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Significant difference of the culturable anaerobic microbial species in GujingTribute pitmud with different ages

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Abstract. Most Chinese liquor belongs to strong-aroma type, and GujingTribute liquor with a history over 1800 years is one of this type. The flavor of strong-aroma Chinese liquor is largely determined by anaerobic bacteria embedded in the pits, especially the bottom area of the old pits. In this study, culturable anaerobic species in the old (50-100 years old) and young (5 years old) pitmuds were isolated with anaerobic cultivation and characterized by near full-length 16S rDNA sequencing. Ruminococcaceae bacterium CPB6 and Lactobacillus acetotolerans were found as the most culturable species in the freshly-sampled old and young pitmud samples, respectively. This result confirmed our previous findings by high throughput next generation sequencing that Ruminococcaceae bacterium CPB6 was more abundant in good quality pitmud while Lactobacillus acetotolerans was more abundant in ordinary or poor quality pitmud.

1. Introduction

Traditional Chinese alcoholic liquor is typically classified into several categories based on aroma characteristics: soy sauce aroma, strong aroma, light aroma, rice aroma, and miscellaneous. The representative liquor brands are Maotai and Langjiu [1-2] for soy sauce aroma type, Luzhou [2], Jiannanchun [3], Wuliangye [3], GujingTribute [4] and Yanghe [5] for strong aroma type; Fen [2] for light aroma type, and Guilin Sanhua [6] for rice aroma type. Examples for the miscellaneous types are combined aroma, herbaceous aroma, sesame aroma, Feng aroma, Te aroma, and fermented-soya-beans aroma; and their typical representatives are Baiyunbian Liquor [7], Dong Liquor [8], JingzhiBaigan [9], Xifeng [10], SiTe [11], and Yubingshao [12], respectively.

GujingTribute liquor has a history over 1800 years, and it is one of the old eight most famous Chinese liquor types in China. In the past a few years, progress on culturable unknown species in GujingTribute pitmud has been limited due to difficulty in anaerobic manipulations [13]. However, some microbes are already deciphered early time in the GujingTribute pit mud, Daqu and Zaopei [14-16]. By now, at least 1100 different species of microbes have been determined for GujingTribute fermentation (data not shown) through different technologies, but many anaerobic bacteria are waiting for isolation and cultivation.

Many anaerobic culture-related literature articles are slow to be updated world widely because little progress has been made methodologically [17]. Researchers in Chinese liquor industry are working hard to reshape the unsatisfactory situation in the anaerobic microbes. Zhu et al [18-19] isolated a species named CPB6 that can highly produce caproic acid from lactate under anaerobic fermentation; Wang et al [20] isolated seven different genus mainly by anaerobic cultivation from the pitmud sample of a strong-aroma liquor. He et al [21] isolated many different species of *Clostridium* from *Zhanggong Lao Jiu* pitmud. Similar studies can be seen in the related reports [22-23], but all the above studies belong to preliminary investigations on anaerobic microbes in the liquor pitmud.

In this study, pitmud itself was employed to be the main part of the culturing medium to isolate colonies from the pitmud samples taken from old (50-100 years) and young (5 years) pits of GujingTribute liquor. Colonies were cultured, recorded with multiple morphological parameters, and statistically classified. The representative colonies were subjected to genome DNA extraction, full-length 16S rDNA amplification and Sanger-sequencing. The results turned out that there were significant

Differences of the culturable anaerobic microbial species in GujingTribute pitmuds with different ages.

2. Materials and Methods

2.1. Medium

In this study, pitmud samples were all freshly collected from 18 pits with different ages: 6 from A plant pits with over 100 years old, 6 from B plant pits with over 50 years old and 6 from C plant pits with only 5 years old. The former 6A + 6B were mixed into one sample as old pitmud, and the latter 6C as yound pitmud. 60g the above pitmud was suspended into 600mL sterile distilled water, centrifuged 10min at 15000r/min for the supernatant. 500mL supernatant was then mixed with glucose 7g, beef extract 1g, peptone 2g, NaCl 2g, yeast extract 0.5g, $MgCl_2$ (1M) 21μl, K_2HPO_4 0.75g, $FeSO_4 \cdot 7H_2O$ 0.05g, L-cysteine 0.25g, and agar 10g, with final volume as 750mL. After 30min autoclaving, the medium was plated in petri dishes within the A35 anaerobic workstation.

2.2. Anaerobic cultivation

Anaerobic microbial species were directly isolated from fresh pitmud by mixing 1g fresh pitmud (old or young, collected as above) into 1ml fresh yellow water from old pits (the two-phase liquid with organic matter and inorganic substance, aggregates during the fermentation and falls down on the pit bottom; The yellow water is a place where lots of aroma molecules are presumably synthesized). 5μl the above stock solution plus 495μl sterile distilled water was then used to inoculate the medium plate and cultured at 32℃in the workstation with the gas condition (N_2:H_2:CO_2=8:1:1) for 4 days. By series of dilution and inoculation, one plate with only 20-40 colonies growing on it was obtained for the old pitmud and one similar plate was obtained for the young pitmud after several days (Fig.2). Colonies were backed up two copies on the solid medium plates, one for further growing in the workstation, and another for colony sequencing.

The experiments were repeated three times and similar morphological features of the colonies were observed. The more accurate dominance was calculated with colony number multiplied by the volume of the same colony. The volume of each colony $V = \pi d^3/6$, where the d means the diameter of the colony; and the dominance (D) = VN, where the N means the number of the colonies with the same sequencing results. So some small colonies, if their number is large, can be still counted as culturable species under this specific cultivation condition.

2.3. Genome extraction and 16S rDNA full-length amplification/sequencing

This part was undertaken as previously [16, 24]. Briefly, Genomic DNA was extracted from individual colonies using Solarbio D2600 kit. Each 20mg or around colony sample generated 100μL genome DNA. The 16s rDNA amplification was undertaken using universal primers 27F (5'- AGA GTT TGA

TCC TGG CTC AG-3') and 1492R (5'TAC GGY TAC CTT GTT ACG ACT T3'). The PCR system had 1 μL of template DNA, 6μL of NPK02 2× buffer, 0.8μL of each primer (2 μM), 0.2μL Taq DNA polymerase (5U/μL), and 3.2 μL of distilled water. In the negative control, the template DNA was replaced by the same volume of water. The reactions started with an initial denaturing step at 95°C for 5min, followed by 34 cycles of 95°C for 30s, 60°C for 60s, and 72°C for 60s, and were appended with a 4 min elongation step at 72°C. Amplified target bands (about 1500bp) were gel-purified using SangonSanPrep kit (Cat#: SK8132), then subjected to Sanger sequencing using both 27F and 1492R.

2.4. Sequence analysis

The raw sequences were assemblied by CExpress and edited by EditSeq to make sure of correct orientation [24]. Before doing BLAST analysis, the 16S sequence was analyzed by Chimera (https://decipher.cee.wisc.edu/FindChimeras.html). Successfully assemblied DNA was subjected to Basic Local Alignment Search Tool (BLAST) analysis at the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) in order to get species-level taxonomic information. The same sequences were also input in the RDP database for further classification. The sequences were then compared with our previous Genbank input (PopSet: 1050229180) that already had some CPB6 strain information [18-19].

2.5. Colony storage approach

Permanent storage of anaerobic colonies is a problem because of the low recover rate after some time under -80 degree. Fresh colonies were picked up and suspended into the liquid medium with the final 35-45% glycerol in a 1.5mL tube. Then the tube was put into a slow-freezing apparatus (Ruomeng Maxhall) so that the microbes can be frozen gradually at a rate of -1 degree per min.

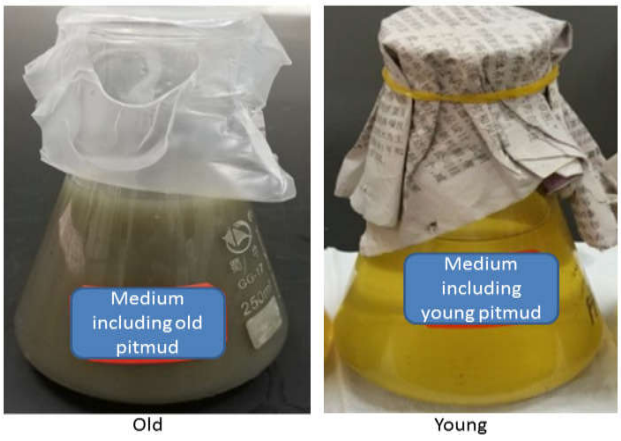


Figure 1. Liquid media made from pitmud suspensions from old and young pits in different plants.

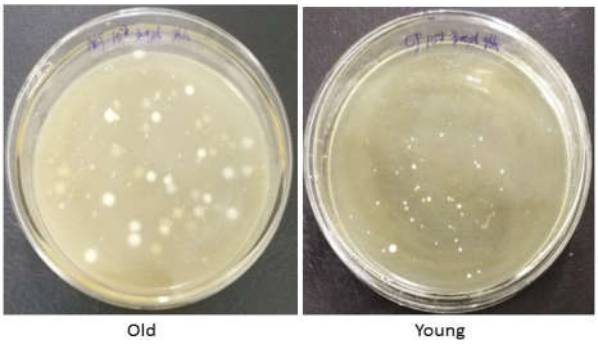


Figure 2. Four-day growth of anaerobic colonies.

3. Results and Discussion

3.1. Isolation of anaerobic colonies

The approach in this study would make sure that culturable anaerobic species are isolated under the specific conditions. Though the culturable species isolated in this study may be not the real culturable ones (because the growing conditions may be not optimal for the real ones), the obtained results were already encouraging (also see below results). Microbes of old and young pitmuds grew on the media made of their own pitmuds. Apparently, the color of the medium from the old pitmud was deeper than that of the young pitmud (Fig.1). Figure 2 displayed one group of the obtained culturable colonies for both old and young pitmud samples.

3.2. DNA sequencing results

In this study, Chimera analysis of 16S sequence and no chimerism in CPB6 strain, the species type was determined by the near full length of the 16S rDNA sequences, though some colonies were actually not able to be determined in this way mainly because there were no matches in the public databases with enough significant similarities. It is not surprising that the pitmud of Chinese strong-aroma liquor holds a plenty of unknown microbes as shown in the literature [14-15,25].Table 1~2 (the * marked the CPB6 strain) demonstrated that among the 37 sequenced colonies, 6 colonies were unknown species with intermediate level of dominance (data not shown). It was very striking that 20 among the 37 colonies were all the strain Ruminococcaceae bacterium CPB6. The CPB6 strain was recently reported [23] to produce high-concentration n-caproic acid from lactate in a fermentation study. Interestingly, this strain had a capacity that it could raise the pH value of the whole reaction system during the transformation of lactate into caproic acid, and this feature is a long-awaited species in the Chinese strong-aroma liquor fermentation. Sequencing results and the colony morphology results were compared and it was observed that the strain had both white and yellow colors for different colonies. Further experiments have to be done in order to confirm the above results, since the commercial DNA sequencing for ribosome RNA genes are easy to make mistakes.

The sequencing results for the young pitmud sample was also surprising in that the 35 among the 36 sequenced colonies were all *Lactobacillus acetotolerans* (data not shown). This is surprising because *Lactobacillus acetotolerans was know*n as the most culturable species in the Zaopei (fermented grains after two to three month of fermentation) sample of GujingTribute liquor. Most pitmud samples from the young pits are not so dominated by the strain, though the strain's dominance is significantly higher than in the old pitmud (data not shown). It was possible that the anaerobic cultivation conditions in this study were very good for the growth of *Lactobacillus acetotolerans*. Another possibility was that the fresh pitmud samples were easily contaminated by the Zaopei liquid since Zaopei was moved right before the pitmud take-up and at this moment, the pit bottom (the pitmud sampling site) was very humid with yellow water mixed with Zaopei stuff.

In our previous sequencing collection sent to Genebank (see: Bacteria 16S ribosomal RNA gene, partial sequence. NCBI PopSet: 1050229180), about 26 input sequence were all exactly the near full length 16S rDNA of Ruminococcaceae bacterium CPB6, confirming that the CBP6 strain may be really important in the old pitmud that highly determines the liquor quality.

3.3. Other culturable species

Previously our group found that *Clostridium tyrobutyricum* was culturable in the same cultivation conditions when the sample was taken from the yellow water [24]. Because the yellow water is just between the bottom pitmud and the lower-layer Zaopei, it is surprising that the strain *Clostridium tyrobutyricum* was not seen in this study.

Table 1. Sequencing BLAST results for the colonies from old pitmud sample

Colony No.	Description
1*	Ruminococcaceae bacterium CPB6, complete genome
2*	Ruminococcaceae bacterium CPB6, complete genome
3	chimera
4	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: 20JB45
5*	Ruminococcaceae bacterium CPB6, complete genome
6	No significant similarity found
7*	Ruminococcaceae bacterium CPB6, complete genome
8*	Ruminococcaceae bacterium CPB6, complete genome
9*	Ruminococcaceae bacterium CPB6, complete genome
10*	Ruminococcaceae bacterium CPB6, complete genome
11	Uncultured compost bacterium partial 16S rRNA gene, clone PS2614
12*	Ruminococcaceae bacterium CPB6, complete genome
13*	Ruminococcaceae bacterium CPB6, complete genome
14*	Ruminococcaceae bacterium CPB6, complete genome
15	Uncultured Clostridium sp. gene for 16S rRNA, partial sequence, clone: CXZX182
16	Clostridium liquoris strain BEY10 16S ribosomal RNA, partial sequence
17*	Ruminococcaceae bacterium CPB6, complete genome
18	Muricomes intestini strain 2PG-424-CC-1 16S ribosomal RNA, partial sequence
19*	Ruminococcaceae bacterium CPB6, complete genome
20*	Ruminococcaceae bacterium CPB6, complete genome
21*	Ruminococcaceae bacterium CPB6, complete genome
22*	Ruminococcaceae bacterium CPB6, complete genome
23*	Ruminococcaceae bacterium CPB6, complete genome
24	Uncultured bacterium clone S7_M51 16S ribosomal RNA gene, partial sequence
25	Lachnospiraceae bacterium BTY6 16S ribosomal RNA gene, partial sequence
26	Lachnospiraceae bacterium BTY6 16S ribosomal RNA gene, partial sequence
27*	Ruminococcaceae bacterium CPB6, complete genome
28	No significant similarity found
29	No significant similarity found
30	Uncultured compost bacterium partial 16S rRNA gene, clone PS2614
31	No significant similarity found
32	Uncultured compost bacterium clone 0B11 16S ribosomal RNA gene, partial sequence
33*	Ruminococcaceae bacterium CPB6, complete genome
34	No significant similarity found
35	No significant similarity found
36	chimera
37*	Ruminococcaceae bacterium CPB6, complete genome

Table 2. Sequencing BLAST results for the colonies from old pitmud sample

Colony No.	E value	Query cover	Identy	Accession
1*	0	98%	99%	CP020705.1
2*	0	98%	99%	CP020705.1
3	--	--	--	--
4	0	99%	98%	AB826017.1
5*	0	98%	96%	CP020705.1
6	--	--	--	--
7*	0	99%	98%	CP020705.1
8*	0	98%	97%	CP020705.1
9*	0	99%	97%	CP020705.1
10*	0	99%	96%	CP020705.1
11	0	98%	96%	FN667389.1
12*	0	99%	97%	CP020705.1
13*	0	99%	97%	CP020705.1
14*	0	98%	98%	CP020705.1
15	0	98%	97%	LC055608.
16	0	98%	82%	NR_148617.1
17*	1E-160	74%	76%	CP020705.1
18	0	97%	95%	NR_144617.1
19*	0	94%	82%	CP020705.1
20*	0	98%	98%	CP020705.1
21*	2E-73	15%	89%	CP020705.1
22*	0	98%	98%	CP020705.1
23*	0	98%	92%	CP020705.1
24	0	95%	99%	KX672689.1
25	0	98%	98%	KC331157.2
26	0	98%	98%	KC331157.2
27*	0	98%	99%	CP020705.1
28	--	--	--	--
29	--	--	--	--
30	0	97%	96%	FN667389.1
31	--	--	--	--
32	0	98%	91%	DQ345464.1
33*	0	92%	93%	CP020705.1
34	--	--	--	--
35	--	--	--	--
36	--	--	--	--
37*	0	99%	99%	CP020705.1

3.4. Liquid cultivation and storage of anaerobic colonies

Anaerobic liquid cultivation of the isolated 37 colonies (Table 1) using the same medium (without agar) as the solid cultivation was tested and it was found that most of them cannot be simply cultured this way, though some of the colonies grew a little in several days, and grew a little more after about 10 days. The potential reasons for the growth difficulty included that the cultivation environment may be not strictly anaerobic, or some colonies have to be co-cultured with other microbes. Besides, the storage process of the 37 colonies also encountered difficulty in that most colonies were hard to recover alive after storage in the -80 degree (fresh colony in the liquid medium with the final 40% glycerol). However, most colonies can be recovered well through the solid cultivation (Fig.3).

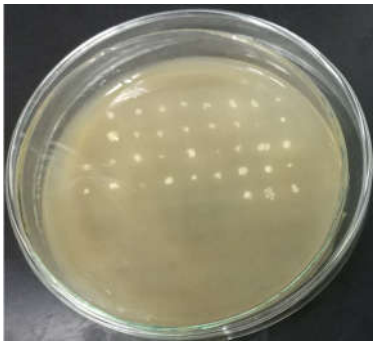


Figure 3. Back-up solid cultivation of 37 anaerobic colonies.

3.5. qPCR quantitation of two isolated key anaerobic strains in different fermentation samples

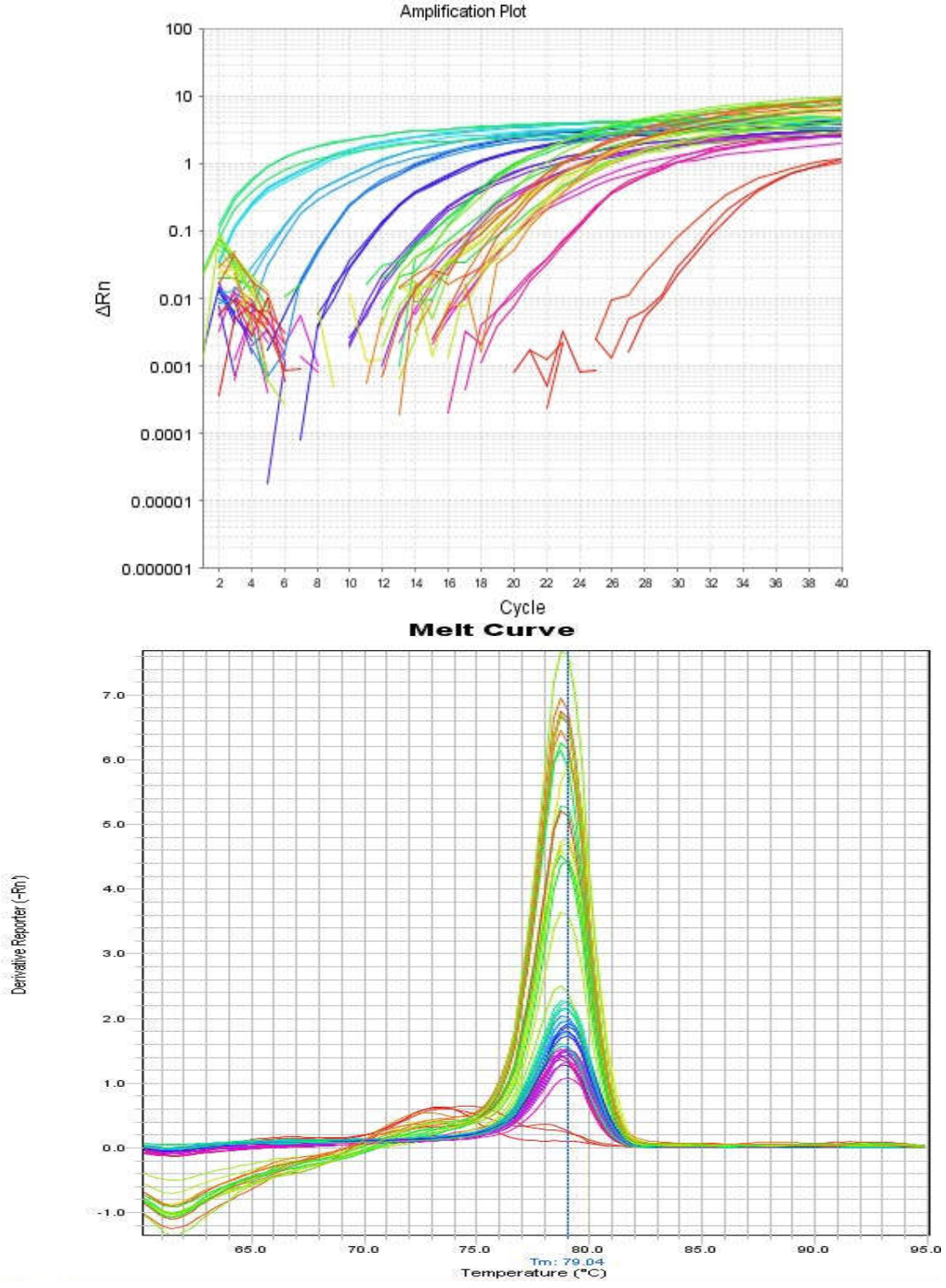
The cultivating medium used in this study was originally designed for *Clostridium*, but it was found that the medium can grow several other genera except for *Clostridium* colonies. Actually this study isolated much more pure colonies than the above 37 (data not shown). Distinct differences were seen between old pit muds (50-100 year) and new pit muds (5 year) in that the dominant colonies in the new pit mud were mainly *Lactobacillus*, while the dominant strains in the old pit mud provide more diversity including *Clostridium, Lactobacillus, Lachnospiraceae, Ruminiclostridium, and Eubacterium*, though some samples from the old pit mud also were dominant by *Lactobacillus*, indicating the microbial distribution in the old pit bottom may be not even. Notebly, Ruminococcaceae bacterium CPB6 is one strain seen in the old pit mud, while *Lactobacillus acetotolerans* is the main strain in the new pit mud, though it is also often seen in the old pit mud with less dominance.

Species-specific primers were designed using Primer-Blast method for the two important strains, *Lactobacillus acetotolerans* and Ruminococcaceae bacterium CPB6. The design process treated all other known bacterial strains in GujingTribute fermentation system as the background, especially those with known whole genome sequences (data not shown). The specificity of the primers, two pairs for each strain, was checked out and confirmed with PCR amplification, Sanger sequencing and TA cloning/colony sequencing. QPCR was employed to detect the relative concentrations of *Lactobacillus acetotolerans* and Ruminococcaceae bacterium CPB6 in a series of fermentation samples from new and old pits. The contents of *Lactobacillus acetotolerans* were apparently higher in Zaopei than pit mud (data not shown), and higher in the new pit mud than the old pit mud (Fig.4). The contents of Ruminococcaceae bacterium CPB6 were higher in the old pit mud than the new pit mud (Fig.5).

Two pairs of primers for *Lactobacillus acetotolerans* were as follow: LA2F: 5'-GTG CAG CTT AAG GGC TTC CT-3' (Tm: 60.32℃), LA2R: 5'-ATC TGT GCA GAT TGT TCC CTT-3' (Tm: 57.55℃), with the amplicon size of 194bp; LA3F:5'-AGA GTG TCC CGA GTA GTC CC-3'(Tm: 60.03℃), LA3R:5'-AAC TGC TCA AGA AGG GAC CG-3' (Tm: 59.96℃), with the amplicon size of 150 bp; Two pairs of primers for Ruminococcaceae bacterium CPB6 were as follow: CPB6-1F: 5'-GGT GAA ACG CCG AAA ACG AA-3' (Tm: 59.97℃), CPB6-1R: 5'-CCA AAA AGG CCA CTG TCT GC-3' (Tm: 59.97℃), with the amplicon size of 118 bp; CPB6-2F: 5'-CGC ACT ATG AGC AAA TGG GC-3' (Tm: 59.97℃), CPB6-2R: 5'-CAT CAA TGC GCT TCA GAG CC-3' (Tm: 59.97℃), with the amplicon size of 211 bp. Quality assessment of the above four pairs of primers showed that two pairs of primers for *Lactobacillus acetotolerans* were all good for qPCR, but only one pair of primer LA3 was chosen for displaying qPCR results (Fig.4); meanwhile, only one pair of primers (CPB6-1) for Ruminococcaceae bacterium CPB6 had satisfying qPCR presentation (Fig.5), and the primer pair (CPB6-2) showed a skewed curve for Tm analysis (data not shown).

It is interesting to note that even in the pit mud samples from new pits, the strain content of Lactobacillus *acetotolerans* is sometimes similar as, even lower than, the old pit mud (Fig.4), though in most cases, *Lactobacillus acetotolerans* was much more dominant in the new pit mud than in the old pit mud samples. The behind mechanisms may take long time to decipher. Besides, the mutual

relationship between *Lactobacillus acetotolerans* and Ruminococcaceae bacterium CPB6 is of great value to tackle, since the former is capable of producing large amount of acetic acid, while the latter was found to be able to transfer lactate into ethyl caproate through still not fully characterized processes [18-19], given that acetic acid and lactate are not far away from each other in the basic cellular metabolic pathways.



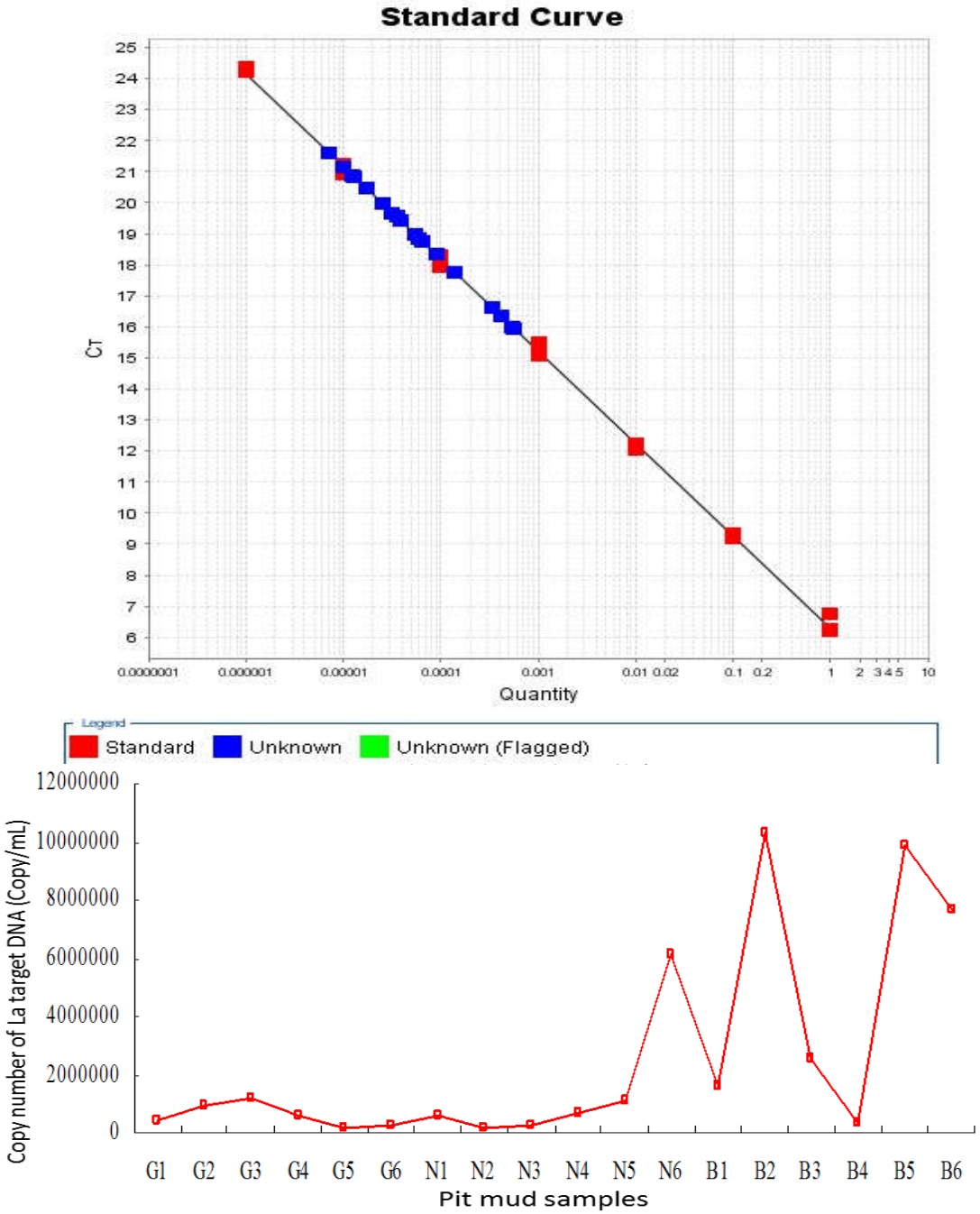
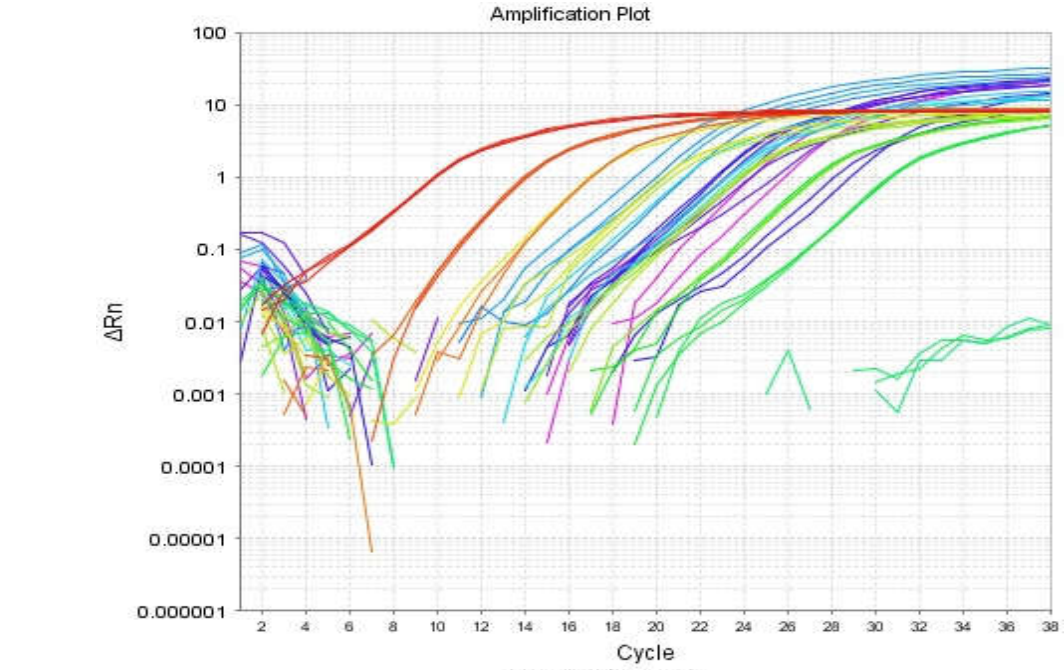


Figure 4. qPCR quantification results of *Lactobacillus acetotolerans* in different fermentation samples. qPCR amplification curves; Tm analysis of amplicons; The standard curve; Quantification results of the strain in 18 pit mud samples (G for A plant; N for B plant and B for C plant).


Amplification Plot
100
10
1
0.1
0.01
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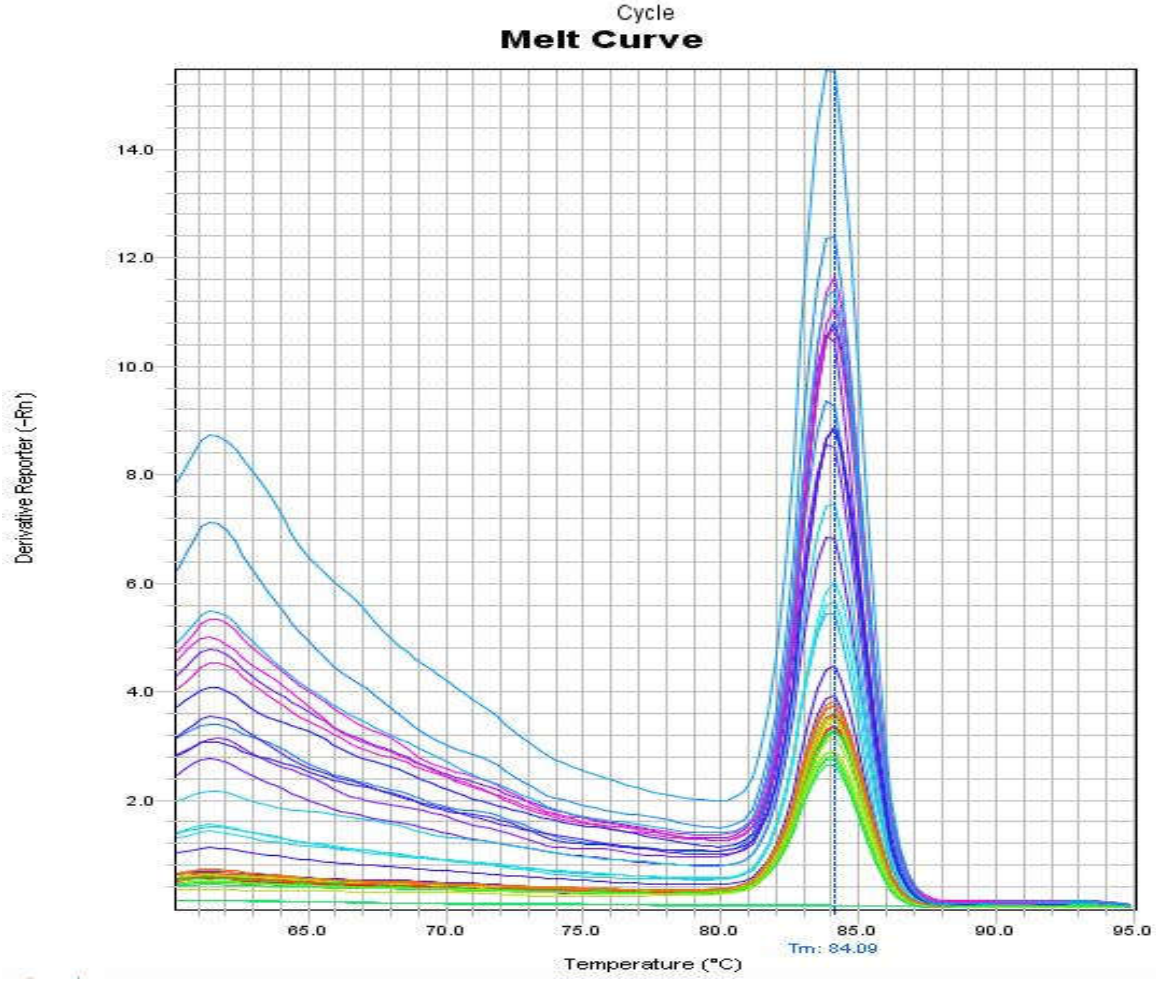
0.000001
ΔRn
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Cycle


Melt Curve
14.0
12.0
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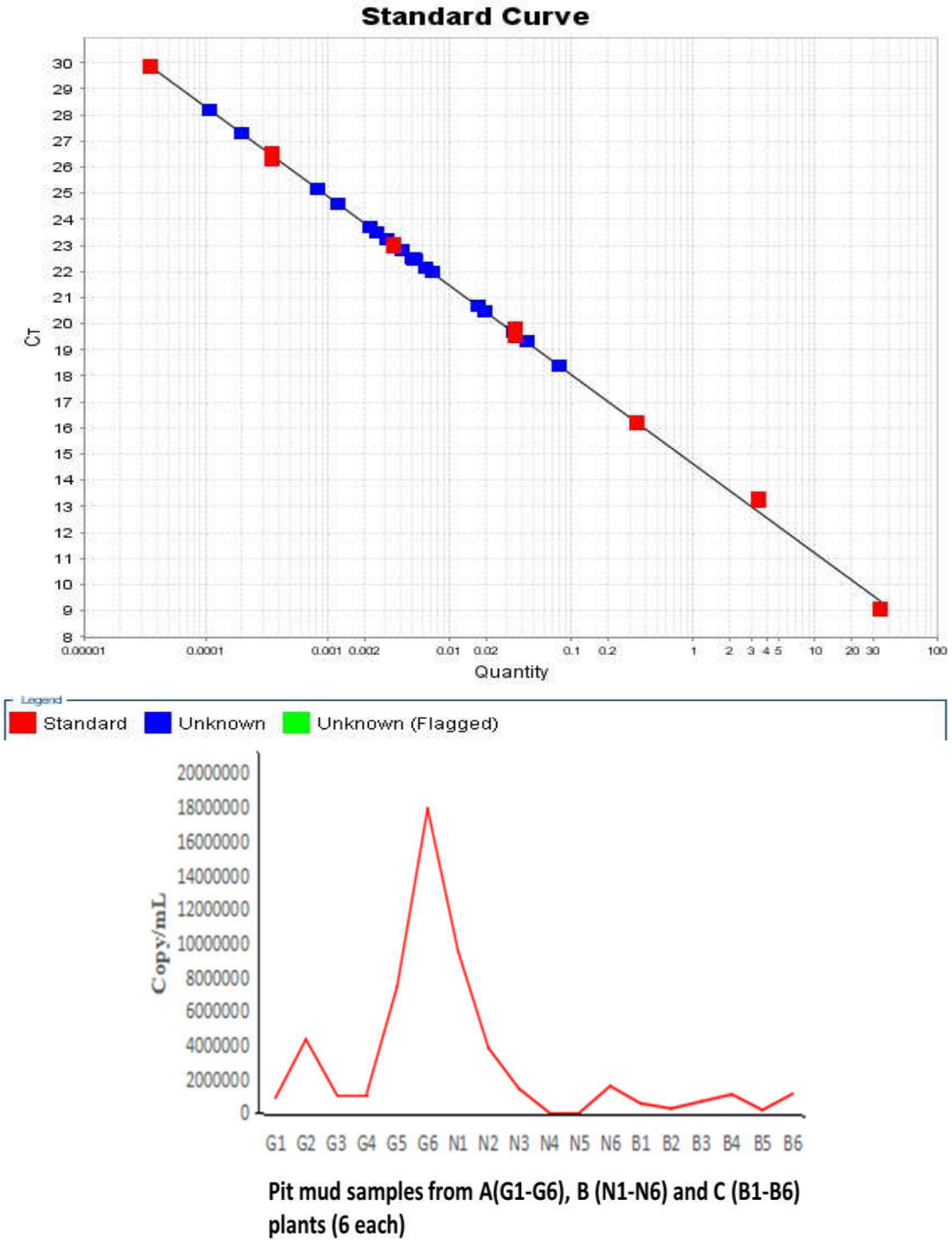

Figure 5. qPCR quantification results of Ruminococcaceae bacterium CPB6 in different fermentation samples. qPCR amplification curves; Tm analysis of amplicons; The standard curve; Quantification results of the strain in 18 pit mud samples.

4. Conclusion

In this study, culturable anaerobic species in the old (50-100 years old) and young (5 years old) pitmuds were isolated with anaerobic cultivation and characterized by near full-length 16S rDNA sequencing. Ruminococcaceae bacterium CPB6 and *Lactobacillus acetotolerans* were found as the

most culturable species in the freshly-sampled old and young pitmud samples, respectively. This result confirmed our previous findings by high throughput next generation sequencing (NCBI PopSet: 1050229180) that Ruminococcaceae bacterium CPB6 was more abundant in good quality pitmud while *Lactobacillus acetotolerans* was more abundant in ordinary or poor quality pitmud. In general, the old pit mud provided more microbial diversity for anaerobic species including *Clostridium, Lactobacillus, Lachnospiraceae, Ruminiclostridium, and Eubacterium*, while the new pit mud was highly dominant by *Lactobacillus*.

Acknowledgments
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