupied distinct habitats with differing environmental conditions during Baijiu fermentation (Fig. 1). The accumulation of large amounts of lactic acid characterized the low pH environment of jiupei, while the pH of pit mud was close to neutral, and these findings were similar to those reported previously (Liu, Tang, Guo, et al., 2017; Zhang et al., 2017). Interestingly, a recent study showed that the pH of pit mud at the base of the fermentation cellar increased with increasing depth (Zhang et al., 2020).

Bacteria were the major microbial consortia during Baijiu fermentation, although their composition and abundance were significantly different in jiupei and pit mud (Fig. 3). Lactic acid bacteria, mainly distributed in the genus *Lactobacillus*, were the most abundant bacterial group in jiupei throughout fermentation, which was recently confirmed by quantitative profiling (Du, Wu, & Xu, 2020). In the in vitro study, *Lactobacillus* was the predominant bacteria at the end of fermentation in the jiupei module (Fig. 6D). It is interesting to note that *Lactobacillus* also dominated the microbial consortia of other Chinese liquors that had different types of flavor (Liu & Miao, 2020; Zhang et al., 2020). This may be due to the similar fermentation substrates used (sorghum and wheat) and purpose of the fermentation ecosystem, i.e., the conversion of starchy raw materials into ethanol and flavor compounds. Metatranscriptomics analysis of jiupei on days eight and 15 indicated the pivotal roles of *Lactobacillus* for the metabolism of flavor compounds and their precursors in strong-flavor Baijiu (Du et al., 2020). *Lactobacillus* was also shown to be the core functional microorganism responsible for the accumulation of lactic acid during the production of Chinese soy sauce-aroma Baijiu (Song, Du, Zhang, & Xu, 2017). Amongst the *Lactobacillus* OTUs, OTU0 was the most abundant and increased during fermentation (Fig. 4C), especially in the mid and late phases. OTU0 was assigned to *Lactobacillus jinshani* (growth pH 3.0–5.0) (Yu et al., 2020), which was previously isolated from acidic vinegar fermentation materials and further reclassified to *Acetilactobacillus jinshanensis* (Zheng et al., 2020). The relative abundance of *Acetobacter* belonging to Proteobacteria increased to 40.4% on day one, reducing to <1.0% by day 16 as the oxygen in jiupei was consumed in 5 d (Figs. 1D & 2B). The microbial community analysis of jiupei on day seven showed that the relative abundances of *Lactobacillus* and *Acetobacter* were significantly lower at the RNA level than the DNA level (Hu et al., 2020). Further systematic studies at the transcriptional and protein levels should be performed to elucidate the microbial community functional diversity in jiupei during fermentation.

In the pit mud module, the most abundant bacterial population was Clostridia, and the α-diversity of the bacterial community structure was relatively stable during fermentation compared with that of jiupei. Interestingly, the number of reads for unclassified OTUs (801 of 2,719 OTUs) at the genus level according to taxonomic assignment against the current databases represented 2.6% and 14.1% of the total reads for jiupei and pit mud, respectively. It could be inferred that the pit mud microbial community was more complex than that of jiupei, which corroborated the results from the α-diversity analysis (Shannon index and species richness). The two most abundant Clostridial populations were found in the genera *Hydrogenispora* and *Sedimentibacter*. While this agreed with previous sequencing studies using primers specifically amplifying Clostridial 16S rRNA gene amplicons (Chai, Xu, et al., 2019), their roles in Baijiu fermenataion remain unclear. Bioaugmentation with *Hydrogenispora ethanolica* LX-B, a representative strain of only one valid species, dramatically enhanced the abundance of *Clostridium* and the production of butyric acid and hydrogen (Yang et al., 2016), indicating that *Hydrogenispora* microbes may function in Baijiu flavor formation by interspecies interactions with *Clostridium*. The relative abundance of *Sedimentibacter* was shown to increase with the increasing quality of pit mud (Hu et al., 2016). The third abundant Clostridial genus was *Caproiciproducens*, which was first reported as a novel genus in 2015 and belongs to family Oscillospiraceae (basonym of Ruminococcaceae) (Kim et al., 2015). The metabolic end products of anaerobic fermentation of *Caproiciproducens galactitolivorans* (JCM 30532), using galactitol as the carbon source included ethanol, acetic acid, butyric acid and hexanoic acid (Kim et al., 2015). An increased abundance of *Caproiciproducens* and accumulation of hexanoic acid in pit mud was also associated with increasing fermentation cellar age (Liu, Tang, Guo, et al., 2017). Numerous experiments using culture-independent and -dependent methods demonstrated that butyric acid- and hexanoic acid-producers mainly inhabited the pit mud in strong-flavor Baijiu production (Chai, Xu, et al., 2019; Tao et al., 2017; Zou et al., 2018).

The roles of the microbial consortia from these two habitats in the biosynthesis of organic acids, especially hexanoic acid, were validated using sequential in vitro jiupei-pit mud fermentation experiments.

The in vitro validation experiments showed that the microbial consortium of the pit mud module could utilize the metabolites from the jiupei module to produce fatty acids (mainly butyric and hexanoic acids). The community composition of the jiupei module resembled that of the in situ single cellar at the end of fermentation, while the microbial composition of the pit mud module was different. This difference was mainly manifested in the disappearance of the Bacteroides and the significant increase of *Caproiciproducens* in the pit mud. Different from the actual fermentation process in cellar, sequential fermentation was conducted in the validation assay. As a result, lactic and acetic acids produced by jiupei microbial community could not be used by microbes inhabiting pit mud in time, which might be the main reason for the difference of community structure of in situ and in vitro pit mud samples. Tao et al. (2017) constructed preliminary metabolic pathways for the main organic acids in pit mud using metagenomic sequencing. They deduced that *Clostridium* cluster IV was a major contributor to the synthesis of hexanoic acid from lactic acid via the chain elongation pathway. At the end of the in vitro fermentation of pit mud, the relative abundance of *Caproiciproducens* was 35.0%, which was 10 times more abundant than that in the fermentation cellar. It suggested that lactic acid, as carbon source, was conducive to the proliferation of *Caproiciproducens* in this system. This inference was consistent with the study of Zhu et al. (2018), who found that *Clostridium* cluster IV (i.e., *Caproiciproducens*) occupied a predominant position in the pit mud bacterial community after 90 d incubation in the leachate of jiupei. The results of both in situ and in vitro experiments showed that although it seems to be independent from the spatial structure for jiupei and pit mud, material exchange existed between them through the leachate of jiupei.

Our data also found that *Caproiciproducens* had a high diversity at the species level as 25 and 33 OTUs were detected in the pit mud and fermentation broth samples, respectively. Based on the concept of synthetic ecology, redundant members that differ in phylogeny within a community provides insurance when faced with changing environmental conditions (Ben Said & Or, 2017; Stenuit & Agathos, 2015). This redundancy phenomenon was also observed in the distribution of *Lactobacillus* in jiupei samples. OTU3 and OTU0 were the two most abundant species assigned to *Lactobacillus*, and dominated the early and later phases of fermentation, respectively (Fig. 4C). This furnished further evidence that selection from a pool of populations, with iterate functions adapted to specific environments, was widespread. This feature plays an important role in the robustness of microbial community structure and function (Konopka, Lindemann, & Fredrickson, 2015). Results from the in situ and validation experiments showed that *Lactobacillus* became the dominant genus when carbohydrate raw materials were abundant, thereby accumulating lactic acid. Environmental and chemical analyses and 16S rRNA gene sequencing showed that lactic acid in the fermentation broth was conducive to the enrichment of

hexanoic acid-producing microbes. Niche differentiation between the jiupei and pit mud consortia (complementary resource utilization) was another important factor for ecosystem function (Konopka et al., 2015).

In summary, this study has revealed that jiupei and pit mud comprised two spatially linked microbial habitats with significantly different environmental conditions in the mud cellar. Metagenomic sequencing showed that the structures of the microbial communities in jiupei and pit mud were significantly different during fermentation. *Lactobacillus* dominated the microbiota of jiupei while Clostridia was the major bacteria in pit mud, comprising mainly *Hydrogenispora*, *Sedimentibacter* and *Caproiciproducens*. In situ fermentation in the cellar and in vitro experiments demonstrated that lactic acid and acetic acid were mainly produced by the jiupei microbiota and microbes inhabiting the pit mud were responsible for the synthesis of hexanoic acid and butyric acid; these were the four key organic acids associated with strong-flavor Baijiu. This study has demonstrated that cooperation within the microbial consortia of jiupei and pit mud drives the formation of key flavor compounds during Baijiu fermentation.

CRediT authorship contribution statement

Wei Qian: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Visualization. **Zhen-Ming Lu:** Conceptualization, Funding acquisition, Writing - review & editing. **Li-Juan Chai:** Funding acquisition, Methodology, Writing - review & editing, Visualization. **Xiao-Juan Zhang:** Methodology. **Qi Li:** Writing - review & editing. **Song-Tao Wang:** Methodology. **Cai-Hong Shen:** Conceptualization. **Jin-Song Shi:** Writing - review & editing. **Zheng-Hong Xu:** Conceptualization, Writing - review & editing, Funding acquisition, Project administration, 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.

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Appendix A. Supplementary material

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References

Ben Said, S., & Or, D. (2017). Synthetic microbial ecology: Engineering habitats for modular consortia. *Frontiers in Microbiology, 8*, 1125. https://doi.org/10.3389/fmicb.2017.01125.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303.

Chai, L. J., Lu, Z. M., Zhang, X. J., Ma, J., Xu, P. X., Qian, W., … Xu, Z. H. (2019). Zooming in on butyrate-producing Clostridial consortia in the fermented grains of baijiu *via* gene sequence-guided microbial isolation. *Frontiers in Microbiology, 10*, 1397. https://doi.org/10.3389/fmicb.2019.01397.

Chai, L. J., Xu, P. X., Qian, W., Zhang, X. J., Ma, J., Lu, Z. M., … Xu, Z. H. (2019). Profiling the Clostridia with butyrate-producing potential in the mud of Chinese liquor fermentation cellar. *International Journal of Food Microbiology, 297*, 41–50. https://doi.org/10.1016/j.ijfoodmicro.2019.02.023.

Dennis, K. L., Wang, Y. W., Blatner, N. R., Wang, S. Y., Saadalla, A., Trudeau, E., … Khazaie, K. (2013). Adenomatous polyps are driven by microbe-instigated focal inflammation and are controlled by IL-10–producing T cells. *Cancer Research, 73*(19), 5905–5913. https://doi.org/10.1158/0008-5472.CAN-13-1511.

Du, R., Wu, Q., & Xu, Y. (2020). Chinese liquor fermentation: Identification of key flavor-producing *Lactobacillus* spp. by quantitative profiling with indigenous internal standards. *Applied and Environmental Microbiology, 86*, e00456–e520. https://doi.org/10.1128/AEM.00456-20.

Edgar, R. C., & Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics, 31*(21), 3476–3482. https://doi.org/10.1093/bioinformatics/btv401.

Fang, C., Du, H., Jia, W., & Xu, Y. (2019). Compositional differences and similarities between typical Chinese baijiu and western liquor as revealed by mass spectrometry-based metabolomics. *Metabolites, 9*(1), 2. https://doi.org/10.3390/metabo9010002.

Fierer, N., Jackson, J. A., Vilgalys, R., & Jackson, R. B. (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Applied and Environmental Microbiology, 71*(7), 4117–4120. https://doi.org/10.1128/AEM.71.7.4117-4120.2005.

Gao, Z., Wu, Z., & Zhang, W. (2020). Effect of pit mud on bacterial community and aroma components in yellow water and their changes during the fermentation of Chinese strong-flavor liquor. *Foods, 9*(3), 372. https://doi.org/10.3390/foods9030372.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology, 2*(2), 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x.

Green, J. L., Bohannan, B. J., & Whitaker, R. J. (2008). Microbial biogeography: From taxonomy to traits. *Science, 320*(5879), 1039–1043. https://doi.org/10.1126/science.1153475.

He, G., Huang, J., Zhou, R., Wu, C., & Jin, Y. (2019). Effect of fortified daqu on the microbial community and flavor in Chinese strong-flavor liquor brewing process. *Frontiers in Microbiology, 10*, 56. https://doi.org/10.3389/fmicb.2019.00056.

Hu, X., Du, H., Ren, C., & Xu, Y. (2016). Illuminating anaerobic microbial community and cooccurrence patterns across a quality gradient in Chinese liquor fermentation pit muds. *Applied and Environmental Microbiology, 82*(8), 2506–2515. https://doi.org/10.1128/AEM.03409-15.

Kim, B. C., Jeon, B. S., Kim, S., Kim, H., Um, Y., & Sang, B. I. (2015). *Caproiciproducens galactitolivorans* gen. nov., sp. nov., a bacterium capable of producing caproic acid from galactitol, isolated from a wastewater treatment plant. *International Journal of Systematic and Evolutionary Microbiology, 65*(12), 4902–4908. https://doi.org/10.1099/ijsem.0.000665.

Konopka, A., Lindemann, S., & Fredrickson, J. (2015). Dynamics in microbial communities: Unraveling mechanisms to identify principles. *The ISME Journal, 9*(7), 1488–1495. https://doi.org/10.1038/ismej.2014.251.

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., … Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology, 31*(9), 814–821. https://doi.org/10.1038/nbt.2676.

Liu, P., & Miao, L. (2020). Multiple batches of fermentation promote the formation of functional microbiota in Chinese miscellaneous-flavor baijiu fermentation. *Frontiers in Microbiology, 11*, 75. https://doi.org/10.3389/fmicb.2020.00075.

Liu, H., & Sun, B. (2018). Effect of fermentation processing on the flavor of Baijiu. *Journal of Agricultural and Food Chemistry, 66*(22), 5425–5432. https://doi.org/10.1021/acs.jafc.8b00692.

Liu, M. K., Tang, Y. M., Guo, X. J., Zhao, K., Tian, X. H., Liu, Y., … Zhang, X. P. (2017). Deep sequencing reveals high bacterial diversity and phylogenetic novelty in pit mud from Luzhou Laojiao cellars for Chinese strong-flavor Baijiu. *Food Research International, 102*, 68–76. https://doi.org/10.1016/j.foodres.2017.09.075.

Liu, M., Tang, Y., Zhao, K., Liu, Y., Guo, X., Ren, D., … Zhang, X. (2017). Determination of the fungal community of pit mud in fermentation cellars for Chinese strong-flavor liquor, using DGGE and Illumina MiSeq sequencing. *Food Research International, 91*, 80–87. https://doi.org/10.1016/j.foodres.2016.11.037.

Lu, S., Liu, X., Ma, Z., Liu, Q., Wu, Z., Zeng, X., … Gu, Z. (2016). Vertical segregation and phylogenetic characterization of ammonia-oxidizing bacteria and archaea in the sediment of a freshwater aquaculture pond. *Frontiers in Microbiology, 6*, 1539. https://doi.org/10.3389/fmicb.2015.01539.

Roller, B. R., Stoddard, S. F., & Schmidt, T. M. (2016). Exploiting rRNA operon copy number to investigate bacterial reproductive strategies. *Nature Microbiology, 1*(11), 1–7. https://doi.org/10.1038/nmicrobiol.2016.160.

Seedorf, H., Fricke, W. F., Veith, B., Brüggemann, H., Liesegang, H., Strittmatter, A., … Gottschalk, G. (2008). The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. *Proceedings of the National Academy of Sciences, 105*(6), 2128–2133. https://doi.org/10.1073/pnas.0711093105.

Song, Z., Du, H., Zhang, Y., & Xu, Y. (2017). Unraveling core functional microbiota in traditional solid-state fermentation by high-throughput amplicons and metatranscriptomics sequencing. *Frontiers in Microbiology, 8*, 1294. https://doi.org/10.3389/fmicb.2017.01294.

Spirito, C. M., Richter, H., Rabaey, K., Stams, A. J., & Angenent, L. T. (2014). Chain elongation in anaerobic reactor microbiomes to recover resources from waste. *Current Opinion in Biotechnology, 27*, 115–122. https://doi.org/10.1016/j.copbio.2014.01.003.

Stenuit, B., & Agathos, S. N. (2015). Deciphering microbial community robustness through synthetic ecology and molecular systems synecology. *Current Opinion in Biotechnology, 33*, 305–317. https://doi.org/10.1016/j.copbio.2015.03.012.

Tao, Y., Wang, X., Li, X., Wei, N., Jin, H., Xu, Z., … Zhu, X. (2017). The functional potential and active populations of the pit mud microbiome for the production of Chinese strong-flavour liquor. *Microbial Biotechnology, 10*(6), 1603–1615. https://doi.org/10.1111/1751-7915.12729.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology, 73*(16), 5261–5267. https://doi.org/10.1128/AEM.00062-07.

Wang, X., Du, H., & Xu, Y. (2017). Source tracking of prokaryotic communities in fermented grain of Chinese strong-flavor liquor. *International Journal of Food Microbiology, 244*, 27–35. https://doi.org/10.1016/j.ijfoodmicro.2016.12.018.

Xiao, C., Lu, Z. M., Zhang, X. J., Wang, S. T., Ao, L., Shen, C. H., … Xu, Z. H. (2017). Bio-heat is a key environmental driver shaping the microbial community of medium-temperature Daqu. *Applied and Environmental Microbiology, 83*, e01550–e1617. https://doi.org/10.1128/AEM.01550-17.

Xiao, C., Lu, Z., Zhang, X., Wang, S., Ao, L., Shen, C., … Xu, Z. (2018). Succession of the fungal community on fermented grains of Luzhou-flavor baijiu through fermentation. *Chinese Journal of Applied and Environmental Biology, 24*(05), 1081–1086. https://doi.org/10.19675/j.cnki.1006-687x.2017.12057.

Xiao, C., Lu, Z., Zhang, X., Wang, S., Li, D., Shen, C., … Xu, Z. (2019). Bacterial community succession in fermented grains of Luzhou-flavor baijiu. *Acta Microbiologica Sinica, 059*(001), 195–204. https://doi.org/10.13343/j.cnki.wsxb.20180123.

Yang, Z., Guo, R., Shi, X., He, S., Wang, L., Dai, M., … Dang, X. (2016). Bioaugmentation of *Hydrogenispora ethanolica* LX-B affects hydrogen production through altering indigenous bacterial community structure. *Bioresource Technology, 211*, 319–326. https://doi.org/10.1016/j.biortech.2016.03.097.

Yu, Y., Li, X., Zhang, J., Chai, L. J., Lu, Z. M., & Xu, Z. H. (2020). *Lactobacillus jinshani* sp. nov., isolated from solid-state vinegar culture of Zhenjiang aromatic vinegar. *Antonie van Leeuwenhoek, 113*(1), 43–54. https://doi.org/10.1007/s10482-019-01316-1.

Zhang, G., Tian, J., Jiang, N. A., Guo, X., Wang, Y., & Dong, X. (2008). Methanogen community in Zoige wetland of Tibetan plateau and phenotypic characterization of a dominant uncultured methanogen cluster ZC-I. *Environmental Microbiology, 10*(7), 1850–1860. https://doi.org/10.1111/j.1462-2920.2008.01606.x.

Zhang, H., Wang, L., Wang, H., Yang, F., Chen, L., Hao, F., … Xu, Y. (2020). Effects of initial temperature on microbial community succession rate and volatile flavors during Baijiu fermentation process. *Food Research International, 109887*. https://doi.org/10.1016/j.foodres.2020.109887.

Zhang, Y., Zhu, X., Li, X., Tao, Y., Jia, J., & He, X. (2017). The process-related dynamics of microbial community during a simulated fermentation of Chinese strong-flavored liquor. *BMC Microbiology, 17*(1), 196. https://doi.org/10.1186/s12866-017-1106-3.

Zheng, X. W., & Han, B. Z. (2016). Baijiu (白酒), Chinese liquor: History, classification and manufacture. *Journal of Ethnic Foods, 3*(1), 19–25. https://doi.org/10.1016/j.jef.2016.03.001.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M., Harris, H. M., Mattarelli, P., … Lebeer, S. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology, 70*(4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107.

Zou, W., Ye, G., Zhang, J., Zhao, C., Zhao, X., & Zhang, K. (2018). Genome-scale metabolic reconstruction and analysis for *Clostridium kluyveri*. *Genome, 61*(8), 605–613. https://doi.org/10.1139/gen-2017-0177.

Zou, W., Zhao, C., & Luo, H. (2018). Diversity and function of microbial community in Chinese strong-flavor baijiu ecosystem: A review. *Frontiers in Microbiology, 9*, 671. https://doi.org/10.3389/fmicb.2018.00671.