

## Profiling prokaryotic community in pit mud of Chinese strong-aroma type liquor by using oligotrophic culturing

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### ABSTRACT

Pit mud microbiota plays a key role in flavour production for Chinese strong-aroma type liquor. However, the pit mud microbiota cannot be cultured in laboratory. In this study, an oligotrophic medium with acetate as carbon source was used to enrich pit mud microbiota. The 16S rRNA gene amplicon sequencing was applied to examine the microbial dynamics of the enrichment consortia. Both methanogens and bacteria were simultaneously enriched. Euryarchaeota, Bacteroidetes and Firmicutes were the top 3 enriched phyla, and 31 genera were successfully enriched. More specifically, 11 genera (65%) out of the 17 dominant genera in pit mud were successfully enriched, including *Petrimonas*, *Proteiniphilum*, *Anaerocella*, *Hydrogenispora*, *Methanosarcina*, *Fermentimonas*, LNR\_A2-18, *Sedimentibacter*, *Lutispora*, *Syntrophomonas* and *Aminobacterium*. Furthermore, 20 rare genera in the analyzed pit mud samples were also enriched. Aceticlastic *Methanosaeta* and *Methanosarcina* were found to be dominant methanogens in the enrichment consortia. Metagenomic sequencing was then applied to the enriched microbial consortia to explore the metabolic potentials of pit mud microbes. Aceticlastic methanogenesis pathway of *Methanosaeta* was reconstructed. Furthermore, 26 high-quality metagenome-assembled genomes (MAGs) were obtained based on the metagenomic binning analysis. Moreover, nutrients in pit mud were found to be crucial to sustain the methanogenesis of the enriched microbial consortia. These results suggested that the enrichment approach by using oligotrophic culturing can effectively cultivate the pit mud microbiota. Combined with metagenomics, the oligotrophic culturing will be greatly helpful to decipher the community composition and metabolic potentials of pit mud microbiota.

### 1. Introduction

Chinese strong-aroma type liquor, a traditional fermented beverage, is typically produced by solid-state fermentation process. This fermentation process is initiated by a variety of microbial communities originating from *Daqu* (a kind of starter) as well as pit mud. *Daqu* provides saccharifying enzymes from filamentous fungi, and provides yeasts for ethanol production (Wang et al., 2017). Although *Daqu* is key to initiate the fermentation for strong-aroma type liquor production, the formation of key aromas of strong-aroma type liquor still needs other microbes. As key aromas, the short- and medium-chain fatty acids, e.g., butyrate and caproate, and their corresponding esters shape the typical flavour of strong-aroma type liquor. It has been reported that the short- and medium-chain fatty acids are produced by pit mud microbes, among which clostridial species are the major contributors (Hu et al., 2016). For

instance, *Ruminococcaceae* bacterium CPB6, isolated from a fermentation cellar, was identified as an important caproate producer (Zhu et al., 2015, 2017). Another study demonstrated that 19 clostridial butyrate-producing species were isolated from pit mud (Chai et al., 2019). Besides clostridial species, other prokaryotes such as Bacteroidetes species and methanogens are believed to be critical to maintain the normal structural and function of pit mud (Hu et al., 2016). Moreover, methanogens are thought to be able to indirectly enhance caproate production through syntrophic metabolism with caproate-producing bacteria (Tao et al., 2014, 2017; Yan and Dong, 2018). However, many microbes other than clostridial species remain uncultivable in laboratory. This limits our knowledge about how pit mud microbiota works during Chinese strong-aroma type liquor fermentation. Therefore, cultivating pit mud microbes as many as possible has been an urgent task.

Our previous study using 16S rRNA gene amplicon sequencing

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indicated that around 225 genera exist in pit mud, among which at least 31 genera were identified as high-abundance microbes (Hu et al., 2016). However, just like other microbes from natural environment, cultivating pit mud microbes derived from the environment of Chinese liquor fermentation in laboratory confronts difficulties. One reason is that microbes from natural environments often grow under oligotrophic or fastidious conditions, resist media containing complex organic carbon sources and thus remain dormant (Overmann et al., 2017). In recent years, researchers found microbes that resist cultivation in laboratory might grow under culture conditions of simulating natural environments. For instance, through application of techniques such as diffusion chambers (Kaeberlein et al., 2002), isolation chip (Nichols et al., 2010) or supplementing soil extracts (Nguyen et al., 2018), uncultured bacteria can be effectively obtained.

Although omics approaches such as metagenomics and metatranscriptomics have been widely used to examine the functions of complex microbial communities in anaerobic habitats (Lillington et al., 2019), these two meta-omics techniques have not been broadly employed in studies of Chinese liquor fermentation for the difficulty in obtaining high-quality DNA as well as RNA from microbial community of fermented samples. The fermentation process of Chinese liquor usually reaches up to two weeks or even several months (Jin et al., 2017). What's more, the recycling fermentation technique is usually used for strong-aroma liquor production. The recycling and long-term fermentation causes the release of fragmental DNA and RNA from dead cells, causing difficulty in applying metagenomics and metatranscriptomics to pit mud microbes. Only limited studies in the field of Chinese liquor fermentation employing metagenomics or metatranscriptomics have been reported up to now (Guo et al., 2014; Song et al., 2017). Therefore, we need find a way to culture the pit mud microbiota in laboratory and then develop a stable and reliable metagenomics approach to reveal the metabolic characteristics of pit mud microbes.

In this study, an oligotrophic medium was chosen for the enrichment culturing of pit mud microbiota. Furthermore, 16S rRNA gene amplicon sequencing and metagenomics analysis were combined to investigate the microbial diversity and metabolic characteristics of the enriched microbial consortia. The aim of this study is to find the unculturable microbes in pit mud, and initially analyze their metabolic capability in enriched consortia.

## 2. Materials and methods

### 2.1. Pit mud sample collection

Pit mud samples were collected from two fermentation pits in a strong-aroma type liquor distillery located in Jiangsu Province, China on December 2, 2018. Triplicate subsamples were collected in each pit and mixed immediately. The samples were immediately transferred to sterile anaerobic bags (Mitsubishi Gas Chemical Co., Inc., Japan) and transported to laboratory for inoculation at the same day.

### 2.2. Media and culturing conditions

A medium described by McInerney et al. (1979) was used with several modifications. The medium contained the components as follows: 5.0% (v/v) of Pfennig's mineral solution, 0.1% (v/v) of Pfennig's metal solution, 0.5% (v/v) of B-vitamin mixture, 7% (v/v) of NaHCO<sub>3</sub> solution, 2% (v/v) of Na<sub>2</sub>S solution; 5 g/L of NaAc·3H<sub>2</sub>O. The growth-stimulating factors were replaced by 0.1% (w/v) yeast extract and 0.1% (w/v) tryptone instead of 5.0% (v/v) rumen fluid. Acetate was used as major carbon source. Inorganic salt compositions were used for the preparation of Pfennig's mineral solution and Pfennig's metal solution. NaHCO<sub>3</sub>, Na<sub>2</sub>S and NaAc·3H<sub>2</sub>O were purchased from Sinopharm Reagent Co., Ltd. (Shanghai, China). Vitamin components were purchased from Sangon Biotech (Shanghai, China). Yeast extract and tryptone were purchased from Oxoid (UK). All the components except

for vitamin solution, Na<sub>2</sub>S and NaHCO<sub>3</sub>, were mixed and then subjected to autoclave. The pH of the medium was adjusted to 7.4 ± 0.2, and then the medium was anaerobically aliquoted into 100 mL-serum bottles. Subsequently, the bottles were sealed and autoclaved at 121 °C for 20 min. Na<sub>2</sub>S solution was autoclaved separately. NaHCO<sub>3</sub> solution and vitamin solution were sterilized via filtration using microporous membrane (0.22-μm, aqueous phase). Then the above three solutions were mixed to make a working volume of 90 mL in a serum bottle. Two pre-mixed pit mud samples were inoculated at 10% (w/v) to obtain enrichment cultures. The inoculated cultures were statically incubated at 37 °C. When the carbon source was nearly exhausted, successive passaging was performed. The successive passaging and sampling were performed using syringes. All these procedures were performed in an anaerobic chamber (Forma 1029, Thermo Scientific, USA).

### 2.3. Gas chromatography analysis

Acetate was measured by Gas Chromatography (GC-7890B, Agilent Technologies, USA) equipped with an Altech capillary column (Econo Cap-Wax, 30-m length, 0.25-mm diameter, 0.25-μm film thickness). Methane in the headspace of the culturing bottles was collected via a syringe (2.5 mL, GERSTEL, Germany), and then detected by Gas Chromatography (GC-6890N, Agilent Technologies) equipped with a capillary column (HP-5, 30-m length, 0.32-mm diameter, 0.25-μm film thickness). Methane with 99.9% purity (XinXiYi Co., Ltd., Wuxi, China) was used as external standard for methane analysis.

### 2.4. DNA extraction and amplicon sequencing analysis

DNA was extracted from pit mud and the enrichment culture using a DNeasy PowerSoil Kit (QIAGEN GmbH, Germany) according to the manufacturer's instruction. The concentration and the purity of the purified DNA was assessed by NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The integrity of the purified DNA was determined by electrophoresis in a 1% agarose gel, and the DNA with single band over 10 kb was judged as integrated DNA. The V4 hypervariable regions of the 16S rRNA gene were amplified using the primer set 515FmodF (5'-GTGYCAGCMGCCGCGTAA-3') and 806RmodR (5'-GGACTACNVGGGTWTCTAAT-3') (Walters et al., 2016). The amplified PCR products were subjected to library construction and then sequenced on illumina MiSeq sequencing platform using PE250 strategy. The sequencing data was analyzed on i-sanger cloud platform provided by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). Briefly, the high-quality bases and the paired-end reads were assembled using FLASH (Magoc and Salzberg, 2011). The sequences shorter than 50 bp were removed using Trimmomatic included in QIIME (ver. 1.9.1) (Caporaso et al., 2010). After that, operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE included in Usearch 7.0 (Edgar, 2013). A single representative sequence from each clustered OTU was used to align against the Silva database (ver. 128). The Shannon diversity index and Chao 1 richness index were calculated based on the resulting numbers by the Mothur (ver. 1.30.1) (Lin et al., 2020). The raw sequence data was deposited in the NCBI Sequence Read Archive (SRA) BioProject PRJNA598278 under the accession number of SAMN13702314-SAMN13702321.

### 2.5. Testing the effect of pit mud on phenotype recovering of microbial community

To recover the phenotypes weakened during the successive passaging, pit mud was supplemented to the enrichment culture and together autoclaved with the medium. Experiments were designed as followed: (i) 10% (v/v) enriched culture was inoculated into the pre-reduced basal medium with acetate as carbon source without adding pit mud; (ii) 10% (v/v) enrichment culture was inoculated into medium

adding 3% (m/v) pit mud; (iii) 3% (m/v) pit mud was supplemented to the same medium without seed inoculation of enriched culture. Successive passaging was performed with syringes. All preparations were performed in triplicate.

## 2.6. Metagenomic analysis

For metagenomics analysis, DNA of samples from the fourth and eighth day from enrichment 1 were pooled. The integrity of DNA was examined by electrophoresis in a 1% agarose gel. The DNA were subjected to Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) for library construction and sequencing. A 400 bp's library was constructed with NEXTFLEX™ Rapid DNA-Seq Kit and then subjected to sequencing on Illumina HiSeq 2500 platform with PE150 strategy. The high-quality paired-end reads were obtained by using Seqprep (John, 2016) and Sickle (Joshi and Fass, 2011). The assembly was performed with Megahit (Version 1.1.2) (Li et al., 2015). The metabolic pathway mapping was performed by aligning the non-redundant translated sequences against KEGG GENES database with Diamond (ver. 0.8.35) (Benjamin et al., 2015), and the relative abundance for each KO gene was calculated. The resulting alignments were annotated with KOBAS 2.0 (Xie et al., 2011). The raw sequence data was deposited in the NCBI Sequence Read Archive (SRA) BioProject PRJNA602212 under the accession number SAMN13885704.

## 2.7. Metagenomic binning and phylogenetic analysis

MetaBat2 (Kang et al., 2019) was used for further metagenomic binning on the basis of completeness >90%, contamination <10% and the size of metagenome-assembled genome (MAG) >2Mbp. The phylogeny of each MAG-representative species was determined against the NCBI database (July 2019). The high-quality MAGs were deposited in the NCBI Sequence Read Archive (SRA) BioProject PRJNA605696 under the accession number SAMN14074453 - SAMN14074478.

## 3. Results

### 3.1. Metabolic characteristics of the oligotrophic enrichment cultures

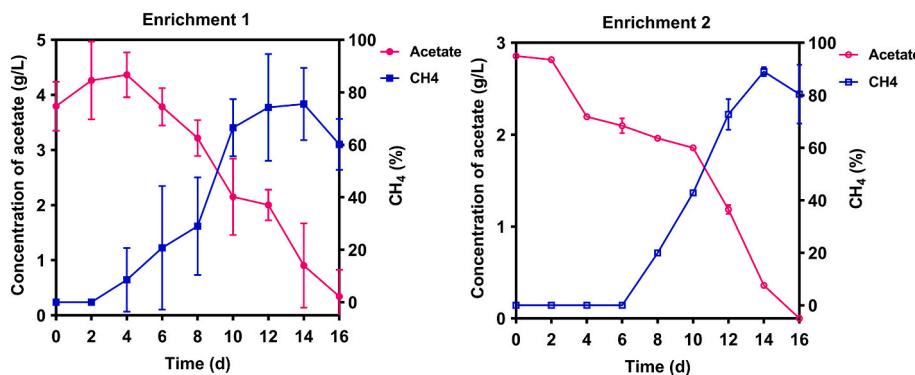
To enrich major aceticlastic methanogens in pit mud, acetate was supplemented as sole carbon source. We performed two batches of enrichment using two mixed pit mud samples derived from two different pits. During the enrichment culturing, acetate was utilized, and methane was gradually generated (Fig. 1). For enrichment 1, we found that methane production was 2 d earlier than the time point of detected acetate consumption (4 d vs. 6 d). Then the relative proportion of methane rapidly increased with the consumption of acetate. Upon the exhaustion of acetate at 14 d, the accumulation of methane reached the

maximum amount. Similar acetate consumption and methane production were observed in enrichment 2 (Fig. 1B). These results suggested that certain methanogens utilizing acetate might be enriched in the oligotrophic cultures.

### 3.2. The diversity of the enriched microbial consortia obtained by oligotrophic enrichment conditions

Alpha diversity was used to evaluate the changes of microbial diversity during the enrichment process (Table 1). Specifically, community richness reflecting the number of species was determined by Chao1 and Ace indices while community diversity was examined by Shannon and Simpson indices. For enrichment 1, both Ace and Chao1 values of enrichment cultures were slightly higher than that of pit mud and increased when the culturing was extended, indicating that the number of species in enrichment cultures increased when comparing with that in pit mud. In addition, the value of Shannon index reached the highest at the fourth day and then decreased at the twelfth day when the culturing was extended. Correspondingly, Simpson index decreased to the lowest at the fourth day and then increased at the twelfth day. The changes of richness and diversity indicated that some species might decline along with the process of enrichment. More significant changes of richness and diversity for the enrichment 2 were observed. Although the richness and diversity of microbiota in pit mud J2 (the sample used in enrichment 2) was only around 55% of that in pit mud J1 (the sample used in enrichment 1), we found that the richness and diversity of microbiotas enriched from pit mud J2 were almost 2-fold as that of microbiotas enriched from pit mud J1. These results regarding to richness and diversity suggested that high species diversity can be obtained by oligotrophic enrichment culturing.

Genera with abundance no less than 1% in either pit mud or enrichment cultures were subjected to the microbial community analysis (Fig. 2 and Table S1). A total of 39 genera were detected in pit mud and two enrichment groups. For enrichment 1, we found that 15 genera were detected in enrichment cultures whereas 24 genera weren't. For enrichment 2, we found that 27 genera were detected whereas 12 genera weren't. An enriched genus was defined according to the following two criterions: i, the abundance of one genus in the enrichment culture was more than 10% of the abundance in the inoculated pit mud sample since a 10% (m/v) inocula of pit mud was used and the dilution factor was 10-fold; ii, the abundance of one genus in the enrichment culture was more than 1% because it will become difficult to isolate pure clones when the relative abundance was too low. Based on the definition of enriched genera, except *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Prevotella* 7, *Kurthia*, *Syntrophaceticus* and *Acholeplasma*, other 31 genera in the 39 detected genera were successfully enriched at least in one enrichment group. Specifically, a total of 15 genera and 27 genera were successfully enriched for enrichment 1 and for enrichment 2,



**Fig. 1.** Acetate consumption and methane production of enrichment cultures. Data were shown as mean  $\pm$  SD from three biological replicates. Accumulation of methane is measured by volume fraction. Each enrichment was performed in triplicates.

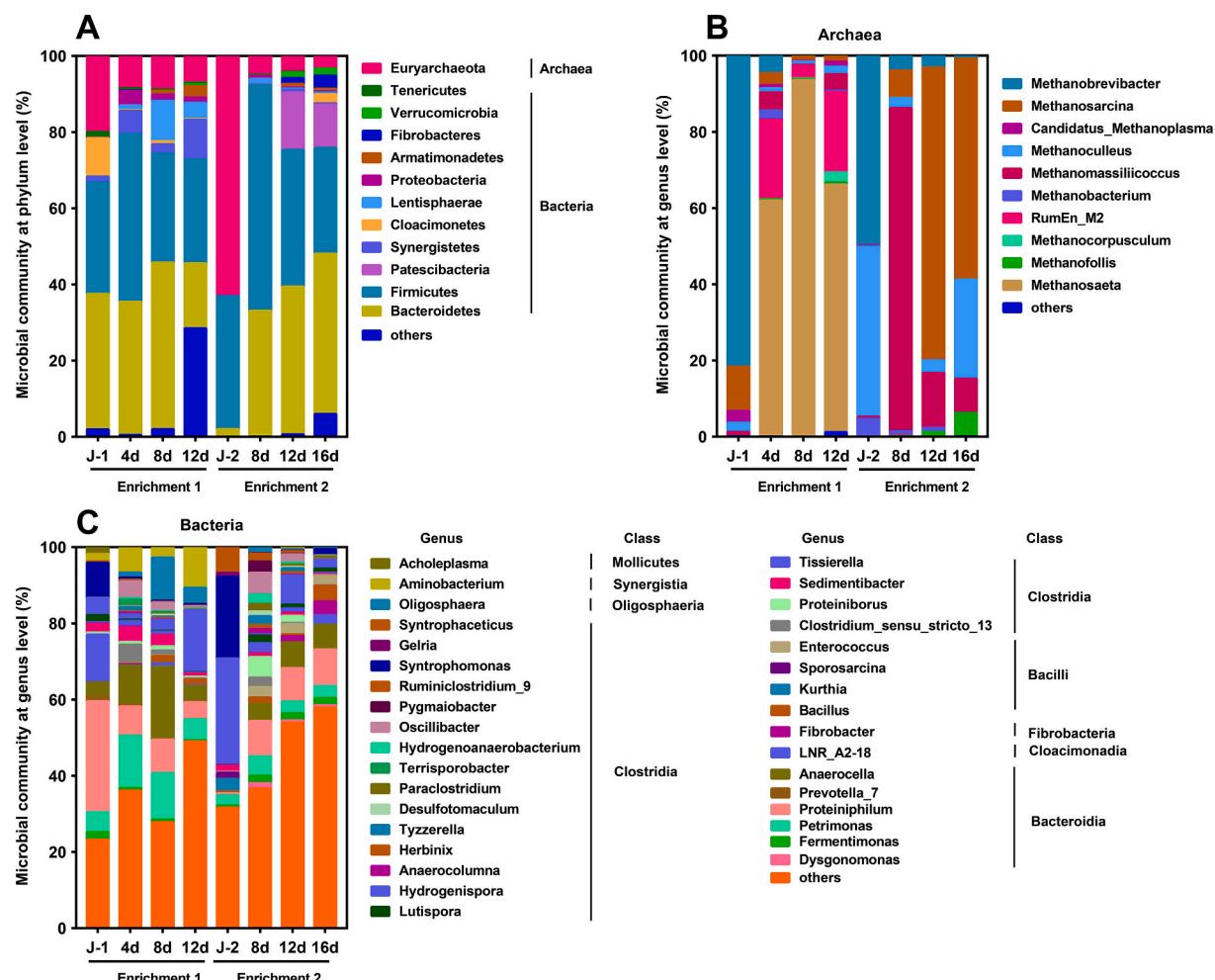
**Table 1**The  $\alpha$ -diversity of communities of typical enrichment cultures and their original pit mud based on 16S rRNA gene amplicon sequencing.

$\alpha$ -Diversity	Enrichment 1				Enrichment 2			
	Pit mud	Enrichment culture post inoculation			Pit mud	Enrichment culture post inoculation		
		J-1	4 d	8 d		J-2	8 d	12 d
<b>Richness</b>								
Ace index	578	679	682	735	320	549	641	627
Chao1 index	580	665	676	726	343	541	632	644
<b>Diversity</b>								
Shannon index	3.24	4.18	3.77	3.21	2.23	4.24	3.95	3.84
Simpson's reciprocal index	11	25	17	9	5	33	20	25

respectively (Fig. 2 and Table S1). Furthermore, 17 dominant genera ( $\geq 1\%$  in any pit mud sample) were detected in both pit mud samples: Euryarchaeota, including *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus* and *Methanosarcina*; Bacteroidetes, including *Fermentimonas*, *Petrimonas*, *Proteiniphilum*, *Prevotella\_7* and *Anaerocella*; LNR\_A2-18 under Fibrobacteres; Firmicutes, including *Kurthia*, *Sedimentibacter*, *Lutispora*, *Hydrogenispora*, *Syntrophomonas*, *Syntrophacetkus* and *Aminobacterium*. Among these 17 genera, four genera (23.5%) including *Petrimonas*, *Proteiniphilum*, *Anaerocella* and *Hydrogenispora* were successfully enriched in both enrichment groups; Seven genera (41.1%) including *Methanosarcina*, *Fermentimonas*, LNR\_A2-18, *Sedimentibacter*, *Lutispora*, *Syntrophomonas* and *Aminobacterium* were successfully

enriched in one of the enrichment groups.

Moreover, not all the enriched microbes were high abundant genera in pit mud. For instance, the abundances of *Tissierella*, *Proteiniphilum* and *Anaerocella* were far below 1% in the enrichment cultures. As shown in Fig. 2 and Table S1, other 20 enriched genera were the low-abundance microbes from pit mud. We referred these low-abundance microbes as rare pit mud microbes. For enrichment 1, nine rare genera including *Methanosaeta*, *RumEn\_M2*, *Bacillus*, *Clostridium\_sensu\_stricto\_13*, *Tissierella*, *Tyzzerella*, *Terrisporobacter*, *Oscillibacter* and *Oligosphaera* were successfully enriched. Except *Tissierella*, other genera were not even detected in the seed pit mud sample. For enrichment 2, 18 rare genera including *Methanomassiliicoccus*, *Dysgonomonas*, *Fibrobacter*, *Bacillus*,



**Fig. 2.** The microbial dynamics of the prokaryote community at phylum level (A), the archaeal community at genus level (B) and the bacterial community at genus level (C) in a typical enrichment culture and the original pit mud. Enrichment 1 and 2 were enrichment groups using two pre-mixed pit mud samples. The microbial compositions of J-1 and J-2 represented the microbial compositions of pre-mixed pit mud. Pooled DNA was used for each enrichment time point.

*Enterococcus*, *Clostridium sensu stricto\_13*, *Proteiniborus*, *Tissierella*, *Anaerocolumna*, *Herbinix*, *Tyzzera*, *Desulfotomaculum*, *Paraclostridium*, *Hydrogenoanaerobacterium*, *Oscillibacter*, *Pygmaeobacter*, *Ruminiclostridium\_9* and *Oligosphaera* were successfully enriched. Therefore, the rare microbes in pit mud can be also effectively enriched.

As indicated in Fig. 2A, at the phylum level, Bacteroidetes, Firmicutes and Euryarchaeota dominated in both pit mud samples. After the enrichment culturing, these three phyla still dominated in enrichment cultures. As shown in Fig. 2B, for enrichment 1, the archaea in pit mud were mainly composed of *Methanobrevibacter* (81.64%), *Methanosarcina* (11.58%), *Candidatus Methanoplasm* (3.15%), *Methanoculleus* (2.44%) and *Methanomassiliicoccus* (1.06%). After the enrichment culturing, *Methanosaeta* dominated in the enrichment cultures, accounting for 61.94 to 93.58%. Besides, *Methanomassiliicoccus*, *Methanoculleus*, *Candidatus Methanoplasm* and *Methanosarcina* were also successfully enriched. For enrichment 2, the archaea in pit mud were mainly composed of *Methanobacterium* (4.68%), *Methanoculleus* (44.63%) and *Methanobrevibacter* (49.79%). After the enrichment culturing, except these three dominant methanogens, *Methanofollis*, *Methanomassiliicoccus*, *Candidatus Methanoplasm* and *Methanosarcina* were also enriched. Although the relative abundance of *Methanomassiliicoccus* reached 84.75% at the 8 d, it then decreased to 8.87% at the end of the culturing. *Methanosarcina* became the dominant methanogen with relative abundance ranging from 7.24% to 76.77%. Therefore, the types of enriched methanogens by using acetate as carbon source depended on the initial types of aceticlastic methanogens in pit mud.

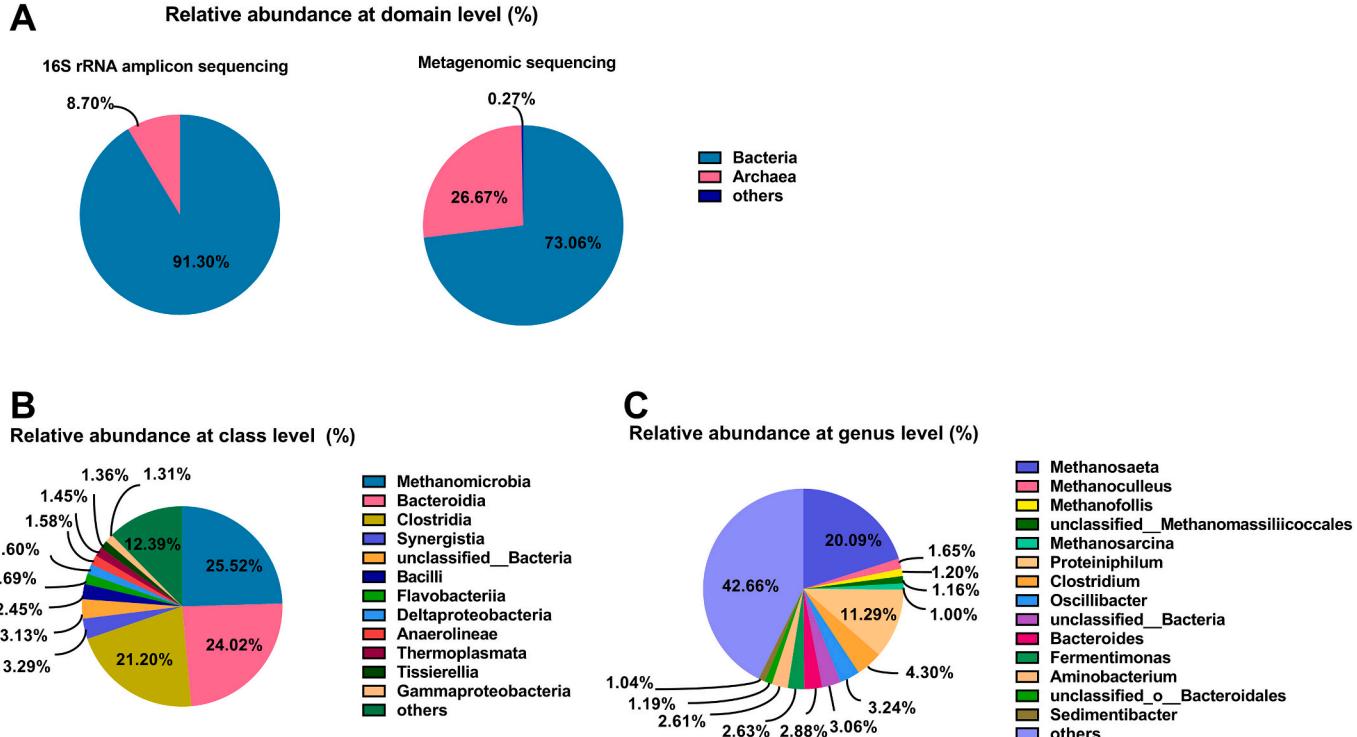
Many rare bacteria from pit mud were enriched. For instance, a total of 19 genera under Clostridia class were detected in both groups, totally accounting for 14.39% to 22.40% in enrichment 1 and 7.12% to 35.20% in enrichment 2 (Table S1). Besides, three other genera under Clostridia class, i.e. *Oscillibacter*, *Tyzzera* and *Clostridium sensu stricto\_13* which were even not detected in pit mud, were also enriched in both enrichment groups.

In addition, we analyzed the dynamics of the archaeal and bacterial communities during the enrichment process. For enrichment 1, we found that 13 genera were enriched at the beginning phase or middle phase during the enrichment (type II in Table S1), whereas 2 genera were enriched at the end-stage of the enrichment (type I in Table S1). For enrichment 2, a total of 17 genera were enriched at the beginning or middle phases during the enrichment (type II in Table S1), while 10 genera were enriched at the end-stage of the enrichment (type I in Table S1). Understanding the dynamic features of the archaeal and bacterial communities is crucial to the following metagenomics analysis as well as for future pure strain isolation.

### 3.3. Microbial structure of the enriched microbial consortia by metagenomics analysis

Here, metagenome sequencing was applied to explore the metagenome and the potential functions of the enriched consortia. DNA from the early- and middle-stage enrichment cultures of enrichment 1 was pooled and then subjected to metagenome sequencing. A total of 92,257,422 clean reads (14.08 Gb's raw data) were yielded by Illumina sequencing. The assembly with Megahit yielded 538 MB's contigs with N50 of 1968 bp. The sequences of these contigs were searched against NCBI nr database to be assigned to corresponding microbial taxa. Of all the contigs, 73.06% belonged to bacteria, and 26.67% belonged to archaea (Fig. 3A). By contrast, the abundance of archaea by 16S rRNA gene amplicon sequencing was only 8.70% (Fig. 2A), suggesting that the abundance of archaea could be underestimated using amplicon analysis via primer 515FmodF/806RmodR which targeted V4 region of the 16S rRNA gene.

At the class level, *Methanomicrobia* (24.52%), *Bacteroidia* (24.02%) and *Clostridia* (21.20%) were the three dominant classes (Fig. 3B). At the genus level, *Methanosaeta* (20.09%) was the major methanogen (Fig. 3C). However, the amplicon analysis indicated that the abundance



**Fig. 3.** The relative abundances of microbes through metagenomic sequencing. (A) showed the comparison of relative abundances of the microbes between 16S rRNA gene amplicon sequencing and metagenomic sequencing at domain level. (B) and (C) showed the relative abundance of metagenomic sequencing at class level and at genus level, respectively. The relative abundance at phylum level of 16S rRNA gene amplicon sequencing was calculated based on the data from the fourth and eighth day.

of *Methanosaeta* was 4.62%, much lower than the abundance obtained by metagenome analysis. It is worth noting that the taxa of 42.66% of the contigs were not unassigned due to the lack of the knowledge on verified species in pit mud.

Metagenomic binning generated 26 high-quality metagenome-assembled genomes (MAGs) (Table S2). The genome sizes of these MAGs were larger than 2 Mbp, ranging in size from 2.01 to 4.53 Mbp, and the completeness was 90 to 100% (Table S2). The MAGs were classified into Euryarchaeota, Firmicutes and Bacteroidetes, the three top abundant phyla in the metagenome. The obtained high-quality MAGs indicated that the oligotrophic enrichment culture could be of greatly helpful to decipher the genome information of pit mud microbes, even for these microbes with low abundances in pit mud.

### 3.4. Metabolic diversity investigation and the methanogenic pathway reconstruction

The predicted open reading fragments (ORFs) of assembled contigs were annotated against KEGG GENES database. The functional genes were mainly related to carbohydrate metabolism (16.99%), amino acid metabolism (15.77%), energy metabolism (11.87%), metabolisms of cofactors and vitamins (9.83%) and nucleotide metabolism (8.80%) (Fig. 4). Particularly, high abundances of genes related to energy metabolism were observed. The energy metabolism subcategory was mostly associated with methane metabolism. Thus, the genes encoding key enzymes involved in methane production and microbial populations were surveyed in detail here. The acetate-utilizing methanogenic pathway in the enrichment cultures was reconstructed (Fig. 5). The genus *Methanosaeta* dominated in methane production. Acetate was converted into acetyl-CoA catalyzed by acetyl-CoA synthetase (ACS). The cleavage of the C–C bond and C–S bond for acetyl-CoA is catalyzed by carbon monoxide dehydrogenase/acetyl-CoA synthetase complex (CODH/ACS), releasing CO<sub>2</sub>, transferring the methyl group to H<sub>4</sub>SPT to generate CH<sub>3</sub>-H<sub>4</sub>SPT. Tetrahydromethanopterin S-methyltransferase (MTR) catalyzed the conversion of CH<sub>3</sub>-H<sub>4</sub>MPT to CH<sub>3</sub>-S-CoM. The CH<sub>3</sub>-

S-CoM reductase (MCR) catalyzed the conversion of CH<sub>3</sub>-S-CoM into CH<sub>4</sub> and CoB-S-S-CoM. CoB-S-S-CoM can be regarded as the terminal electron acceptor of the process, which is reduced by Heterodisulfide reductase (HDR) catalyzed the conversion of CoM-S-S-COB into reduced CoM and CoB. These annotated genes can only be classified to the genera level, indicating that the dominant methanogen was an uncultured *Methanosaeta* species.

### 3.5. The nutrients from pit mud supported methanogenesis of enriched microbial consortia

From the first generation's inoculation of pit mud into the oligotrophic enrichment condition, we found that the abundance of the enriched microbes changed during the culturing process. Subsequently, successive passaging was done. After subculturing several times, both the utilization of acetate and the production of methane became weakened (Fig. 6A & B). Autoclaved pit mud was supplemented. The results showed that the supplement with autoclaved pit mud promoted the utilization of acetate and the production of methane (Fig. 6C & D). In the control groups of subculturing (F4) and autoclaved pit mud, the utilization of acetate and production of methane were not observed (Fig. 6C & D). Therefore, nutrients from pit mud can effectively recover the phenotypes of acetate consumption and methane production. The results suggested that the nutrients from pit mud were at least critical to stabilize methanogenesis.

## 4. Discussion

As an important habitat for anaerobic prokaryotes in Chinese liquor fermentation, pit mud not only provides bacteria for flavour producing, but also contains methanogens to maintain the stability of the pit mud microbiome (Hu et al., 2016; Tao et al., 2014). Culture-independent approaches such as amplicon sequencing revealed that pit mud microbiota is constituted of hundreds of species (Liang et al., 2015; Tao et al., 2017). Most of these species have not been accurately identified at the

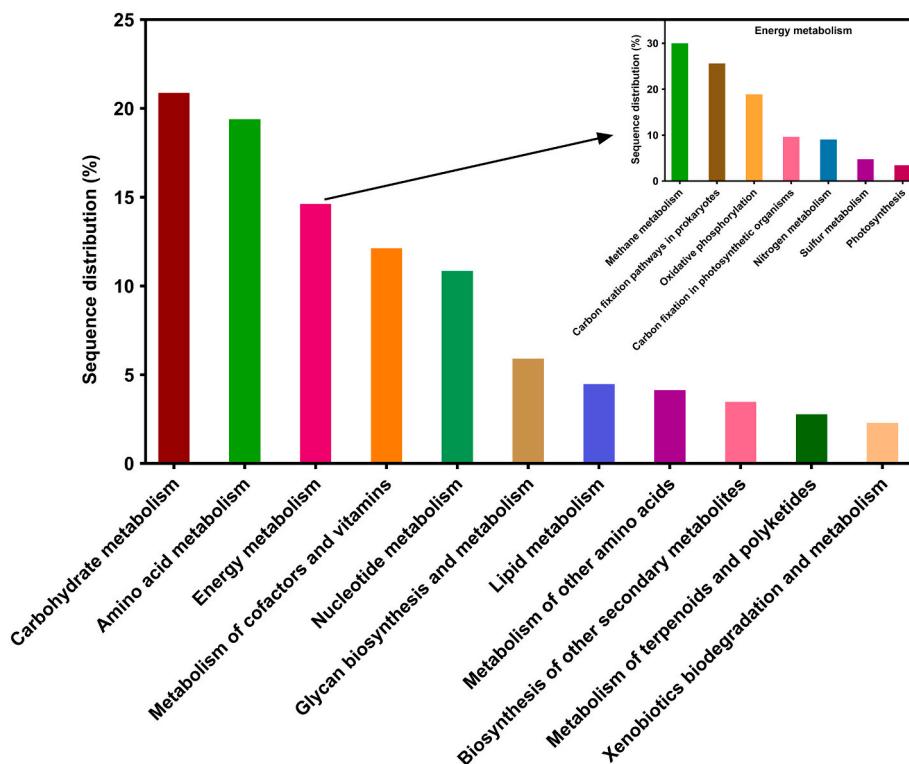
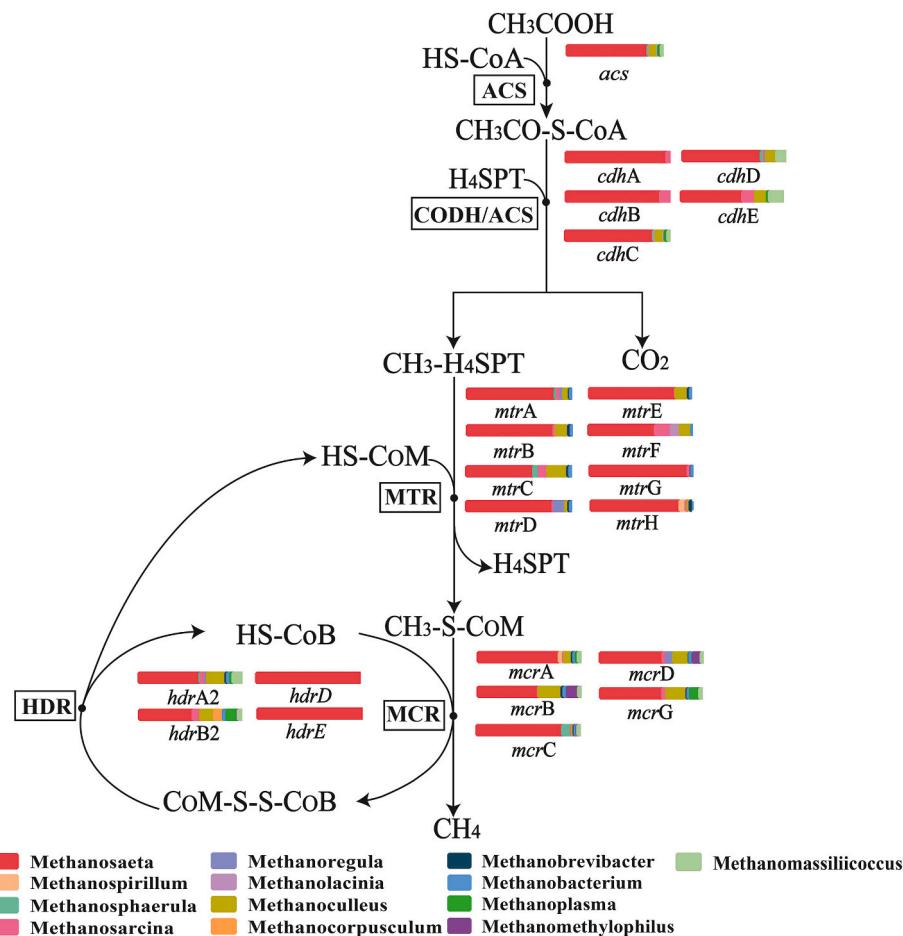


Fig. 4. Metabolic diversity from metagenomic analysis based on annotation of genes of the assembled contigs.



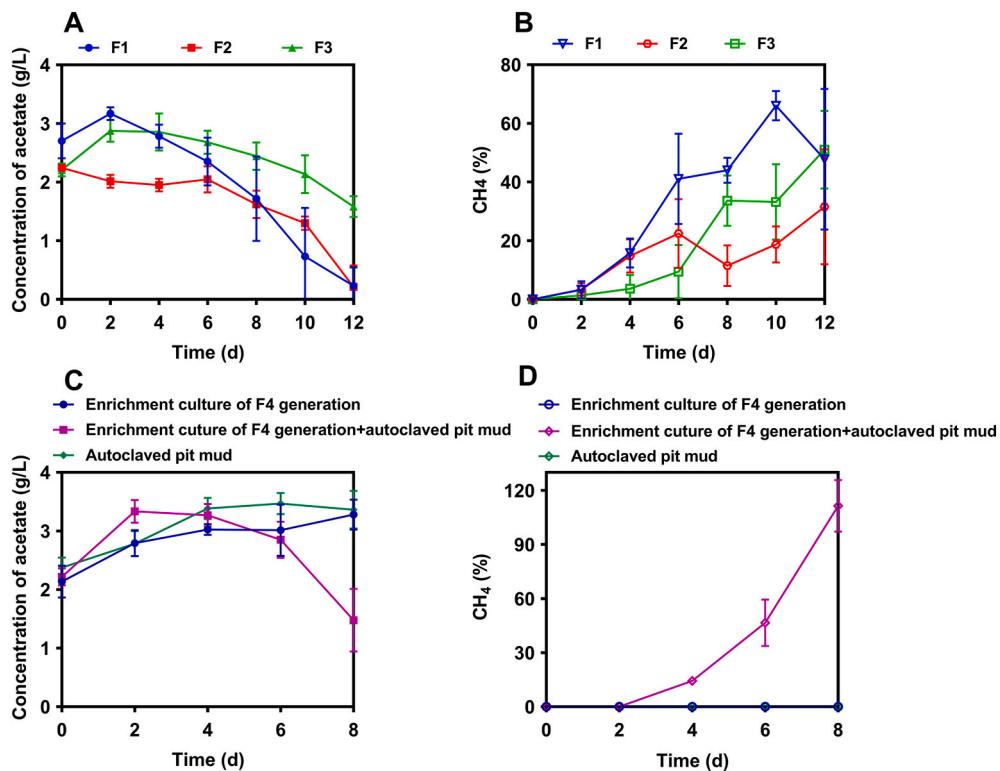
**Fig. 5.** Aceticlastic methanogenesis pathway inferred from the assembled contigs. The colored bars indicate the distribution of methanogens based on relative abundances of genes encoding key enzymes involved in methanogenesis. Abbreviations: ACS, acetyl-CoA synthetase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase complex; MTR, tetrahydromethanopterin S-methyltransferase; MCR, coenzyme-B sulfoethylthiotransferase; HDR, heterodisulfide reductase.

species level since the limitation of omics approaches targeting the in-situ pit mud microbiota. Although the dominant methanogens in pit mud including *Methanobacterium*, *Methanobrevibacter* and *Methanoculleus* can be enriched, they were not the dominant aceticlastic methanogens in the acetate-containing enrichment cultures because the dominant methanogens in pit mud of this study were believed to be hydrogenotrophic. Instead, two types of aceticlastic methanogens, i.e. *Methanosaeta* and *Methanosarcina*, dominated in the enrichment cultures. Interestingly, the relative abundances of the two dominant methanogens were extremely low in the original pit mud (Table S1). And the abundance of *Methanosaeta* was even under the detection limitation of amplicon sequencing. Therefore, the aceticlastic methanogens in pit mud can be cultured by using acetate as carbon source in the oligotrophic medium which only contains limited carbon source and nitrogen source. And most of the enriched species in the two enrichment groups were low-abundance microbes in pit muds. This indicated that oligotrophic enrichment culturing was effective to elevate the relative abundances of minority populations. Moreover, through successive transfer analysis, results showed that the nutrients from pit mud are essential to sustain the stability of methanogenesis phenotype. The effective nutrients supporting the growth of methanogens could be minerals from the pit mud or growth factors generated by pit mud microbiota.

The initial purpose of this study using the enrichment culturing was to enable aceticlastic methanogens culturable. Surprisingly, the relative abundance of bacteria achieved much higher increase than that of archaea within the enrichment cultures. In addition, the diversity of

bacteria in the enrichment cultures was even higher than pit mud microbiota. It was noteworthy that the abundance of each genus changed during the enrichment culturing, suggesting that the sampling time-phase should be carefully considered.

In previous studies, glucose and other carbon sources such as lactate and sucrose were usually used to enrich the microbes from pit mud, strains belonging to class Clostridia producing butyrate or caproate were the obtained species by using regular medium, such as RCM medium and other eutrophic medium (Chai et al., 2019; Ji et al., 2020; Zhu et al., 2015). However, other Clostridia species except for butyrate- and caproate-producing were still difficult to be cultured. In this study, we didn't find obvious accumulation of butyrate or caproate. Hence the medium using acetate as major carbon source here prefers to enable non-butyrate and non-caproate producing microbes to be enriched. As a result, some species under Clostridia class, e.g. *Tissierella* and *Hydrogenispora*, which were not easily enriched previously, were successfully enriched. Besides low concentration of carbon source, another difference of the enrichment approach herein is that low concentration of nitrogen sources (1 g/L of yeast extract and 1 g/L of tryptone) were employed. Before, eutrophic media have been widely used for pit mud microbe screening (Chai et al., 2019; Ji et al., 2020), for instance, Reinforced Clostridial Medium (RCM) medium is rich in glucose (5 g/L), beef extract (10 g/L), peptone (10 g/L), yeast extract (3 g/L) and soluble starch (1 g/L) (Hirsch and Grinsted, 1954). The concentration of these carbon sources and nitrogen sources reaches up to 29 g/L. High concentration of nutrients is useful to obtain fast-growing microbes such as Clostridium species (Chai et al., 2019). However, other microbes



**Fig. 6.** The utilization of acetate (A) and production of methane (B) through the successive transfers of the enrichment cultures. The addition of nutrients from autoclaved pit mud recovered the consumption of acetate (C) and production of methane (D). F1 represented the primary culture inoculated with pit mud at 10% (m/V). F2 to F4 represented successive culture inoculated with an 8d's culture at 10% (v/v). Pit mud (3%, m/v) was supplemented in the medium before autoclaving in the phenotype recovery tests. Data was shown as mean  $\pm$  SD from three biological replicates.

assigned to dominant phyla, such as Bacteroidetes and Proteobacteria, in pit mud are thought to be oligotrophic bacteria (M et al., 2002). Species under the genera of *Anaerocella* and *Aminobacterium* can be cultured in the medium containing limited yeast extracts (Abe et al., 2012; Chertkov et al., 2010). Therefore, we speculate that low concentration of carbon source and nitrogen source is vital to achieve high diversity of bacterial community from pit mud. In addition, various minerals, vitamins and unknown growth factors from pit mud might be important to support high diversity of bacterial community. From microbial ecology concept, microbes can be divided into copiotrophs (r-strategists) and oligotrophs (k-strategists), usually referring fast-growing bacteria and slow-growing bacteria, respectively (Andrews and Harris, 1986; Ho et al., 2017). The species of the microbial consortia achieved by using oligotrophic culturing could be k-strategists.

In summary, this study demonstrated that oligotrophic culturing was effective to obtain microbial consortia with high diversity. The metagenomes of pit mud microbes have not been well examined because of the challenges in extracting high-quality DNA from pit mud. Even if high-quality DNA was obtained, resolving the metagenomes of rare anaerobes in pit mud is still difficult because the limitation of metagenome sequencing and assembly technology for low-abundant microbes. Appropriate enrichment approach is greatly helpful for the rare anaerobes to reach detection threshold by using metagenomics. Combining metagenomic analysis with oligotrophic culturing, exploring functional properties of the uncultured species becomes feasible. Furthermore, culturomics can be used to isolate pure strain from enriched consortia by using the oligotrophic culturing approach. We should also notice that several genera cannot be enriched in this study, more parameters such as carbon sources and other nutrients affecting the culturability should be further investigated. We believe that through the combination of culture-dependent such as enrichment culturing and culture-independent approaches such as metagenomics, the taxonomic classification and the functions of pit mud microbiota for Chinese strong-aroma type liquor fermentation will be completely deciphered.

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## Author contributions

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**Yan Xu:** Writing - Conceptualization, Review & Editing, Supervision, Funding acquisition.

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## Declaration of competing interest

The authors declare no conflict of interest.

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## References

- Abe, K., Ueki, A., Ohtaki, Y., Kaku, N., Watanabe, K., Ueki, K., 2012. *Anaerocella delicata* gen. nov., sp. nov., a strictly anaerobic bacterium in the phylum Bacteroidetes isolated from methanogenic reactor of cattle farms. *J. Gen. Appl. Microbiol.* 58, 405–412.

- Andrews, J.H., Harris, R.F., 1986. R- and K-selection and microbial ecology. In: Marshall, K.C. (Ed.), *Advances in Microbial Ecology*. Springer US, Boston, MA, pp. 99–147.
- Benjamin, B., Chao, X., Daniel, H.H., 2015. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–63.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Hutley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336.
- Chai, L., Xu, P., Qian, W., Zhang, X., Ma, J., Lu, Z., Wang, S., Shen, C., Shi, J., Xu, Z., 2019. Profiling the Clostridia with butyrate-producing potential in the mud of Chinese liquor fermentation cellar. *Int. J. Food Microbiol.* 297, 41–50.
- Chertkov, O., Sikorski, J., Brambilla, E., Lapidus, A., Copeland, A., Glavina Del Rio, T., Nolan, M., Lucas, S., Tice, H., Cheng, J.F., Han, C., Detter, J.C., Bruce, D., Tapia, R., Goodwin, L., Pitluck, S., Liolios, K., Ivanova, N., Mavromatis, K., Ovchinnikova, G., Pati, A., Chen, A., Palaniappan, K., Land, M., Hauser, L., Chang, Y.J., Jeffries, C.D., Spring, S., Rohde, M., Goker, M., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholz, P., Kyrpides, N.C., Klenk, H.P., 2010. Complete genome sequence of Aminobacterium colombiense type strain (ALA-1). *Stand. Genomic Sci.* 2, 280–289.
- Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998.
- Guo, M., Huo, D., Ghai, R., Rodriguez-Valera, F., Shen, C., Zhang, N., Zhang, S., Hou, C., 2014. Metagenomics of ancient fermentation pits used for the production of Chinese strong-aroma liquor. *Genome Announc.* 2, e01045-01014.
- Hirsch, A., Grinsted, E., 1954. Methods for the growth and enumeration of anaerobic spore-formers from cheese, with observations on the effect of nisin. *J. Dairy Res.* 21, 101–110.
- Ho, A., Di Leonardo, D.P., Bodelier, P.L., 2017. Revisiting life strategy concepts in environmental microbial ecology. *FEMS Microbiol. Ecol.* 93, fix006.
- 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. *Appl. Environ. Microbiol.* 82, 2506–2515.
- Ji, M., Du, H., Xu, Y., 2020. Structural and metabolic performance of p-cresol producing microbiota in different carbon sources. *Food Res. Int.* 132, 109049.
- Jin, G., Zhu, Y., Xu, Y., 2017. Mystery behind Chinese liquor fermentation. *Trends Food Sci. Technol.* 63, 18–28.
- John, S.J., 2016. SeqPrep: tool for stripping adaptors and/or merging paired reads with overlap into single reads. <https://github.com/jstjohn/SeqPrep>.
- Joshi, N.A., Fass, J.N., 2011. Sickle: a sliding-window, adaptive, quality-based trimming tool for FastQ files (version 1.33) [software]. Available at: <https://github.com/najoshi/sickle>.
- Kaeberlein, T., Lewis, K., Epstein, S.S., 2002. Isolating “uncultivable” microorganisms in pure culture in a simulated natural environment. *Science* 296, 1127–1129.
- Kang, D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., Wang, Z., 2019. MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 7, e7359.
- Li, D., Liu, C., Luo, R., Sadakane, K., Lam, T.W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31, 1674–1676.
- Liang, H., Li, W., Luo, Q., Liu, C., Wu, Z., Zhang, W., 2015. Analysis of the bacterial community in aged and aging pit mud of Chinese Luzhou-flavour liquor by combined PCR-DGGE and quantitative PCR assay. *J. Sci. Food Agric.* 95, 2729–2735.
- Lillington, S.P., Leggieri, P.A., Heom, K.A., O’Malley, M.A., 2019. Nature’s recyclers: anaerobic microbial communities drive crude biomass deconstruction. *Curr. Opin. Biotechnol.* 62, 38–47.
- Lin, Y., Ye, Y., Wu, C., Hu, Y., Shi, H., 2020. Changes in microbial community structure under land consolidation in paddy soils: a case study in eastern China. *Ecol. Eng.* 145, 105696.
- Magoc, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963.
- McInerney, M.J., Bryant, M.P., Pfennig, N., 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch. Microbiol.* 122, 129–135.
- Nguyen, T.M., Seo, C., Ji, M., Paik, M.J., Myung, S., Kima, J., 2018. Effective soil extraction method for cultivating previously uncultured soil bacteria. *Appl. Environ. Microbiol.* 84, e01145-01118.
- Nichols, D., Cahoon, N., Trakhtenberg, E.M., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K., Epstein, S.S., 2010. Use of iChip for high-throughput in situ cultivation of “uncultivable” microbial species. *Appl. Environ. Microbiol.* 76, 2445–2450.
- Overmann, J., Abt, B., Sikorski, J., 2017. Present and future of culturing bacteria. *Annu. Rev. Microbiol.* 71, 711–730.
- 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. *Front. Microbiol.* 8, 1294.
- Tao, Y., Li, J., Rui, J., Xu, Z., Zhou, Y., Hu, X., Wang, X., Liu, M., Li, D., Li, X., 2014. Prokaryotic communities in pit mud from different-aged cellars used for the production of Chinese strong-flavored liquor. *Appl. Environ. Microbiol.* 80, 2254–2260.
- Tao, Y., Wang, X., Li, X., Wei, N., Jin, H., Xu, Z., Tang, Q., Zhu, X., 2017. The functional potential and active populations of the pit mud microbiome for the production of Chinese strong-flavor liquor. *Microb. Biotechnol.* 10, 1603–1615.
- Walters, W., Hyde, E.R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.A., Jansson, J.K., Caporaso, J.G., Fuhrman, J.A., Apprill, A., Knight, R., 2016. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems* 1, e00009-e00015.
- Wang, X., Du, H., Xu, Y., 2017. Source tracking of prokaryotic communities in fermented grain of Chinese strong-flavor liquor. *Int. J. Food Microbiol.* 244, 27–35.
- Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., Kong, L., Gao, G., Li, C.Y., Wei, L., 2011. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res.* 39, W316–W322.
- Yan, S., Dong, D., 2018. Improvement of caproic acid production in a Clostridium kluyveri H068 and methanogen 166 co-culture fermentation system. *AMB Express* 8, 175.
- Zhu, X., Tao, Y., Liang, C., Li, X., Wei, N., Zhang, W., Zhou, Y., Yang, Y., Bo, T., 2015. The synthesis of n-caproate from lactate: a new efficient process for medium-chain carboxylates production. *Sci. Rep.* 5, 14360.
- Zhu, X., Zhou, Y., Wang, Y., Wu, T., Li, X., Li, D., Tao, Y., 2017. Production of high-concentration n-caproic acid from lactate through fermentation using a newly isolated Ruminococcaceae bacterium CPB6. *Biotechnol. Biofuels* 10, 102.