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Profiling the Clostridia with butyrate-producing potential in the mud of Chinese liquor fermentation cellar

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ABSTRACT

Butyrate and its derivates pertain to the key aroma contributors of strong-flavour baijiu, a kind of Chinese liquors, that is produced from grains by solid-state multispecies anaerobic fermentation in a mud cellar. Microbes inhabiting in the fermentation pit mud largely determines baijiu's flavour and quality. In order to shed light on the microbial functional groups driving butyrate production in pit mud, clone library analysis was firstly performed and the results demonstrated that Clostridia (relative abundance: 50%) and Bacilli (37%) were major groups possessing butyrate kinase (*buk*) pathway and Clostridia (98%) dominated butyryl-CoA:acetate CoA-transferase (*but*) pathway. According to Clostridial specific-16S rRNA gene sequencing analysis, we found the resilience character of Clostridial community in pit mud. Amongst Clostridial groups, 32.0% of the sequences were grouped into Clostridiales incertae sedis, followed by Heliobacteriaceae (18.3%) and Clostridiaceae 1 (8.4%). Moreover, *Hydrogenispora*, *Sedimentibacter* and *Clostridium* were the top three abundant genera. Relative abundance of *Hydrogenispora* was higher in the late days of fermentation, while *Sedimentibacter* exhibited higher proportion in the early days. Different from the previous studies using universal bacterial primer sets, *Hydrogenispora* was first reported as one dominant genus in pit mud. As for the reported potential butyrate producer *Clostridium*, nineteen species were obtained and ten of them were first isolated from the pit mud. Amongst them, *buk* was identified in eleven species by PCR analysis, while *but* was identified in the other seven, indicating the species-specific butyrate synthesis pathways of *Clostridium*. This study provides a perspective on targeting and isolating specific functional microbes in baijiu microbiota with the gene sequence-based medium prediction method.

1. Introduction

Baijiu (https://en.wikipedia.org/wiki/Baijiu) is a kind of distilled spirits which is constituted by three major (sauce-, strong-, and light-flavour) and nine minor categories based on their aroma characteristics (Zheng and Han, 2016). The world consumption of baijiu was over 13 billion liters in 2016, representing a market value of approximately 97.4 billion dollars (Liu and Sun, 2018). Market share of strong-flavour baijiu, also known as Luzhou-flavour baijiu, accounts for > 70% of the total baijiu production in China (Zou et al., 2018a). High-quality baijiu depends on harmonious balance of the numerous aroma compounds. According to the Osme analysis, butyrate (Osme value ≥13, rancid and cheesy aroma) and its derivates, e.g. ethyl butanoate (Osme value ≥14, pineapple aroma) were assigned to the key aroma contributors of strong-flavour baijiu (Fan and Qian, 2006). Osme is a quantitative method in gas chromatography–olfactometry analysis, which can directly measure the aroma intensities of volatile compounds (Plutowska and Wardencki, 2008). Recent studies also showed the significant contribution of butyrate and its major derivate ethyl butanoate in strong-aroma baijiu's flavour due to their relatively high concentration and low olfactory threshold (Fang et al., 2019; Liu and Sun, 2018). Therefore, study of the butyrate producers is conductive to understand the formation mechanisms of strong-flavour baijiu's characteristic flavour.

Abbreviations: buk, butyrate kinase; but, butyryl-CoA:acetate CoA-transferase; SCFA, short-chain fatty acids

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Strong-flavour baijiu is produced via the anaerobic fermentation of grains (mainly sorghum) in a mud pit (also referred as fermentation cellar) under solid state lasting for around 40–60 days. The inner side of the underground fermentation cellar walls is covered with pit mud, providing a suitable habitat for brewing microbes (Wang et al., 2017). Besides pit mud, Daqu (generally considered as fermentation starter) is also a key source for microorganisms as well as the saccharifying and fermentative agent in baijiu fermentation (Xiao et al., 2017). Compared to the brewing technology and other factors, microbial fermentation process plays a critical role in determining the flavour, quality and yield of Chinese liquor (Jin et al., 2017). The microbial community in pit mud dominantly consists of bacterial classes, including Clostridia, Bacteroidia and Bacilli, and two archaeal classes, including Methanobacteria and Methanomicrobia (Ding et al., 2014; Liu et al., 2017). Amongst them, Clostridia is the most abundant class. Furthermore, Clostridial cluster IV, *Clostridium* and *Sedimentibacter* were identified as mainly active populations in pit mud, in which *Clostridium* was most likely involved in butyrate production through metagenomics analysis (Tao et al., 2017). Although some culture-dependent studies have been conducted to reveal the roles of *Clostridium* (Hu et al., 2015; Zou et al., 2018a), it is still difficult to interpret the gap between the microbial community and the butyrate-producing function in pit mud due to the limited information.

Microbial butyrate metabolism is recognized with two alternative pathways based on the final step: butyrate is synthesized from butyryl-CoA via butyrate kinase (EC:2.7.2.7) and phosphate butyryltransferase (EC:2.3.1.19) or butyryl-coenzyme A (CoA):acetate CoA-transferase (EC:2.8.3.8), i.e. buk or but pathway (Supplementary Fig. 1) (Louis and Flint, 2017). Therefore, *buk* and *but* were previously chosen as the functional genes to specify the butyrate-producing pathways of microbes (Louis and Flint, 2009; Vital et al., 2015). Ruminococcaceae and Lachnospiraceae were predominant butyrate-producing families under anaerobic environment, and not all species belonging to these two families have the ability to produce butyrate (Louis and Flint, 2017), indicating the species-specific butyrate-producing feature. The corresponding representative species for each family were *Faecalibacterium prausnitzii* and *Eubacterium rectale*, respectively (Louis and Flint, 2009; Louis and Flint, 2017).

The aim of this study was to shed light on the potential butyrate-producing Clostridia in fermentation pit mud involved in strong-flavour baijiu fermentation and their butyrate synthesis pathways. Clone library analysis were firstly performed to reveal the phylogenetic diversities of *buk* and *but* genes in pit mud. Based on this, Clostridia was identified as the main potential butyrate-producing group, and the succession of its community throughout fermentation process was investigated by Clostridial specific-16S rRNA gene sequencing analysis. Strains of the major potential butyrate-producing genus *Clostridium* were isolated, and their butyrate synthesis ability and butyrate synthesis pathways were evaluated. This work is expected to provide more useful basis to elucidate the butyrate-producing microbial community in pit mud and their contribution to baijiu flavour.

2. Materials and methods

2.1. Pit mud sampling and physiochemical properties analysis

Pit mud samples were obtained from a Chinese strong-flavour baijiu-producing enterprise, Luzhou Laojiao Group Co., Ltd., located in Luzhou city, Sichuan Province, China (105°29′50″ E, 28°53′47″ N). We selected three fermentation cellars (length × width × height = 4.3 m × 3.3 m × 2.3 m) that have been continuously used for approximately 100 years. Sampling was performed on day 1 (beginning of fermentation), 3, 7, 9, 11, 16, 22, 26, 31, 36 and 46 (fermentation finished) during October and November in 2016. In each time point, samples were collected from the center and four corner positions of the cellar bottom (Supplementary Fig. 2A), mixed and wrapped by sterile aluminum foil, transferred immediately to lab, frozen in liquid nitrogen, pulverized homogeneously, and stored at −80 °C for further use.

For each sample, 2 g were suspended in 20 mL sterile distilled water and remained rotational shaking (100 rpm) for 3 h at room temperature. After centrifugation at 10,000 ×*g* for 15 min, the supernatant extract was collected for pH analysis. pH was measured via a Mettler Toledo FiveEasy Plus™ pH/mV meter equipped with an LE438 solid electrode (Mettler Toledo Instruments, Shanghai, China). Moisture content of pit mud was measured immediately once sampling by a gravimetric method after drying in the oven at 105 °C for 4 h. All experiments were carried out in triplicates.

2.2. DNA extraction

Metagenomic DNA was extracted from pit mud using a PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. DNA quality and quantity were assessed by Nanodrop 2000 (Thermo Scientific, USA) and electrophoresis in a 1% agarose gel.

2.3. Construction of clone libraries based on buk and but genes

Microbes having *buk* and/or *but* could be with distant phylogenetic relationships, for example, these microbes could come from different genera or families, even classes, consequently, it is difficult to design sequence-specific primer in order to amplify the conservative fragments of their nucleic acid sequences. Compared with nucleic acid sequences, amino acid sequences are more conservative across microbes scattered in different taxonomic categories. Therefore, in this study, to obtain the degenerate primers of key genes (*buk* and *but*) in butyric acid synthesis pathway (Supplementary Fig. 1), we downloaded the representative amino acid sequences of these two genes in FunGene database (http://fungene.cme.msu.edu/), respectively (Fish et al., 2013).

Degenerate primers were designed using CODEHOP approach (http://blocks.fhcrc.org/codehop.html) (Rose et al., 2003). Parameters were set as follows: maximum core degeneracy, 100; target clamp temperature, 60 °C; genetic code, standard; codon usage table, equal. Forward/reverse primers for *buk* were 5′-AAAATATTAATNATHAAYCCNGG-3′/5′-GCCATTGCTTTTTGATTNARNRCRTG-3′; for *but* were 5′-ATGGGAACTAAGAANHTNTAYGA-3′/5′-TAAATTTACCATTCCRWAYTCNGT-3′. The length of *buk* and *but* PCR products was 500 and 450 bp, respectively.

Buk and *but* were amplified as described by Vital et al. (2013) with some modifications. Equal amounts of DNA from each sample were pooled as the template. Each reaction consisted of 2.0 μL DNA, 2.0 μL 2.5 mM dNTP, 1.0 μL of 10 mM forward and reverse primers, 0.2 μL Ex Taq DNA Polymerase (TAKARA, Dalian, China), 2.5 μL 10 × buffer and 16.3 μL sterile double distilled water. PCR amplification programs were as follows: one cycle of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 47 °C (*buk*) or 49 °C (*but*), 30 s at 72 °C, then a final elongation step of 5 min at 72 °C. After electrophoresis in a 1.2% agarose gel, target products were purified using GK 2043-200 gel extraction kit (GeneRay, Shanghai, China). Purified amplicons were ligated with pMD19-T vector (TAKARA, Dalian, China) and transformed into *Escherichia coli* JM109 competent cells. Respective above 100 recombinant clones of *buk* and *but* were randomly selected and sent to Sangon Biotech (Shanghai, China) for sequencing. Alignment was performed via ClustalW (http://www.genome.jp/tools/clustalw/) and the sequences were trimmed to avoid sequence difference caused by degenerate primer mismatch. Then they were clustered into operational taxonomic units (OTUs) under the threshold of 95% identity by Fungene Pipeline (http://fungene.cme.msu.edu/FunGenePipeline/). The phylogenetic classification and functional definition of each OTU were investigated through Blastx in UniProtKB database (http://www.uniprot.org/blast/).

2.4. Clostridial specific-16S rRNA gene amplicon sequencing analysis

Clone library analysis revealed that Clostridia dominated the butyrate-producing population in pit mud. Consequently, the diversity of Clostridial community was further explored by 16S rRNA gene sequencing. V4–V5 hypervariable region of 16S rRNA gene was amplified using Clostridial specific primers: SJ-F (5′-CGGTGAAATGCGTAGAKATTA-3′) and reverse primer SJ-R (5′-CGAATTAAACCACATGCTCCG-3′) (Hu et al., 2014). PCR products were purified with SanPrep Column PCR Product Purification Kit (Sangon Biotech, Shanghai, China), and assessed by 2% agarose gel electrophoresis. Equimolar content of the purified products was used for amplicon library construction using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). Paired-end (2 × 300 bp) sequencing was carried out on the Illumina MiSeq platform.

Raw reads for each sample were distinguished by barcode and primer sequences, which were trimmed subsequently for raw tags assembly by FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). High quality clean tags were obtained after filtration based on Bokulich et al. (2013), and then further quality-filtering was performed using QIIME (V1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) pipeline (Caporaso et al., 2010). Chimera sequences were removed via the Uchime algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar et al., 2011). Then the obtained effective tags from all samples were classified into OTUs by Uparse (V7.0.1001, http://drive5.com/uparse/) using 97% identity as a cutoff (Edgar, 2013). The taxonomic annotation of each OTU's representative sequence was analysed by SILVA (http://www.arb-silva.de/) database at a confidence level of 80% (Wang et al., 2007). Alpha diversity (e.g. Chao1, Shannon index) and beta diversity (e.g. Bray-Curtis distance) calculation were performed in QIIME (V1.9.1) after rarefying all samples to the same sequencing depth.

2.5. Biomass analysis via quantitative real-time PCR (qRT-PCR)

Dynamic changes of Clostridial biomass in pit mud during brewing was detected using the Clostridial specific primers via qRT-PCR method (Hu et al., 2014). Bacterial biomass was also measured using Eub338 (5′-ACTCCTACGGGAGGCAGCAG-3′) and Eub518 (5′-ATTACCGCGGCTGCTGG-3′) primers (Fierer et al., 2005). qRT-PCR assay was performed by the SYBR™ Select Master Mix (Applied Biosystems, USA) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) according to our previous protocol (Xiao et al., 2017). Standard curve to quantify bacterial biomass in pit mud was created through plotting the Cq value versus the concentration of *E. coli*'s 16S rRNA gene PCR amplification products, which were 10-fold serially diluted from 6.14×10^8 to 6.14×10^4 copies/μL (Supplementary Fig. 2C). The 16S rRNA gene copy numbers were calculated according to Zhang et al. (2008). For enumeration of Clostridia, 16S rRNA gene of *Clostridium kluyveri* was amplified and ligated with pMD19-T vector (TAKARA, Dalian, China), then transformed into *E. coli* JM109 competent cells. After verifying the insertion of target fragment, standard curve was made using the above stated method with PCR products under a 10-fold serial dilution from 1.69×10^9 to 1.69×10^4 copies/μL (Supplementary Fig. 2D). All of the reactions were carried out in three replicates.

2.6. Clostridial isolation and evaluation of their short-chain fatty acids (SCFA)-producing potential

According to the above analysis, Clostridial strains were isolated from the pit mud under an anaerobic system (DG250, Don Whitely Scientific, UK), including 80% N_2, 10% CO_2 and 10% H_2. To improve the isolation efficiency, besides the universal microbial medium (e.g. ethanol/sodium acetate medium, ES; Reinforced Clostridial Medium, RCM), targeted culture medium for different strains were predicted based on the OTU sequences from Clostridia-specific 16S rRNA gene sequencing using the Known Media Database (KOMODO, http://komodo.modelseed.org/) under the default thresholds (Oberhardt et al., 2015).

Genomic DNA of the isolated strains was extracted by Bacterial Genomic DNA Extraction Kit (DP302) (Tiangen, Beijing, China). Approximately full length 16S rRNA gene was amplified using the bacterial 27F/1492R primer set (Zhang et al., 2014). PCR amplification programs were as follows: one cycle of 5 min at 95 °C, followed by 30 cycles of 60 s at 95 °C, 30 s at 55 °C and 90 s at 72 °C, and then the final step of 5 min at 72 °C. PCR products purification and transformation into *E. coli* were performed as described in Section 2.3. Positive clones were selected for sequencing and taxonomic feature of the strains were determined by alignment with EzBioCloud database (Yoon et al., 2017). Subsequently, all the isolates were cultured anaerobically in RCM for 7 days at 37 °C and their metabolic SCFA concentrations, including formic acid, acetic acid, propanoic acid, butyric acid, pentanoic acid and hexanoic acid, were determined by high performance liquid chromatography (HPLC) method (Wu et al., 2017). In brief, 750 μL fermentation culture of each isolate (after cultured anaerobically in RCM for 7 days) was mixed with 300 μL zinc sulphate (300 g/L) and 300 μL potassium ferrocyanide (106 g/L) in a 2 mL centrifuge tube, followed by thorough vortex, centrifugation at 10,000 ×*g* for 10 min, and the supernatant was filtrated through a 0.22 μm nylon syringe filter (ALWSCI, Zhejiang, China). Ten microliters were used for SCFA assessment in a Waters E2695 HPLC system with a 2998 PDA detector. The mobile phase was 2.5 mM H_2SO_4 with 0.6 mL/min flow rate, detection wavelength was 210 nm and the column (SEPAX Carbomix H-NP 5:8, 5 μm, 7.8 × 300 mm) temperature was maintained at 55 °C.

2.7. Sequence deposit

The raw Clostridial specific-16S rRNA gene amplicon sequencing data sets have been deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra) with accession number SRP172813.

3. Results

3.1. Dynamic changes of pit-mud physiochemical properties during fermentation

The moisture content of pit mud fluctuated slightly during baijiu brewing, ranging from 29.2% to 35.5% (Supplementary Fig. 2B). The pH demonstrated a volatility with two troughs respectively identified on day 11 and 26 during fermentation. From day 1 to 11, a downward trend featured the pit-mud's pH, which was downregulated from 8.1 to 6.4, and after day 26, pH increased moderately and reached 7.9 at the end of fermentation.

3.2. The diversity of butyrate-producing bacteria in pit mud

Bacteria harbouring the butyrate metabolic pathways was identified based on the key enzyme-coding genes (*buk* and *but*) clone library analysis. Eleven and thirteen representative sequences were clustered from 105 *buk* and 100 *but* clones, respectively (Supplementary Table 1). Bacteria in *buk* pathway fell into four classes, including Clostridia (52 clones), Bacilli (39 clones), unclassified_Bacteroidetes (11 clones) and Tissierellia (3 clones) (Fig. 1A & C). At the family level, *buk* pathway was scattered in Family_XI, Bacillaceae, unclassified_Clostridia and Planococcaceae, and their relative abundance was 22%, 21%, 21% and 16%, respectively. Relative abundance of OTU1-4 accounted for 77% of all identified *buk* clones, and they were annotated as Clostridia bacterium BRH_c25, *Virgibacillus necropolis*, *Sedimentibacter* sp. B4 and *Sporosarcina psychrophile*, respectively.

With regard to *but* pathway, Clostridia showed its absolute predominance (Fig. 1B & D). However, bacteria with *but*-encoding

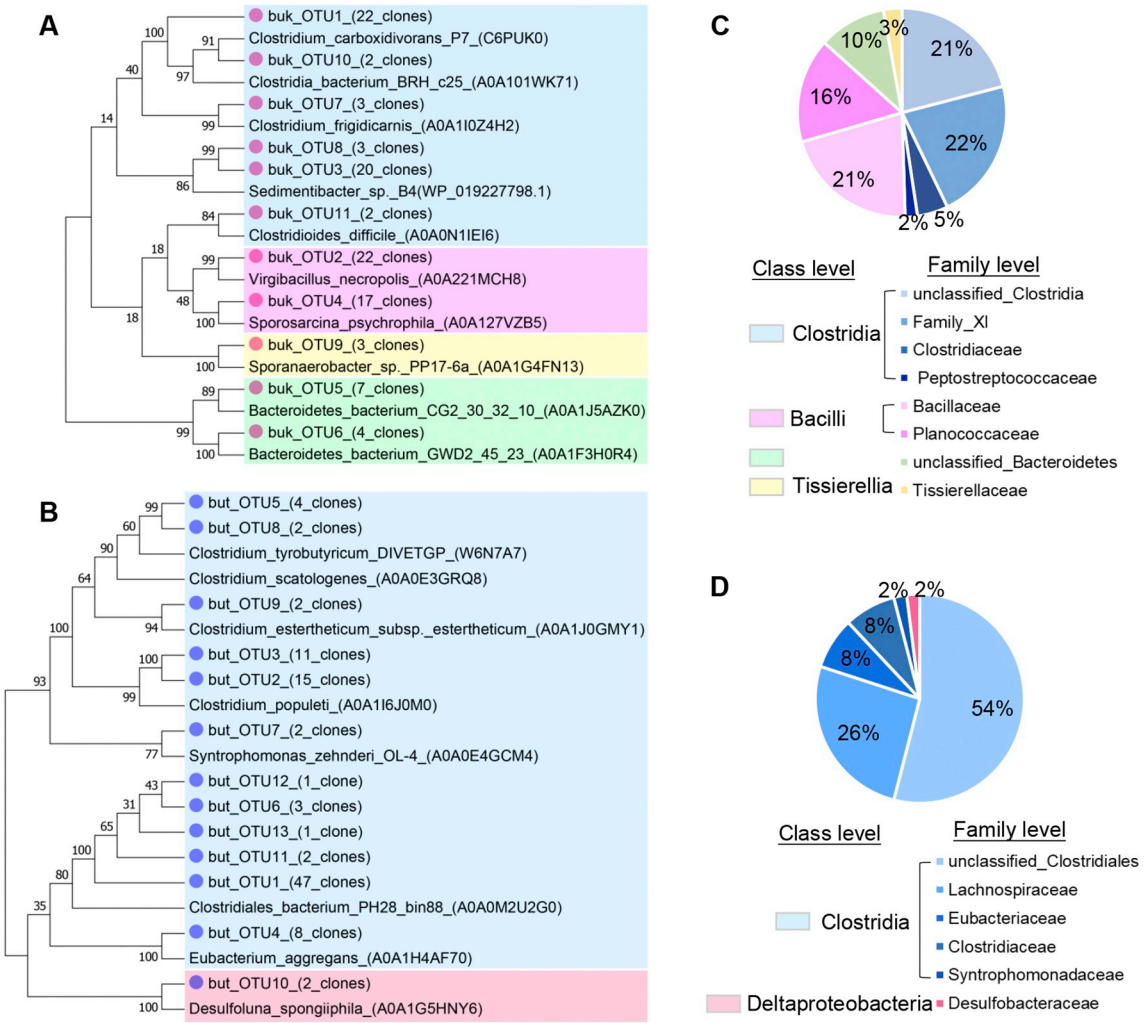

Fig. 1. Taxonomic distributions of *buk* and *but* genes in fermentation pit mud of Luzhou-flavour baijiu based on clone library analysis. Neighbour-joining phylogenetic trees constructed depending on the predicted protein sequences of (**A**) *buk* and (**B**) *but*. Taxonomic distributions of (**C**) *buk* and (**D**) *but* at class and family levels. Blue, pink, yellow, green and light red respectively represent Clostridia, Bacilli, Tissierellia, unclassified Bacteroidetes and Deltaproteobacteria. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

potential was less diverse compared with those possessing *buk* gene in our current results. Except OTU_10 (2 clones), the other 12 OTUs were classified into five families of Clostridia, and unclassified_Clostridiales family with 54% relative abundance ranked first, followed by Lachnospiraceae (26%), Eubacteriaceae (8%), Clostridiaceae (8%) and Syntrophomonadaceae (2%). Based on the present study, we followed in more detail the Clostridia's dynamic taxonomic distribution during brewing via Clostridia-specific 16S rRNA gene sequencing analysis.

3.3. Dynamic changes of Clostridial community overall structure in pit mud during fermentation

At the beginning of Chinese liquor fermentation, biomass of total bacteria was 7.98 lg 16S rRNA gene copies per gram pit mud, followed by a rapid increase and peaked on day 9 (lg value was 9.8, which was 44-fold of that at beginning) (Fig. 2A). Subsequently, a slight downtrend was observed from day 9 to day 46 with 8.25 lg copies/g at the end of fermentation. Clostridial biomass was 5.15 lg copies/g on the first day, then rose to 7.60 lg copies/g on day 3, and maintained the same order of magnitude till day 9. Like that of bacteria, the changes of Clostridial biomass was on the decline after day 9 and reached 5.48 lg copies/g on day 46. The biomass ratio of Clostridia to total bacteria showed rapid increase occurred between day 1 and 7, followed by sharp decrease on day 9, and then kept in a stable range until the end of fermentation (Fig. 2B).

Clostridial specific-16S rRNA gene sequencing resulted in 10,790,679 (99.9%) qualified reads from all pit mud samples and each sample contained 217,908 to 397,753 clean reads (Supplementary Table 2). In total, 2895 OTUs were clustered based on a cutoff of 97% identity of 16S rRNA gene sequences. Amongst them, 1244 OTUs were classified into Clostridia, while the left OTUs were unannotated at the class level probably due to the limited information of present databases. Rarefaction analysis indicated that all communities were well represented since the Chao1, Shannon and observed species rarefaction curves reached the saturation plateau (Supplementary Fig. 3). The Chao1 and Shannon diversity indices, representing the Clostridial alpha diversity in pit mud, displayed undulated changes during brewing, and relatively dramatic variations were mainly detected from day 1 to 7 based on one-way ANOVA test. No significant difference was recognized between the beginning (day 1) and end (day 46) of fermentation. The resilience of Clostridial species richness and diversity in pit mud after a fermentation cycle, reflected the robustness of the Clostridial community in pit mud over the long-time range, and further can be applied to explain the quality stability of baijiu.

Cluster analysis and the constrained principal coordinate analysis (cPCoA) were conducted based on Bray-Curtis approaches to evaluate

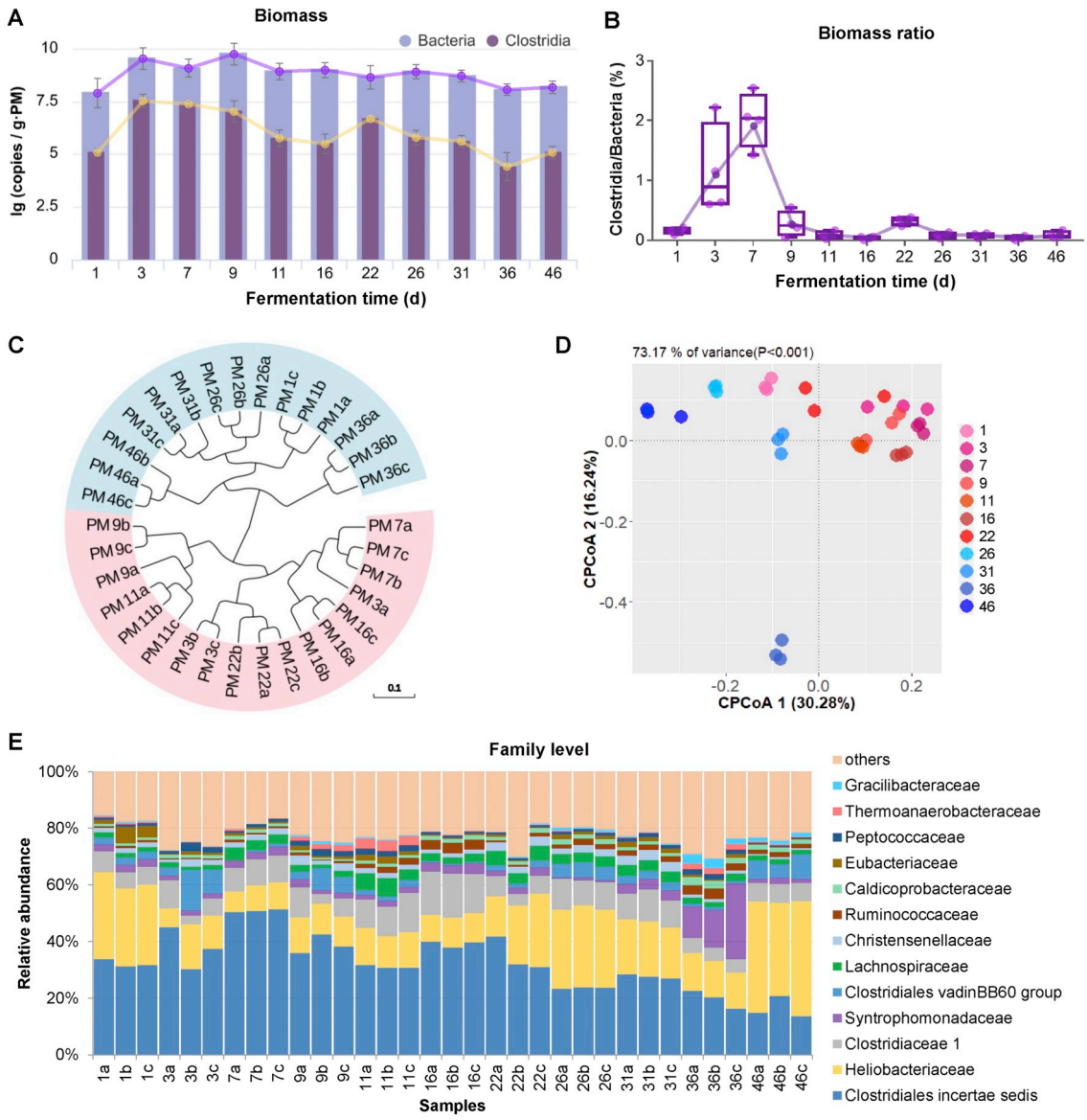

Fig. 2. Temporal dynamics research on the biomass of bacteria and Clostridia and Clostridial community structure in pit mud during brewing. (**A**) Biomass of bacteria and Clostridia; (**B**) The biomass ratio of Clostridia to bacteria; (**C**) Cluster analysis of pit-mud Clostridial community based on Bray-Curtis algorithms; (**D**) Constrained principal coordinate analyses (cPCoA) using the capscale procedure implemented in the vegan package of R (version 3.4.4); (**E**) Distribution of abundant families.

the Clostridial communities' similarities in pit mud during brewing (Fig. 2C & D). The results revealed that the profiles of Clostridial communities could be divided into two phases through fermentation, phase I (day 1 to 22) and phase II (day 22 to 46) (Fig. 2C). In addition, day 1 was clustered together with the samples belonging to phase II, which was further supported the above inference, the “resilience” of pit mud Clostridial community.

3.4. Dynamics of Clostridial taxonomic diversity in pit mud during fermentation

At the order level, Clostridiales dominated in the pit mud throughout Chinese liquor fermentation process, with an average relative abundance in each sample approaching 93.5% (Supplementary Fig. 4A). Most of the sequences were further grouped into Clostridiales incertae sedis, which generally appeared a decrease tendency in relative abundance during brewing, followed by Heliobacteriaceae and Clostridiaceae 1 and their average relative abundance per sample was 32.0%, 18.3% and 8.4%, respectively (Fig. 2E). Clostridiales incertae sedis was further scattered in three groups, including Family XI, XIV and XIII (Supplementary Fig. 4B). Family XI constituted 53.6% to 92.0% (average ratio: 69.8%) of the total Clostridiales incertae sedis abundance in each sample and Family XIV presented higher relative abundance in phase I than in phase II with the average value 12.1% and 2.7%, respectively. However, Family XIII (average ratio: 3.0%) was a small minority compared with Family XI and XIV.

With respect to the genus level, the top 25 genera (relative abundance per sample above 0.1%) constituted 52.6% to 78.5% of the total Clostridial abundance in each sample (Supplementary Fig. 4C). Amongst all genera identified, *Hydrogenispora*, *Sedimentibacter* and *Clostridium* were the top three abundant genera and their proportions in each sample ranged from 6.7% to 40.7%, 8.9% to 34.0% and 2.1% to 9.1%, respectively. However, the changing trends of their abundance and diversity were completely divergent. The species diversity of *Hydrogenispora* fluctuated sharply during brewing based on the Shannon and inverse Simpson indexes (Fig. 3A), while *Sedimentibacter* diversity displayed a gradually slight increasing tendency (Fig. 3C). Moreover, the relative abundance of *Hydrogenispora* was relatively

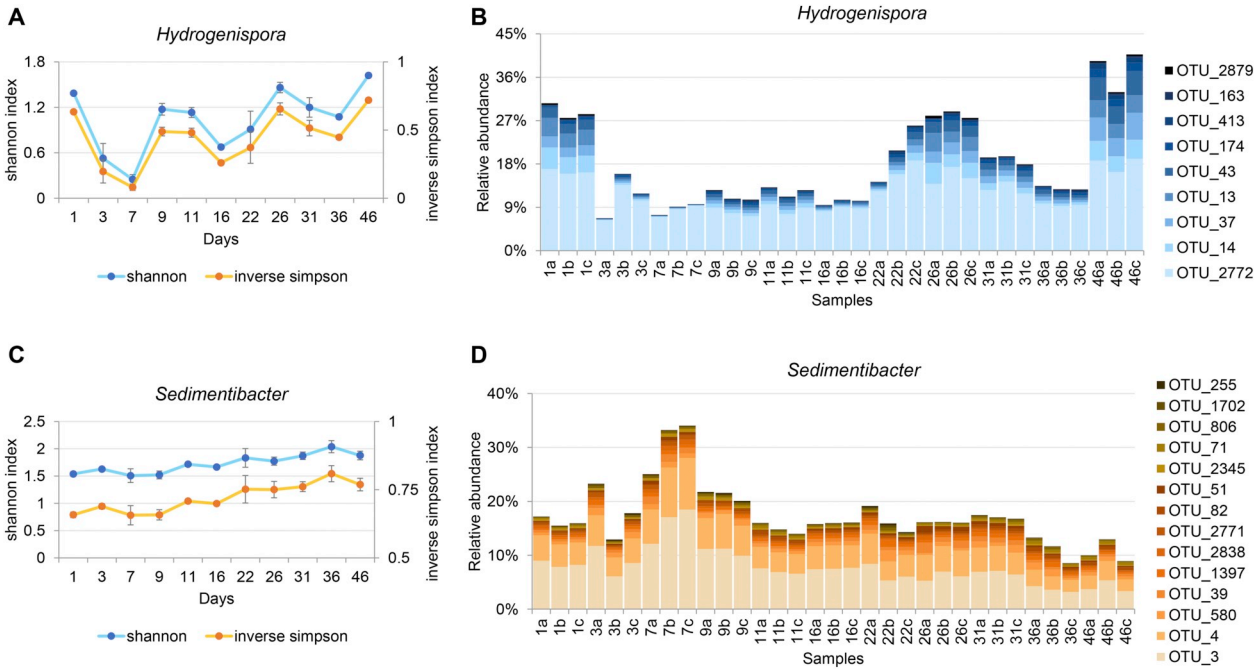

Fig. 3. Dynamic profiles of the top two abundant genera, *Hydrogenispora* and *Sedimentibacter*, in pit mud throughout the fermentation process. (**A**) α-diversity indices and (**B**) OTU-level species distributions of *Hydrogenispora*; (**C**) α-diversity indices and (**D**) OTU-level species distributions of *Sedimentibacter*.

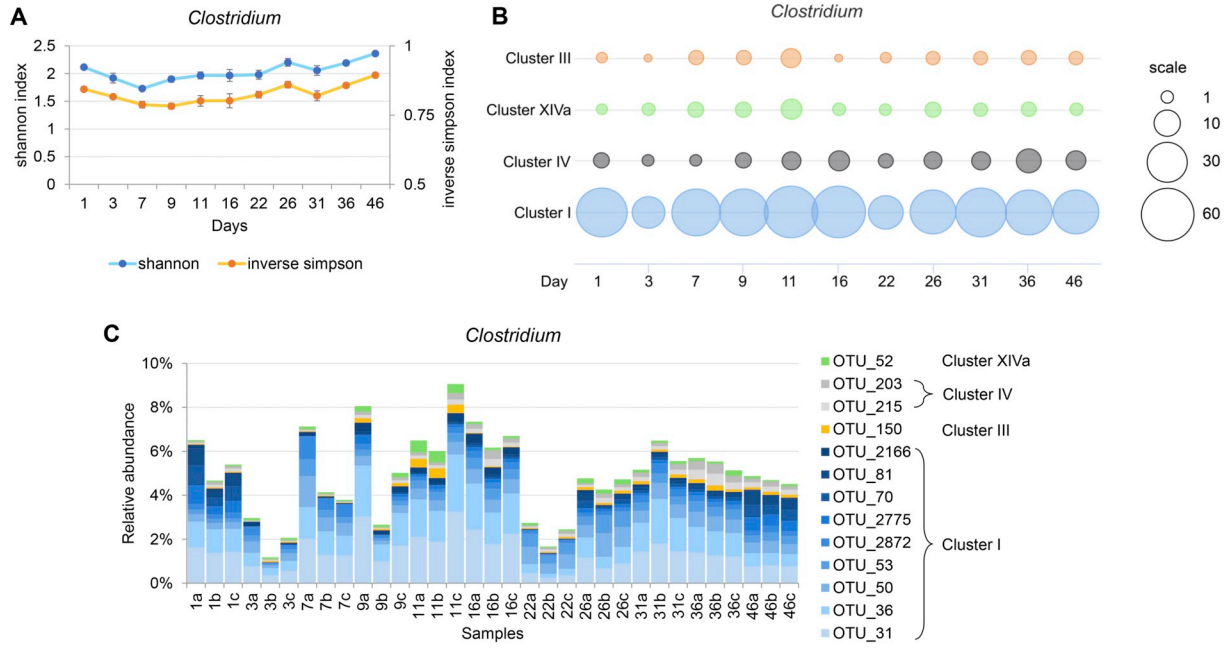

Fig. 4. Dynamic profiles of the third abundant genera, *Clostridium*, in pit mud throughout the fermentation process. (**A**) α-diversity indices; (**B**) Abundance proportions of different *Clostridium* clusters; (**C**) OTU-level species distributions.

higher in the late days of fermentation (Fig. 3B), while *Sedimentibacter* exhibited higher proportion in the early days and peaked on day 7 (30.8%) (Fig. 3D). The genus *Hydrogenispora* mainly fell into nine OTUs and OTU_2772 was commonly predominant in all pit mud samples ranging from 6.4% to 19.1% of the total 16S rRNA gene sequences and over 50% of *Hydrogenispora* abundance. Fourteen OTUs were affiliated with *Sedimentibacter*, dominated by OTU_3 and OTU_4 accounting for 52.0% to 82.3% of the total *Sedimentibacter* abundance per sample. In terms of *Clostridium*, its diversity decreased from day 1 to 7 and then increased till the end of brewing (Fig. 4A). *Clostridium* was further classified into Cluster I, III, IV and XIVa, and Cluster I (9 out of 13 OTUs), i.e. *Clostridium sensu stricto*, was in absolute predominance amongst them, constituting 76.1% to 97.2% of the total *Clostridium* abundance in each sample (Fig. 4B & C). Relative abundance and species diversity of other genera also demonstrated various changing patterns throughout fermentation (Supplementary Figs. 5 & 6). These data indicated that Clostridial community structure was always under dynamic changing during fermentation process.

Table 1
Profiles of short-chain fatty acids (SCFAs) in the fermentation broth of *Clostridium* strains isolated from pit mud.

Strain no.	Hit taxon name	EzBioCloud accession no.	Identity (%)	Butyrate synthesis pathways		Production of SCFAs (g/L)*					
				buk	*but*	Formic acid	Acetic acid	Propionic acid	Butyric acid	Pentanoic acid	Caproic acid
1	***Clostridium homopropionicum***	LHUR01000014	96.8	−	+	nd	3.12 ± 0.03	2.95 ± 0.04	6.50 ± 0.02	2.06 ± 0.04	0.77 ± 0.03
2	***Clostridium sp. ArC5***	X71848	98.7	+	−	nd	0.94 ± 0.00	nd	4.73 ± 0.00	1.71 ± 0.03	0.83 ± 0.03
3	***Clostridium diolis***	AJ458418	99.8	+	−	nd	1.50 ± 0.02	nd	4.14 ± 0.03	0.33 ± 0.00	0.69 ± 0.01
4	***Clostridium acetireducens***	LZFO01000074	99.9	+	−	nd	0.27 ± 0.00	nd	3.49 ± 0.05	1.32 ± 0.04	1.20 ± 0.02
5	***Clostridium senegalense***	JF824801	99.3	−	+	0.50 ± 0.00	2.62 ± 0.72	2.62 ± 0.02	2.04 ± 0.56	2.20 ± 0.08	0.87 ± 0.03
6	***Clostridium pasteurianum***	CP013018	100	−	+	1.94 ± 1.03	0.77 ± 0.11	2.04 ± 0.03	1.75 ± 0.01	2.05 ± 0.20	0.37 ± 0.03
7	*Clostridium kluyveri*	CP000673	96.7	−	+	1.97 ± 0.05	0.69 ± 0.03	1.75 ± 0.00	1.62 ± 0.01	1.89 ± 0.08	0.60 ± 0.03
8	*Clostridium tyrobutyricum*	APMH01000060	100	−	+	0.24 ± 0.00	1.60 ± 0.03	1.36 ± 0.24	1.53 ± 0.01	1.89 ± 0.09	0.75 ± 0.03
9	*Clostridium amylolyticum*	jgi.1107657	100	+	−	nd	1.64 ± 0.00	nd	1.52 ± 0.01	2.30 ± 0.01	3.55 ± 0.02
10	***Clostridium guangxiense***	KT000268	99.0	−	+	nd	1.22 ± 0.03	1.59 ± 0.02	1.44 ± 0.01	0.41 ± 0.02	0.35 ± 0.03
11	***Clostridium magnum***	LWAE01000001	95.0	+	+	0.02 ± 0.01	1.31 ± 0.03	2.37 ± 0.22	1.40 ± 0.01	2.54 ± 0.15	0.71 ± 0.03
12	*Clostridium swellfunianum*	AB838978	96.2	+	−	0.24 ± 0.01	2.28 ± 0.03	1.58 ± 0.10	1.09 ± 0.01	1.92 ± 0.06	1.28 ± 0.03
13	***Clostridium scatologenes***	CP009933	99.9	+	−	0.14 ± 0.00	2.23 ± 7.03	1.65 ± 0.04	1.06 ± 7.01	nd	1.28 ± 7.03
14	*Clostridium luticellarii*	KP342256	96.3	−	+	0.30 ± 0.00	0.92 ± 0.03	1.30 ± 0.03	1.06 ± 0.01	1.56 ± 0.05	0.66 ± 0.03
15	***Clostridium aciditolerans***	DQ114945	99.9	+	−	0.52 ± 0.01	1.32 ± 0.03	0.94 ± 0.01	0.98 ± 0.01	0.38 ± 0.01	0.10 ± 0.03
16	*Clostridium carboxidivorans*	ACVI01000229	98.4	+	−	0.12 ± 0.03	1.81 ± 0.03	0.92 ± 0.65	0.94 ± 0.01	2.77 ± 0.03	0.91 ± 0.03
17	*Clostridium sporogenes*	JFBQ01000001	98.7	+	−	0.14 ± 0.00	0.63 ± 0.17	1.88 ± 0.26	0.76 ± 0.21	0.24 ± 0.07	4.24 ± 0.07
18	*Clostridium celerecrescens*	X71848	98.7	+	−	0.11 ± 0.01	1.89 ± 0.03	1.14 ± 0.01	0.57 ± 0.01	2.08 ± 0.12	0.66 ± 0.03
19	*Clostridium ultunense*	AZSU01000006	94.8	+	−	nd	1.21 ± 0.03	1.37 ± 0.01	0.43 ± 0.01	1.77 ± 0.01	0.42 ± 0.03

Species highlighted by bold fonts were isolated from the pit mud of Chinese strong-flavour liquor for the first time according to the review by Zou et al. (2018a). *, The SCFAs concentrations were measured after the isolates cultured anaerobically in RCM for 7 days at 37 °C. "+"/"−" represents visible electrophoresis band around correct size was/wasn't observed. nd, non-detected or the peak area is over 100-fold smaller than internal standard.

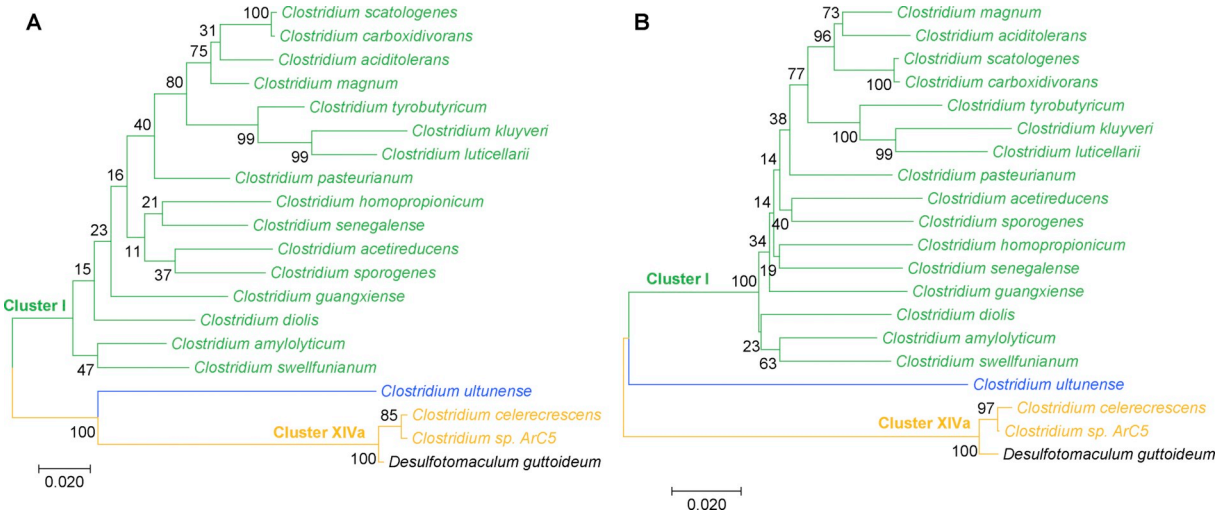

Fig. 5. (**A**) Maximum-likelihood and (**B**) neighbour-joining phylogenetic trees with 1000 bootstrap replicates were developed showing the relationships between the isolated *Clostridium* strains. The branches highlighted by green and orange represent *Clostridium* Cluster I and XIVa, respectively. Strain *Desulfotomaculum guttoideum* DSM 4024^{T} was used as the outgroup. The scale bar represents 2% sequence divergence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Isolation of *Clostridium* and evaluation of their butyrate synthesis capacity

Although *Hydrogenispora* and *Sedimentibacter* were the top abundant genera, merely one and four species with valid names were published respectively according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) and EzBioCloud databases (Parte, 2018; Yoon et al., 2017), impeding taxonomic identification of the isolates. Therefore, we mainly introduced the isolates identified as *Clostridium* species here. Also, *Clostridium* was deduced as the most likely potential butyrate producer (Tao et al., 2017). A total of nineteen *Clostridium* species with divergent butyric acid production capacity were isolated from the pit mud (Table 1). Amongst them, ten species indicated in bold fonts in Table 1 were for the first time isolated from the pit mud of strong-flavour baijiu according to the previous reports (Zou et al., 2018a). Maximum-likelihood and neighbour-joining phylogenetic trees showed that sixteen out of nineteen species were grouped into *Clostridium* Cluster I (family Clostridiaceae 1), i.e. *Clostridium sensu stricto*, *C.* sp. ArC5 and *C. celerecrescens* fell into Cluster XIVa (Lachnospiraceae) and *C. ultunense* fell into Clostridiales incertae sedis Family XI (Fig. 5). Three strains produced over 4 g/L butyric acid after cultured anaerobically in RCM for 7 days, including *C. homopropionicum*, *C.* sp. ArC5 and *C. diolis*. Additionally, all these isolates produced acetic acid, pentanoic acid (except *C. scatologenes*) and caproic acid as well. Formic acid and propionic acid were also detected in the fermentation broth of more than two-thirds isolates. *C. sporogenes* and *C. amylolyticum* with low butyrate production (0.76 and 1.52 g/L, respectively) generated high amounts of caproic acid (4.24 and 3.55 g/L, respectively), however, the top three high-yield butyric acid strains produced about six-fold lower caproic acid compared with their corresponding butyric acid concentration (Table 1). As for the butyrate synthesis genes, *buk* could be amplified in eleven species amongst the isolates via PCR analysis, while *but* in the other seven. Both *buk* and *but* could be identified in *C. magnum*.

4. Discussion

Solid-state pit mud in the fermentation cellar provides a suitable habitat for microorganisms that play pivotal roles in the flavour formation of strong-aroma Chinese liquor, termed baijiu (Jin et al., 2017). Butyrate and its major derivate, ethyl butanoate, are the representative aroma compounds in strong-flavour baijiu (Zheng and Han, 2016). Previous reports showed that Clostridia, the most abundant class in pit mud, was the main potential butyrate producer (Tao et al., 2017). Nevertheless, it is still unclear how these Clostridial microbes participate in the formation of butyrate. According to the *buk* and *but* clone library analysis, we found that butyrate-producing Clostridia were divergent at the species level between *buk* and *but* pathways (Fig. 1). Additionally, some OTUs in the two libraries, especially *but* (54%), could not be annotated at the order or family level owing to the low identity (< 80%) to reference sequences in current databases. Except the unclassified sequences, most of the OTUs identified in *buk* pathway were grouped into Clostridiales incertae sedis Family XI, while Lachnospiraceae dominated *but* pathway, which was consistent with the results of previous studies on human colonic microbiota (Louis and Flint, 2017). Moreover, it is noteworthy that Bacilli was the second predominant class related with *buk* pathway, indicating its non-negligible effects on butyrate formation, although it is not abundant in pit mud (Hu et al., 2016). Bacilli was dominant in the fermented grains (also called “Zaopei”) (Zou et al., 2018b), which directly affected the flavour of baijiu (Lang et al., 2015).

To illustrate the contribution of microorganisms to baijiu flavour, bacterial diversities in different ages and qualities pit mud were primarily detected by both next-generation sequencing and culture-dependent methods (Hu et al., 2015; Hu et al., 2016; Tao et al., 2014). However, little attention has been paid to elucidate the dynamic profiles of microbial community structure in pit mud during fermentation process to date. Considering the results of clone library analysis in this work, we focused on investigating the succession of Clostridial community, the major butyrate-producing group in pit mud. *Hydrogenispora* was the most abundant genus in the tested samples according to the Clostridial specific-16S rRNA gene sequencing analysis (Fig. 3), which has never been reported in strong-flavour ecosystem using either culture-independent or culture-dependent approaches (Zou et al., 2018b). This difference arises from many reasons: on the one hand, the pit mud samples were from different-aged fermentation cellars (Tao et al., 2014) or with different quality (Hu et al., 2016); on the other hand, amplification using Clostridial specific primers could be conducive to detect non-abundant Clostridia or some microorganisms coexisting with others, that were difficult to be distinguished using universal bacterial primer sets (Hu et al., 2014; Hung et al., 2008). There is merely one species with the valid name in *Hydrogenispora* according to the LPSN and EzBioCloud databases so far (Parte, 2018; Yoon et al., 2017). The predominant end-product from glucose by *Hydrogenispora ethanolica*

was hydrogen, followed by acetate and ethanol (Liu et al., 2014), and bioaugmentation of this species significantly increased *Clostridium* abundance and enhanced butyrate formation coupled with a higher hydrogen yield (Yang et al., 2016). Some *Clostridium* species could utilize H_2 to balance hydrogen pressure and produce SCFA (Liew et al., 2016; Zou et al., 2018a). This previous information may help to explain the increasement of *Clostridium* abundance after *Hydrogenispora* reaching its peaks in our results.

Sedimentibacter was one of the abundant genera in pit mud (Fig. 3D) and positively correlated with its quality and fermentation cellar ages (Hu et al., 2016; Tao et al., 2014). Our data also demonstrated that large amounts of OTUs pertained to *Sedimentibacter* (Fig. 4). *Sedimentibacter* could utilize amino acids and pyruvate to produce volatile and non-volatile fatty acids, e.g. acetate, butyrate and lactate in the presence of yeast extract (Breitenstein et al., 2002; Imachi et al., 2016). These fatty acids played important roles in the formation of baijiu flavour (Fan and Qian, 2006). Thus, the content of amino acid in pit mud was also suggested as a potential indicator to assess pit-mud quality besides the generally-used ammonium nitrogen concentration (Liu et al., 2017). The latest published species (four in total based on LPSN), *Sedimentibacter acidaminivorans*, was isolated from an enriched methanogenic microbial community (Imachi et al., 2016). Similarly, methanogenic archaea were also identified in pit mud (Ding et al., 2014), contributing to the SCFA production likely by interspecies hydrogen transfer (Tao et al., 2017). Based on these studies, we predicted that there might be underlying interactions between *Sedimentibacter* and methanogens, which needs to be further investigated to elucidate the important roles of *Sedimentibacter* in baijiu flavour formation.

Compared with *Hydrogenispora* and *Sedimentibacter*, *Clostridium* attracted more attention due to their abilities to produce organic acids and short chain fatty acids (Hu et al., 2016; Tao et al., 2017; Yin et al., 2016), the characteristic aroma compounds of baijiu. To date, approximately 231 *Clostridium* species are published with valid names in LPSN database (Parte, 2018) and 123 species belong to *Clostridium* cluster I, the largest group of Clostridia and regarded as the true *Clostridium*, i.e. *Clostridium sensu stricto* (Gupta and Gao, 2009; Lawson, 2016), based on the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/) (Cole et al., 2013). In this study, of the nineteen butyrate-producing isolates, sixteen species were affiliated with *Clostridium* cluster I (Fig. 5) and their functions in baijiu production were not well understood, even ten of them were first isolated from pit mud in our work according to the review by Zou et al. (2018a). Metagenomics analysis of the pit mud microbiome revealed that *Clostridium* was one of the groups mainly expressed *buk* at genus level (Tao et al., 2017). Many *Clostridium* species identified in human colon also prefer the buk rather than but enzyme for the final step in butyrate biosynthesis, while *but* pathway dominated butyrate formation in the human colonic ecosystem (Louis and Flint, 2017). Besides *buk* matching to most isolates, *but* was identified in approximately half of the isolates (Table 1), highlighting the remarkable functional diversity at species level of butyrate synthesis in *Clostridium*, which was consistent with the results of clone library assay (Fig. 1).

In summary, this work revealed that Clostridia was the predominant butyrate-producing group in both *buk* and *but* pathways. The Clostridial specific-16S rRNA gene sequencing results demonstrated the resilience of Clostridial species richness and diversity in pit mud after a fermentation cycle. This work also helped us to zoom in on the top three abundant genera, *Hydrogenispora*, *Sedimentibacter* and *Clostridium*, and further targeted the potential butyrate-producing bacteria. Based on culture-dependent methods, nineteen *Clostridium* species with butyrate formation ability were isolated from the pit mud, displaying species-specific features in butyrate biosynthesis pathways. The present data offer a useful basis for future studies to investigate the metabolic function of these potentially butyrate-producing species. Further studies will aim at illustrating the butyrate metabolism pathways in pit mud using next-generation sequencing approaches coupled with culture omics, especially isolating representative species of *Hydrogenispora* and *Sedimentibacter*.

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Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.02.023.

References

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10, 57.

Breitenstein, A., Wiegel, J., Haertig, C., Weiss, N., Andreesen, J.R., Lechner, U., 2002. Reclassification of *Clostridium hydroxybenzoicum* as *Sedimentibacter hydroxybenzoicus* gen. nov., comb. nov., and description of *Sedimentibacter saalensis* sp. nov. Int. J. Syst. Evol. Microbiol. 52, 801–807.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, 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, T.J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2013. Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42, D633–D642.

Ding, X.F., Wu, C.D., Zhang, L.Q., Zheng, J., Zhou, R.Q., 2014. Characterization of eubacterial and archaeal community diversity in the pit mud of Chinese Luzhou-flavor liquor by nested PCR–DGGE. World J. Microbiol. Biotechnol. 30, 605–612.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.

Fan, W., Qian, M.C., 2006. Identification of aroma compounds in Chinese "Yanghe Daqu" liquor by normal phase chromatography fractionation followed by gas chromatography/olfactometry. Flavour Fragr. J. 21, 333–342.

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, 2.

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. Appl. Environ. Microbiol. 71, 4117–4120.

Fish, J.A., Chai, B., Wang, Q., Sun, Y., Brown, C.T., Tiedje, J.M., Cole, J.R., 2013. FunGene: the functional gene pipeline and repository. Front. Microbiol. 4, 291.

Gupta, R.S., Gao, B., 2009. Phylogenomic analyses of clostridia and identification of novel protein signatures that are specific to the genus *Clostridium sensu stricto* (cluster I). Int. J. Syst. Evol. Microbiol. 59, 285–294.

Hu, X.L., Wang, H.Y., Wu, Q., Xu, Y., 2014. Development, validation and application of specific primers for analyzing the clostridial diversity in dark fermentation pit mud by PCR-DGGE. Bioresour. Technol. 163, 40–47.

Hu, X.L., Du, H., Xu, Y., 2015. Identification and quantification of the caproic acid-producing bacterium *Clostridium kluyveri* in the fermentation of pit mud used for Chinese strong-aroma type liquor production. Int. J. Food Microbiol. 214, 116–122.

Hu, X., Du, H., Ren, C., Xu, Y., 2016. Illuminating anaerobic microbial community and co-occurrence patterns across a quality gradient in Chinese liquor fermentation pit muds. Appl. Environ. Microbiol. 82, 2506–2515.

Hung, C.H., Cheng, C.H., Cheng, L.H., Liang, C.M., Lin, C.Y., 2008. Application of *Clostridium*-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. Int. J. Hydrogen Energ. 33, 1586–1592.

Imachi, H., Sakai, S., Kubota, T., Miyazaki, M., Saito, Y., Takai, K., 2016. *Sedimentibacter acidaminivorans* sp. nov., an anaerobic, amino-acid-utilizing bacterium isolated from marine subsurface sediment. Int. J. Syst. Evol. Microbiol. 66, 1293–1300.

Jin, G., Zhu, Y., Xu, Y., 2017. Mystery behind Chinese liquor fermentation. Trends Food Sci. Tech. 63, 18–28.

Lang, Z.W., Lu, Z.M., Gong, J.S., Wang, S., Shen, C., Shi, J., 2015. Compositional discrepancy of volatile compounds in fermented grains and distilled grains of Luzhou-flavor liquor. Food Ferment. Ind. 41, 34–37.

Lawson, P.A., 2016. The taxonomy of the genus *Clostridium*: current status and future perspectives. Microbiol. China 43, 1070–1074.
Liew, F., Martin, M.E., Tappel, R.C., Heijstra, B.D., Mihalcea, C., Köpke, M., 2016. Gas fermentation—a flexible platform for commercial scale production of low-carbon-fuels and chemicals from waste and renewable feedstocks. Front. Microbiol. 7, 694.
Liu, H., Sun, B., 2018. Effect of fermentation processing on the flavor of Baijiu. J. Agr. Food Chem. 66, 5425–5432.
Liu, Y., Qiao, J.T., Yuan, X.Z., Guo, R.B., Qiu, Y.L., 2014. *Hydrogenispora ethanolica* gen. nov., sp. nov., an anaerobic carbohydrate-fermenting bacterium from anaerobic sludge. Int. J. Syst. Evol. Microbiol. 64, 1756–1762.
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 Res. Int. 102, 68–76.
Louis, P., Flint, H.J., 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol. Lett. 294, 1–8.
Louis, P., Flint, H.J., 2017. Formation of propionate and butyrate by the human colonic microbiota. Environ. Microbiol. 19, 29–41.
Magoč, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957–2963.
Oberhardt, M.A., Zarecki, R., Gronow, S., Lang, E., Klenk, H.P., Gophna, U., Ruppin, E., 2015. Harnessing the landscape of microbial culture media to predict new organism–media pairings. Nat. Commun. 6, 8493.
Parte, A.C., 2018. LPSN-list of prokaryotic names with standing in nomenclature (bacterio. net), 20 years on. Int. J. Syst. Evol. Microbiol. 68, 1825–1829.
Plutowska, B., Wardencki, W., 2008. Application of gas chromatography–olfactometry (GC–O) in analysis and quality assessment of alcoholic beverages–a review. Food Chem. 107, 449–463.
Rose, T.M., Henikoff, J.G., Henikoff, S., 2003. CODEHOP (COnsensus-DEgenerate hybrid oligonucleotide primer) PCR primer design. Nucleic Acids Res. 31, 3763–3766.
Tao, Y., Li, J., Rui, J., Xu, Z., Zhou, Y., Hu, X., Li, X., 2014. Prokaryotic communities in pit mud from different-aged cellars used for the production of Chinese strong-flavor liquor. Appl. Environ. Microbiol. 80, 2254–2260.
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. Microb. Biotechnol. 10, 1603–1615.
Vital, M., Penton, C.R., Wang, Q., Young, V.B., Antonopoulos, D.A., Sogin, M.L., Morrison, H.G., Raffals, L., Chang, E.B., Huffnagle, G.B., Schmidt, T.M., Cole, J.R., Tiedje, J.M., 2013. A gene-targeted approach to investigate the intestinal butyrate-producing bacterial community. Microbiome 1, 8.
Vital, M., Gao, J., Rizzo, M., Harrison, T., Tiedje, J.M., 2015. Diet is a major factor governing the fecal butyrate-producing community structure across Mammalia, Aves and Reptilia. ISME J 9, 832–843.
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. Appl. Environ. Microbiol. 73, 5261–5267.
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.
Wu, L.H., Lu, Z.M., Zhang, X.J., Wang, Z.M., Yu, Y.J., Shi, J.S., Xu, Z.H., 2017. Metagenomics reveals flavour metabolic network of cereal vinegar microbiota. Food Microbiol. 62, 23–31.
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 microbial community of medium-temperature Daqu. Appl. Environ. Microbiol. 83, e01550-17.
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. Bioresour. Technol. 211, 319–326.
Yin, Q., Tao, Y., Zhu, X., Zhou, Y., He, X., Cheng, L., Li, D., 2016. *Clostridium liquoris* sp. nov., isolated from a fermentation pit used for the production of Chinese strong-flavoured liquor. Int. J. Syst. Evol. Microbiol. 66, 749–754.
Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J., 2017. Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. Int. J. Syst. Evol. Microbiol. 67, 1613–1617.
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. Environ. Microbiol. 10, 1850–1860.
Zhang, J., Fang, F., Chen, J., Du, G., 2014. The arginine deiminase pathway of koji bacteria is involved in ethyl carbamate precursor production in soy sauce. FEMS Microbiol. Lett. 358, 91–97.
Zheng, X.W., Han, B.Z., 2016. Baijiu (白酒), Chinese liquor: history, classification and manufacture. J. Ethnic Foods 3, 19–25.
Zou, W., Ye, G., Zhang, K., 2018a. Diversity, function, and application of *Clostridium* in Chinese strong flavor Baijiu ecosystem: a review. J. Food Sci. 83, 1193–1199.
Zou, W., Zhao, C., Luo, H., 2018b. Diversity and function of microbial community in Chinese strong-flavor Baijiu ecosystem: a review. Front. Microbiol. 9, 671.