

Analysis of Microbial Community Structure and Volatile Compounds in the Pit Mud Used for Manufacturing Taorong-type Baijiu Based on High-Throughput Sequencing

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Abstract

In this study, the pit mud used in manufacturing Taorong-type Baijiu was collected from the upper, middle, lower and bottom layers of pits in Henan Yangshao Liquor Co., LTD. Besides, high-throughput sequencing (HTS) technology was adopted to analyze the microbial community structure of the pit mud. In addition, the volatile compounds in the pit mud were subjected to preliminarily qualitative analysis through headspace-solid phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS). The results of HTS demonstrated that there were 5, 3, 5 and 5 dominant bacterial phyla (including 11, 11, 9 and 8 dominant bacterial genera) and 3, 3, 3 and 3 dominant fungal phyla (including 4, 7, 7 and 5 dominant fungal genera) in the pit mud from F-S (upper), G-Z (middle), H-X (lower) and I-D (bottom), respectively. The qualitative analysis results of volatile compounds demonstrated that a total of 78 kinds of volatile compounds were detected in the pit mud, including 46, 45, 39 and 49 kinds in the pit mud from F-S, G-Z, H-X and I-D, respectively. Ester and acid were the two main components in the pit mud. Meanwhile, the correlation between microorganisms and main volatile compounds in the pit mud was analyzed. Moreover, *Lentimicrobium*, *Syner-01* and *Blvii28_wastewater-sludge group* were found for the first time in the pit mud used for manufacturing Taorong-type Baijiu. The findings of this study could provide a theoretical foundation for improving the quality of pit mud and the flavor of Taorong-type Baijiu.

Introduction

Baijiu has a long history in China, and it is one of China's national cultural characteristics^[1]. As an innovative flavor type in this industry in China, Taorong-type Baijiu is uniquely characterized by "yellowish and transparent color, delicate, mellow and harmonious flavor and lasting fragrance"^[2]. There is an old saying, namely, "pits throughout years and distillers' grains throughout centuries". Taorong-type Baijiu is fermented in a solid anaerobic environment^[3]. Pit mud is the basis for the fermentation of traditional solid-state Baijiu^[4]. As one of the crucial influencing factors, pit mud determines the quality and flavor of Baijiu produced to a great extent^[5], and plays a vital role in the brewing process of Taorong-type Baijiu^[6]. Besides, pit mud provides a comfortable habitat for fermentation microorganisms^[5], who need to grow and reproduce in pit mud^[7]. In addition, different microbial communities constitute a complex ecosystem^[8]. The flavor substances in Taorong-type Baijiu are mainly generated in pit mud. There are different living environments for the microorganisms in different spatial positions of the pits^[9]. Various microbial communities contribute to generating specific flavor compounds, which determine the flavor and quality of Baijiu^[10].

The microbial diversity of pit mud has a significant influence on the flavor of Baijiu^[11]. Bacteria and fungi are important functional flora, which can produce abundant enzymes and flavor substances contained in Baijiu, thus bestowing it with unique flavor^[12]. Bacterial metabolism in pit mud would generate important organic acids (such as butyric acid and caproic acid) in Taorong-type Baijiu, which will increase its flavor and reduce its stimulation. Fungi, such as *Saccharomyces cerevisiae* (*S. cerevisiae*) can be involved in

alcoholic fermentation, and *Aspergillus* can be involved in the generation of glucoamylase. The composition and quantity of microorganisms in pit mud are two of the factors affecting the flavor of Taorong-type Baijiu^[13]. Pit mud in different spatial positions could affect the reproduction and metabolism of microorganisms and the flavor composition of Taorong-type Baijiu^[14]. Therefore, it is necessary to explore the microbial community in pit mud.

Traditionally, isolation and culture methods are commonly adopted in the study of microorganisms^[15]. However, the species and quantity of isolated microorganisms are limited, many microbial species are usually missed, and even key functional microorganisms may be omitted^[16]. Due to the fact that the closed environment of pit mud is strictly anaerobic in essence, many microorganisms cannot be cultivated or it is difficult to cultivate them. The non-culture method can be employed to detect more microorganisms compared with the culture method, which objectively reflects the microbial composition of samples^[17]. In contrast, high-throughput sequencing (HTS) technology is the preferred method for the analysis of pit mud^[18, 19]. HTS methods have been extensively used, in an attempt to obtain a more comprehensive analysis of microbial diversity^[20]. HTS, also known as the next-generation sequencing (NGS) technology, features high throughput, high sensitivity, high resolution, low cost and simple operation, which can be employed to generate plenty of data in a short time^[21], and can comprehensively present the microbial community composition and diversity information of samples. Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) is an advanced technology^[22], which can be considerably effective in the analysis of volatile substances^[23]. Solid-phase microextraction (SPME) is a rapid analysis technology, which integrates the pretreatment, adsorption and extraction of samples. Owing to the emergence of different types of adsorbents with multiple polarities, it is possible to separate trace compounds with different substrates, which has been used in a series of fields. Besides, it is suitable for the detection and analysis of trace components, process monitoring and so forth in Baijiu and related production process^[24]. Gas chromatography-mass spectrometry (GC-MS) has been regarded as the gold standard for the analysis of many compounds^[25]. Further, it is a common and mature technique in the analysis of volatile and semi-volatile aroma components in the world. This technology has such advantages as high sensitivity, high separation efficiency, high selectivity and rapid analysis. It has a high and excellent performance in the separation, detection and data processing, and hence it can be applied in the accurate qualitative analysis^[2].

Currently, there is no report on the microbial community structure, volatile compounds and their correlation in the pit mud used for manufacturing Taorong-type Baijiu. Therefore, HTS technology was adopted in this study to analyze the microbial community structure of the upper, middle, lower and bottom layers of these pits used in manufacturing Taorong-type Baijiu. Moreover, the volatile compounds in the pit mud from different layers were investigated by a combined method with HS-SPME and GC-MS. Meanwhile, the correlation between the microbial community structure and volatile compounds was explored, which provided resources for establishing the microbial information database of Taorong-type Baijiu. Additionally, it could provide theoretical support for improving the quality of pit mud, cultivating

artificial pit mud, and improving the flavor and quality of Baijiu, which conduces to thoroughly understanding the contribution of microbes in pit mud to the flavor during the brewing of Baijiu.

Materials And Methods

Materials

The pit mud samples adopted in this experiment were from the 30-year-old pit mud in Henan Yangshao Liquor Co., LTD. Three pits were randomly selected, and 10g pit mud was collected from the center of the four pit walls of the upper layer (F-S, 50cm away from the pit mouth), the middle layer (G-Z, the pit center) and the lower layer (H-X, 50cm away from the pit bottom) of each pit, as well as the center of the bottom layer (I-D). Subsequently, they were mixed evenly. These collected samples were marked as F-S-1, F-S-2, F-S-3, G-Z-1, G-Z-2, G-Z-3, H-X-1, H-X-2, H-X-3, I-D-1, I-D-2 and I-D-3, respectively. They were stored in a refrigerator at -20°C^[26].

Reagents and Instruments

Reagent: D3141 HiPure Soil DNA Kits (Soil DNA Extraction Kit) were purchased from Guangzhou Magen Biotechnology Co., Ltd.; PCR related reagents were purchased from TOYOBO (SHANGHAI) BIOTECH CO., LTD.; AMPure XP magnetic beads were purchased from Beckman Coulter, USA; Anhydrous ethanol was purchased from Guangzhou Chemical Reagent Factory (GCRF); Agarose (BiowestAgarose) was purchased from Beijing Mengyimei Business Center; The goldview (Goldview I) was purchased from Beijing Mengyimei Business Center; NaCl was purchased from Tianjin Kemiou Chemical Reagent Co., Ltd.

Instruments: The centrifuge (Eppendorf 5427R) was purchased from Eppendorf AG, Germany; The pipette (Eppendorf) was purchased from Eppendorf AG, Germany; The ultrapure water instrument (Mingche TM-D) was purchased from RephiLe Bioscience Ltd., Shanghai; The refrigerator (-80°C) (DW-HL528S) was purchased from Zhongke Meiling Cryogenics Co., Ltd.; The vortex oscillator (mix-28+) was purchased from Guangzhou Wego Instrument Co., Ltd.; Nanorop spectrophotometer (NanoDrop 2000) was purchased from Thermo Fisher Scientific, USA; Agarose gel electrophoresis instrument (DYY-6C) was purchased from BEIJING LIUYI BIOTECHNOLOGY CO., LTD. Gel imaging system (Tanon-2500) was purchased from Tanon (Shanghai); PCR instrument (ETC811) was purchased from EASTWIN SCIENTIFIC EQUIPMENTS INC.; Qubit 3.0 was purchased from Thermo Fisher Scientific; Gas chromatography-mass spectrometry (GCMS-QP2010 Ultra) was purchased from Shimadzu, Japan; The solid-phase microextraction device was purchased from Merck & Co., Inc., USA.

Experimental methods

Sample DNA Extraction

According to the instruction manual for HiPure Soil DNA Kits (Soil DNA Extraction Kit) of Guangzhou Magen Biotechnology Co., Ltd., the genomic DNA of bacteria was extracted, and the DNA integrity was detected by 1% agarose gel electrophoresis.

PCR Amplification

The primers for bacteria were 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTATCTAAT-3'); The primers for fungi were ITS3_KYO2F (5'-GATGAAGAACGYAGYRAA-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3').

The First Round of Amplification

The first amplification system included 10×Buffer KOD, 5μL; 2mM dNTPs, 5μL; 25mM MgSO₄, 3μL; Primer F (10μM), 1.5μL; Primer R (10μM), 1.5μL; KOD enzyme, 1μL; Template, 4μL (100ng); H₂O, up to 50μL. The procedure for the first round of amplification was 94°C/2min, 98°C/10s, 62-66°C/30s, and 68°C/30s (30 cycles); 68°C/5min. The product purification after the first round of amplification: PCR product was purified by AMPure XP Beads and quantified by Qubit 3.0. After the first round of PCR amplification, the second round of PCR amplification was performed.

The Second Round of Amplification

The second amplification system included 10×Buffer KOD, 5μL; 2mM dNTPs, 5μL; 25mM MgSO₄, 1μL; Index Primer (10μM), 1μL; Universal PCR Primer (10μM), 1μL; KOD enzyme, 1μL; Template, 4μL (100ng); H₂O, up to 50μL. The procedure for the second round of amplification was 94°C/2min, 98°C/10s, 65°C/30s, and 68°C/30s (12 cycles); 68°C/5min. After the amplification, PCR products were detected by agarose gel electrophoresis.

Library Quantification and Sequencing

AMPure XP Beads were adopted to purify the products of the second round of amplification, and ABI StepOnePlus Real-Time PCR System (Life Technologies) was adopted to conduct the quantification. Relying on Guangzhou Gene Denovo Biotechnology Co., Ltd., the products were sequenced according to PE250 mode pooling of Novaseq 6000.

Pretreatment of Pit Mud Samples

1g pit mud was put into a headspace bottle, and 2g NaCl and 5mL distilled water were added. Subsequently, the stopper was tightened and the mixture was shaken properly.

Conditions of Headspace Solid-phase Microextraction (HS-SPME)

1g pit mud sample was weighed and placed in a headspace bottle, which was preheated in a water bath at 50°C for 10min. Solid-phase CAR/PDMS (75μm CAR/PDMS, carbon molecular sieve/polydimethylsilane) extraction fiber head was inserted into the silica gel stopper of the headspace bottle, and inserted into the sample for headspace adsorption for 30min.

Conditions of Gas Chromatography-Mass Spectrometry (GC-MS)

Conditions of gas chromatography: HP-FFAP chromatographic column (30m×0.32mm×0.25µm); No shunt, flow rate 1.21mL/min; Temperature at sample injection port: 250°C; Heating procedure: 40°C for 3min, 5°C/min for 60°C without holding, and 8°C/min to 230°C for 7min;

Conditions of mass spectrometry: The interface temperature was 220°C, the ionization mode is electron ionization (EI) source, the electron energy is 70eV, and the ion source temperature is 200°C.

Results

Basic Sequencing Data and Alpha Diversity Analysis

Table 1
Basic Sequencing Data of the Pit Mud from F-S, G-Z, H-X and I-D

Sample	16S				ITS			
	Effective sequence	Shannon index	Chao 1 index	Coverage rate	Effective sequence	Shannon index	Chao 1 index	Coverage rate
F-S-1	112576	5.2948	826	0.9976	128083	2.4599	86	0.9999
F-S-2	122754	5.3320	842	0.9978	120498	2.4471	91	0.9999
F-S-3	114750	5.3160	812	0.9979	120712	2.6153	92	0.9998
G-Z-1	127751	5.4721	785	0.9983	117690	3.1094	89	0.9998
G-Z-2	119701	5.4386	773	0.9982	117107	2.5827	87	0.9999
G-Z-3	119528	5.3970	762	0.9981	117972	2.2831	61	0.9999
H-X-1	117698	5.2033	815	0.9978	126473	3.6627	101	0.9998
H-X-2	116475	5.2284	754	0.9978	123248	3.5273	93	0.9999
H-X-3	113759	5.1491	768	0.9976	121489	3.4006	98	0.9999
I-D-1	125031	4.3747	815	0.9979	126599	3.1670	93	0.9999
I-D-2	120550	4.5248	800	0.9978	117278	3.2523	102	0.9999
I-D-3	120392	4.4931	804	0.9979	126217	3.1045	100	0.9999

Note: F-S: upper layer; G-Z: middle layer; H-X: lower layer; I-D: bottom layer.

As can be seen from Table 1, effective sequences are obtained by the quality control of original sequences and the removal of chimeras. The average remaining effective sequences of pit mud samples in F-S, G-Z, H-X and I-D are 116693, 122327, 115977 and 121991, respectively. The coverage rate is more than 0.99, thus indicating that the sequencing depth is sufficient, the sequences in the samples are

basically completely detected, and the results are true and reliable, which can be used for the subsequent analysis.

According to the Alpha diversity analysis of pit mud samples, Chao 1 index is mainly related to the abundance of samples, and the larger the index, the higher the abundance; Shannon index is mainly related to the diversity information of samples, which not only reflects the abundance of species, but also reflects the evenness of species. The larger the index, the higher the diversity. As can be seen from Table 1, in terms of the sequencing of 16S rDNA sequence, Chao 1 index can be ranked as F-S<I-D<H-X<G-Z, and Shannon index can be ranked as G-Z<F-S<H-X<I-D; In terms of the sequencing of ITS sequence, Chao 1 index can be ranked as I-D<H-X<F-S<G-Z, and Shannon index can be ranked as H-X<I-D<G-Z<F-S.

Venn Diagram Analysis

Figure 1 presents a Venn diagram based on OTUs (Operational Taxonomic Units). The overlapping part between graphs in the Venn diagram represents the number of common species, while the non-overlapping part represents the number of unique species of samples, which can clearly show the similarities and differences among samples. As can be seen from Fig. 3, the OTU number of the pit mud from F-S, G-Z, H-X and I-D are 691, 651, 629 and 662, respectively. The total number of common OTUs shared by four pit mud samples is 334, which indicates that 334 bacterial species exist simultaneously in the pit mud from F-S, G-Z, H-X and I-D; The number of unique OTUs in the pit mud from F-S, G-Z, H-X and I-D is 153, 120, 100 and 128, respectively.

Analysis of Bacterial Community Structure at the Phylum Level

As can be seen from Fig. 2, at the phylum level, with the relative abundance >0.5% as the threshold, there are 5 dominant bacterial phyla in the pit mud from F-S, including Firmicutes (52.8%), Bacteroidetes (29.6%), Synergistetes (6.4%), Chloroflexi (2.1%) and Spirochaetes (0.6%); There are 3 dominant bacterial phyla in the pit mud from G-Z, including Firmicutes (67.4%), Bacteroidetes (25.5%) and Synergistetes (0.9%); There are 5 dominant bacterial phyla in the pit mud from H-X, including Bacteroidetes (48.2%), Firmicutes (34.4%), Synergistetes (8.0%), Chloroflexi (2.7%) and Cloacimonetes (2.6%); There are 5 dominant bacterial phyla in the pit mud from I-D, including Firmicutes (66.7%), Bacteroidetes (14.7%), Synergistetes (10.3%), Kiritimatiellaeota (0.9%) and Chloroflexi (0.9%).

Analysis of Fungal Community Structure at the Phylum Level

As can be seen from Fig. 3, at the phylum level, with the relative abundance >0.5% as the threshold, the unclassified fungi (Unclassified) in the pit mud from F-S account for 0.06%, and there are 3 dominant fungal phyla, including Ascomycota (70.5%), Mucoromycota (28.9%) and Basidiomycota (0.6%); The unclassified fungi (Unclassified) in the pit mud from G-Z account for 0.2%, and there are 3 dominant fungal phyla, including Ascomycota (52.2%), Mucoromycota (44.6%) and Basidiomycota (3.0%). The unclassified fungi (Unclassified) in the pit mud from H-X account for 0.9%, and there are 3 dominant

fungal phyla, including Ascomycota (53.2%), Mucoromycota (44.1%) and Basidiomycota (1.8%); The unclassified fungi (Unclassified) in the pit mud from I-D account for 2.0%, and there are 3 dominant fungal phyla, including Mucoromycota (58.5%), Ascomycota (37.4%) and Basidiomycota (2.0%).

Analysis of Bacterial Community Structure at the Genus Level

As can be seen from Fig. 4, at the genus level, with the relative abundance >1.0% as the threshold, the unclassified bacteria (Unclassified) in the pit mud from F-S account for 14.02%, and there are 11 dominant bacterial genera, including *Hydrogenispora* (23.67%), *Petrimonas* (12.75%), *Caproiciproducens* (7.32%), *Proteiniphilum* (6.61%), *Ruminofilibacter* (5.15%), *Aminobacterium* (4.06%), *Lentimicrobium* (3.27%), *Christensenellaceae R-7 group* (2.60%), *Syner-01* (2.16%), *Sedimentibacter* (1.53%) and *Syntrophomonas* (1.49%); The unclassified bacteria (Unclassified) in the pit mud from G-Z account for 17.43%, and there are 11 dominant bacterial genera, including *Caproiciproducens* (28.00%), *Lactobacillus* (10.87%), *Lentimicrobium* (8.02%), *Petrimonas* (7.46%), *Proteiniphilum* (6.58%), *Fermentimonas* (2.13%), *Hydrogenispora* (1.64%), *Herbinix* (1.46%), *Caldicoprobacter* (1.22%), *Sedimentibacter* (1.12%), and *Syntrophomonas* (1.07%); The unclassified bacteria (Unclassified) in the pit mud from H-X account for 19.44%, and there are 9 dominant bacterial genera, including *Proteiniphilum* (16.10%), *Blvii28_wastewater-sludge group* (14.27%), *Petrimonas* (10.21%), *Aminobacterium* (7.78%), *Hydrogenispora* (7.63%), *Caproiciproducens* (3.92%), *LNR_A2-18* (2.67%), *Fermentimonas* (1.76%) and *Syntrophomonas* (1.62%). The unclassified bacteria (Unclassified) in the pit mud from I-D account for 21.54%, and there are 8 dominant bacterial genera, including *Hydrogenispora* (36.92%), *Petrimonas* (10.33%), *Aminobacterium* (10.04%), *Proteiniphilum* (3.22%), *Sedimentibacter* (1.98%), *Sporosarcina* (1.68%), *Syntrophomonas* (1.56%) and *Caproiciproducens* (1.31%).

Analysis of Fungal Community Structure at the Genus Level

As can be seen from Fig. 5, at the genus level, with the relative abundance >1.0% as the threshold, the unclassified fungi (Unclassified) in the pit mud from F-S account for 1.5%, and there are 4 dominant fungal genera, including *Thermomyces* (42.7%), *Rhizopus* (28.8%), *Aspergillus* (19.6%) and *Thermoascus* (3.7%); The unclassified fungi (Unclassified) in the pit mud from G-Z account for 18.7%, and there are 7 dominant fungal genera, including *Rhizopus* (44.0%), *Acremonium* (8.5%), *Cyphellophora* (7.4%), *Thermomyces* (5.3%), *Aspergillus* (4.6%), *Trichosporon* (3.3%) and *Thermoascus* (1.4%); The unclassified fungi (Unclassified) in the pit mud from H-X account for 8.3%, and there are 7 dominant fungal genera, including *Rhizopus* (43.8%), *Aspergillus* (31.0%), *Thermoascus* (4.1%), *Cladosporium* (1.6%), *Thermomyces* (1.6%), *Pseudeurotium* (1.3%) and *Penicillium* (1.2%); The unclassified fungi (Unclassified) in the pit mud from I-D account for 18.5%, and there are 5 dominant fungal genera, including *Rhizopus* (57.7%), *Aspergillus* (10.2%), *Thermoascus* (3.3%), *Penicillium* (1.2%) and *Hyphopichia* (1.0%).

Composition and Content of Volatile Compounds in Pit Mud

The composition and content of volatile compounds in pit mud are listed in Table 2.

Table 2
Composition and Content of Volatile Compounds in Pit Mud

Compound Type	No.	Compound Name	Relative Content/%			
			Pit mud from F-S	Pit mud from G-Z	Pit mud from H-X	Pit mud from I-D
Esters	1	Ethyl 2-hydroxypropionate	5.43	5.65	1.05	0.35
	2	Butyl caproate	0.55	0.12	0.05	0.7
	3	Hexyl butyrate	0.17	-	-	0.21
	4	Ethyl caprylate	1.47	0.64	0.16	3.07
	5	Isoamyl caproate	0.1	-	-	0.63
	6	Butyl lactate	0.46	-	-	-
	7	Hexyl hexanoate	0.3	0.27	0.27	10.68
	8	Ethyl caprate	0.14	0.33	0.1	0.44
	9	Diethyl succinate	0.34	0.33	0.08	0.02
	10	Ethyl methoxyacetate	0.28	0.24	0.04	0.07
	11	Ethyl phenylacetate	0.67	0.46	0.3	0.26
	12	Ethyl 3-Phenylpropionate	0.75	0.33	0.29	0.3
	13	Ethyl methyl-4-pentenoate	0.27	0.04	-	-
	14	Ethyl tetradecanoate	0.16	0.18	0.14	0.16
	15	Ethyl pentadecanoate	0.24	0.09	0.1	0.04
	16	Ethyl hexadecanoate	0.91	0.77	0.49	0.78
	17	Ethyl 9-octadecenoic acid	0.26	0.1	-	0.13
	18	Ethyl 9, 12-octadecadienoic acid	0.34	0.21	-	-
	19	Ethylene acetate	0.27	-	-	-
	20	Dibutyl phthalate	0.23	-	-	-
	21	Ethyl caproate	0.67	3.94	0.06	9.23
	22	Ethyl heptanoate	-	0.14	0.06	0.65
	23	Butyl 2-hydroxypropanoate	-	0.17	-	-

	24	Caproic acid 2,2-Dimethylhexanoic acid	-	0.1	-	-
	25	Ethyl 13-methyl-tetradecanoate	-	0.13	-	-
	26	Caproic acid 4-octyl ester	-	-	0.04	-
	27	Allyl 2-ethylbutyrate	-	-	0.2	-
	28	Ethyl tridecanoate	-	-	0.11	-
	29	Amyl hexanoate	-	-	-	0.66
	30	Propyl caprylate	-	-	-	0.06
	31	Ethyl nonanoate	-	-	-	0.12
	32	2-ethylbutyric acid, 4-heptyl ester	-	-	-	0.05
	33	Heptyl acetate	-	-	-	1.04
	34	N-hexyl caprylate	-	-	-	1.75
	35	Furfuryl acetate	-	-	-	0.05
	36	Allyl 2-ethylbutyrate	-	-	-	0.08
	37	Octyl pelargonate	-	-	-	0.1
	38	Octyl octanoate	-	-	-	0.07
	39	9-Hexadecenoicacid, ethylester	-	-	-	0.04
Alcohols	1	1-hexanol	2.56	1.83	0.41	0.95
	2	1-octanol	0.31	0.15	-	0.18
	3	6-hendecanol	0.09	-	-	-
	4	Benzyl alcohol	0.15	-	-	-
	5	Phenethyl alcohol	1.31	1.75	0.64	0.18
	6	Triethylene glycol	0.09	-	-	-
	7	1-butanol	-	0.63	-	-
	8	3-methyl-1-butanol	-	0.37	-	-
	9	1-decyl alcohol	-	-	-	0.04
Acids	1	2-methylpropionic acid	1.45	0.69	0.54	0.06
	2	Acetic acid	3.76	3.65	2.15	1.1

	3	Butyric acid	16.83	12.11	12.53	1.77
	4	3-methylbutyric acid	0.06	1.9	2.45	0.28
	5	3-methylpentanoic acid	1.94	-	-	-
	6	Valeric acid	2.78	3.06	11.28	0.93
	7	α -methyl phenylpropionic acid	0.05	-	-	-
	8	4-methylpentanoic acid	0.14	0.18	0.19	-
	9	Caproic acid	43.82	50.43	50.63	39.99
	10	Heptanoic acid	1.04	1.52	4.25	4.72
	11	Caprylic acid	1.86	2.22	3.57	14.36
	12	Nonanoic acid	0.1	0.07	0.15	0.44
	13	Capric acid	0.09	0.15	0.18	0.79
	14	Benzoic acid	0.33	0.11	0.11	-
	15	Tetradeconic acid	0.12	-	-	-
	16	Palmitic acid	2.36	0.14	0.11	-
	17	Propionic acid	-	0.06	0.58	0.07
	18	Phenylacetic acid	-	0.07	0.08	-
	19	2-methylbutyric acid	-	-	0.01	-
	20	2-methylpentanoic acid	-	-	0.04	-
	21	2-phenethyl hexanoic acid	-	-	-	0.19
	22	Cis10-heptadecenoic acid	-	-	-	0.14
Others	1	Phenol	0.19	0.32	0.3	0.32
	2	P-methylphenol	1.49	2.17	4.77	0.82
	3	Cresol	-	0.05	-	-
	4	3-methylphenol	-	-	-	0.03
	5	2,4-di-tert-butylphenol	-	-	-	0.16
	6	Tetramethylpyrazine	-	0.38	0.14	-
	7	D-limonene	-	0.16	-	-

A total of 78 volatile compounds are detected from the pit mud used for manufacturing Taorong-type Baijiu, including 39 esters, 9 alcohols, 22 acids and 7 others. Esters and acids are two dominant components in pit mud. There are significant differences in esters and acids, as well as their content in different layers of pit mud; while, the differences in alcohols are not significant. The total content of volatile compounds in pit mud shows an upward-downward-upward trend with the pit depth. There are 46 kinds of volatile compounds in the pit mud from F-S, 45 kinds from G-Z, 39 kinds from H-X and 49 kinds from I-D. Besides, there are also differences in the relative contents of various components in the pit mud from F-S, G-Z, H-X and I-D. Ester compounds are the most volatile compounds with the highest content and variety in pit mud, 39 of which are the main contributors to the aroma of Taorong-type Baijiu, and there are 21, 20, 17 and 28 kinds in the pit mud from F-S, G-Z, H-X and I-D, respectively. Ethyl caproate ranks first with respect to the content of esters, with its content in the pit mud from F-S, G-Z, H-X and I-D being 0.67%, 3.94%, 0.06% and 9.23% respectively. It is mainly generated under the synergistic action of various bacteria and enzymes^[11]. Ethanol and acetic acid combine to form butyric acid, followed by the synthesis of caproic acid through esterase. Subsequently, caproic acid is synthesized through ethanol^[27]. Ethyl caproate is considered as the key component contributing to the flavor and quality of Baijiu^[28]. Ethyl caprylate and ethyl heptanoate ranks second and third with respect to the content of esters. It can be found that the content of ethyl esters in pit mud is the highest, and there are 13, 15, 12 and 14 kinds of ethyl esters in the pit mud from F-S, G-Z, H-X and I-D, respectively, which are the main esters in Taorong-type Baijiu. There is no significant difference in ethanol compounds in pit mud, and 9 kinds of ethanol compounds are detected, including 6, 5, 2 and 4 kinds in the pit mud from F-S, G-Z, H-X and I-D, respectively. 1-Hexanol is the ethanol compound with the highest content in pit mud, with its relative contents in the pit mud from F-S, G-Z, H-X and I-D being 2.56%, 1.83%, 0.41% and 0.95%, respectively. There are abundant acid compounds in pit mud, and there are significant differences in different layers of pit mud. A total of 22 kinds of acid compounds are detected, including 16, 25, 17 and 13 kinds in the pit mud from F-S, G-Z, H-X and I-D, respectively. Among them, caproic acid, butyric acid, acetic acid, valeric acid, caprylic acid and heptanoic acid have a higher content. The content of caproic acid is extremely high, with its relative contents in the pit mud from F-S, G-Z, H-X and I-D being 43.82%, 50.43%, 50.63% and 39.99%, respectively. Under the coupling action of *Caproiciproducens* and Methanogen, acetic acid is produced by ethanol oxidation, and then ethanol reacts with butyric acid to produce caproic acid^[29]. Caproic acid and ethyl caproate produced by pit mud fermentation are the main aroma components of Taorong-type Baijiu^[11]. A large amount of caproic acid is produced during fermentation, and then reacts with ethanol to produce ethyl caproate, the main aroma component of Baijiu^[30]. Acetic acid, butyric acid, heptanoic acid and caprylic acid have a second higher content, and they are the main organic acid components of Taorong-type Baijiu.

Correlation between Microbes and Main Volatile Compounds in Pit Mud

Note

Z-A: ethyl 2-hydroxypropionate; Z-B: butyl caproate; Z-C: ethyl caprylate; Z-D: hexyl hexanoate; Z-E: ethyl caprate; Z-F: ethyl phenylacetate; Z-G: ethyl 3-phenylpropionate; Z-H: ethyl hexadecanoate; Z-I: ethyl caproate; Z-J: ethyl heptanoate; Z-K: heptyl acetate; Z-L: N-hexyl caprylate; C-A: 1-hexanol; C-B: 1-octanol; C-C: 6-hendecanol; C-D: benzyl alcohol; C-E: phenethyl alcohol; C-F: triethylene glycol; C-G: 1-butanol C-H: 3-methyl-1-butanol C-I: 1-decyl alcohol; S-A: 2-methylpropionic acid; S-B: acetic acid; S-C: butyric acid; S-D: 3-methylbutyric acid; S-E: 3-methylpentanoic acid; S-F: valeric acid; S-G: caproic acid; S-H: heptanoic acid; S-I: caprylic acid; S-J: nonanoic acid; S-K: capric acid; S-L: palmitic acid; Q-A: phenol; Q-B: p-methylphenol; Q-C: cresol; Q-D: 3-methylphenol; Q-E: 2,4-di-tert-butylphenol

The correlation analysis between the main volatile compounds and bacteria and fungi in pit mud was conducted respectively, and the correlation heat map was obtained. As can be seen in Fig. 6, the bacterial genera that are closely correlated with the main volatile compounds in pit mud include *Hydrogenispora*, *Aminobacterium*, *Lentimicrobium*, *Sedimentibacter*, *Ruminofilibacter*, *Christensenellaceae_R-7_group* and *Syner-01*. As can be seen from Fig. 7, the fungal genera that are closely correlated with the main volatile compounds in pit mud include *Rhizopus*, *Thermomyces*, *Monascus* and *Penicillium*. Ethyl caproate is the main aroma substance in Baijiu, which has the highest correlation with *Sedimentibacter* and *Monascus*, followed by *Hydrogenispora* and *Rhizopus*. *Sedimentibacter* can synthesize caproic acid, butyric acid, acetic acid, hexanol, ethanol and butanol with carbon source and protein as substrates, and can generate ethyl caproate. Acetic acid is positively correlated with *Syner-01*, *Ruminofilibacter*, *Lentimicrobium*, *Caproiciproducens* and *Thermomyces*, and negatively correlated with *Aminobacterium*, *Monascus* and *Penicillium*. Hexanol, octanol, 6-hendecanol and benzyl alcohol are positively correlated with *Syner-01*, *Christensenellaceae_R-7_group*, *Ruminofilibacter* and *Thermomyces*. Therefore, the microbial community structure in pit mud has a certain influence on the flavor and quality of Baijiu.

Conclusion And Discuss

In this study, HTS technology was employed to analyze the bacterial community structure of the pit mud used for manufacturing Taorong-type Baijiu in Henan Yangshao Liquor Co., LTD. The volatile compounds in the pit mud used for manufacturing Taorong-type Baijiu were analyzed qualitatively by a combined method of HS-SPME and GC-MS, in an attempt to understand the composition of volatile compounds in pit mud.

There are 5, 3, 5 and 5 dominant bacterial phyla in the pit mud from F-S, G-Z, H-X and I-D, respectively, and the common dominant bacterial phyla shared in all layers include Firmicutes, Bacteroidetes and Synergistetes. Firmicutes is the dominant bacterial phyla in the pit mud from F-S, G-Z and I-D, and Bacteroidetes is the dominant bacterial phyla in the pit mud from H-X. At the level of phylum, there is no significant difference in the bacterial community structure in pit mud at different spatial positions. With an increase in the depth of the pits, the content of Firmicutes shows an upward-downward-upward trend, reaching 66.7% in I-D. These bacteria have cell wall structure, and most of them can produce endophytic spores and resist extreme environments; Besides, the flora of Firmicutes has the functions of producing caproic acid and reducing lactic acid^[31]; The content of Synergistetes shows a decrease-increase trend,

with the highest content of 10.3% in I-D. These bacteria could degrade amino acids, and some of them are specific anaerobic. Deng et al.^[32] adopted HTS technology to explore the microbial community structure in the 5-year-old and 30-year-old pit mud from a wine company in Sichuan, and they found that Firmicutes, Bacteroidetes, Synergistetes, Spirochaetes and Chloroflexi were the dominant bacterial genera. There were 3 dominant fungal phyla in the pit mud from F-S, G-Z, H-X and I-D, including Ascomycota, Mucoromycota and Basidiomycota. It can be seen that, at the phylum level, there is the same structure composition of fungal phyla in different structure layers in pit mud. The content of Ascomycota and Mucoromycota is higher, and they are absolutely dominant fungal phyla in each layer of pit mud. On the whole, with an increase in the depth of pit mud, the relative abundance of Ascomycota shows a downward trend, while that of Mucoromycota shows an upward trend.

There are 11, 11, 9 and 8 dominant bacterial genera in the pit mud from F-S, G-Z, H-X and I-D, respectively. The dominant microbial genera shared in these layers include *Hydrogenispora*, *Petrimonas*, *Caproiciproducens*, *Proteiniphilum* and *Syntrophomonas*. *Hydrogenispora* is the absolutely dominant microbial genus in the pit mud from F-S and I-D; *Caproiciproducens* is the absolutely dominant microbial genus in the pit mud from G-Z; *Proteiniphilum* is the absolutely dominant microbial genus in the pit mud from H-X. *Syner-01*, *Pelotomaculum*, *Ruminofilibacter* and *Ruminiclostridium 9* are the uniquely dominant microbial genera in the pit mud from F-S; *Lactobacillus*, *Herbinix*, *Ruminiclostridium*, *Caldicoprobacter* and *Fastidiosipila* are the uniquely dominant microbial genera in the pit mud from G-Z; LNR_A2-18 and *Prevotella_7* are the uniquely dominant microbial genera in the pit mud from H-X; *Sporosarcina* is the uniquely dominant microbial genus in the pit mud from I-D.

At the genus level, there is spatial heterogeneity in the microbial community structure of pit mud at different spatial levels. *Hydrogenispora*, *Caproiciproducens*, *Sedimentibacter* and *Syntrophomonas* belong to Clostridia, Clostridia flora can synthesize caproic acid, butyric acid, acetic acid, hexanol, ethanol and butanol with carbon source and protein as substrates, and it can also generate ethyl caproate^[33]. In the pits, Clostridia and Bacteroidia have a considerably higher proportion (51.1% and 29.5%, respectively), and the content of Clostridia is the highest in the pit mud from I-D, reaching 63.86%, indicating that the tested pit mud samples are in the mature state. Clostridia can use ethanol and acetic acid to generate acetoacetic acid^[34], and it is one of the key microbial groups to promote the synthesis of short-chain and medium-chain fatty acids, such as butyric acid and caproic acid^[35]. These microbes can be easily screened from pit mud. *Hydrogenispora*, *Sedimentibacter*, *Petrimonas*, *Syntrophomonas* and *Aminobacterium* have a high content in the pit mud from I-D. *Hydrogenispora* can produce acetate, ethanol and H₂^[36]. *Sedimentibacter* and *Aminobacterium* can ferment amino acids^[33] to generate ammonium nitrogen through metabolism, which could provide nitrogen sources for the growth of other microbes^[37]. In addition, they have the function of degrading lactic acid; Most members of *Petrimonas* can use glucose to produce H₂ and CO₂, or acetic acid and propionic acid. *Aminobacterium*, *Syntrophomonas* and *Petrimonas* play a positive role in the maturing of pit mud^[38]. *Caproiciproducens* is a significant flora in pit mud. Due to the fact that the caproic acid produced by *Caproiciproducens* can inhibit lactic acid bacteria, and the quality of pit mud is closely correlated with pH, the degradation of

lactic acid bacteria can improve the quality of pit mud. *Lactobacillus* is the uniquely dominant bacterial genera in the pit mud from G-Z, and its metabolism can produce aroma substances of Nongxiang-type (strong aroma) Baijiu. However, the content of *Lactobacillus* in the pit mud from G-Z is too high, reaching 10.87%. The accumulation of lactic acid will increase the content of ethyl lactate in Baijiu and reduce its quality^[30]. Besides, the content of *Lactobacillus* in mature pit mud will decrease significantly^[39]. Hu et al.^[40] also found that the content of *Lactobacillus* in high-quality pit mud decreased significantly, and the content of such core bacteria as *Aminobacterium* increased significantly. Therefore, there should be a moderate amount of lactic acid bacteria in pit mud^[37].

There are 4, 7, 7 and 5 dominant fungal genera in the pit mud from F-S, G-Z, H-X and I-D, respectively. *Thermomyces* is the absolutely dominant fungal genera in the pit mud from F-S, and its content decreases with the depth increase of pit mud. *Rhizopus* is the absolutely dominant fungal genera in the pit mud from G-Z, H-X and I-D, and its content shows an upward trend with the depth increase of pit mud. *Thermomyces* is the dominant mold in the pit mud used for manufacturing Daqu jiu and the brewing environment. It has favorable comprehensive enzyme activity characteristics, can be used as an important microbial index for brewing environment evaluation, monitoring, storage period and quality evaluation of Daqu products^[41]. In addition, it has strong thermal stability and can maintain stable catalytic efficiency under the condition of high temperature in the fermentation process of Baijiu^[42]; *Rhizopus* is the main contributor to protein analysis in metabonomics. It plays a saccharification role by secreting three glycosidases and two glycosyltransferases^[43]. It is the crucial flora for the saccharification of distiller's yeast and contributes to improving the saccharification agent for food fermentation. *Aspergillus* has been extensively applied in the brewing industry. It has certain acid resistance and a strong ability to produce amylase, protease and metabolize organic acids, and could secrete glucoamylase^[44]. Therefore, it plays an important role in the production and aroma generation of Baijiu.

A total of 78 volatile compounds are detected from these pit mud samples, including 46, 45, 39 and 49 kinds in the pit mud from F-S, G-Z, H-X and I-D, respectively. These volatile compounds mainly include esters, acids and alcohols. Esters and acids are two main components in these pit mud samples. There are significant differences in esters and acids, as well as their content in different layers of pit mud; while, the differences in alcohols are not significant. Ethyl caproate, 1-hexanol and caproic acid are esters, alcohols and acids with the highest content in these pit mud samples, respectively. Meanwhile, the correlation analysis between microbes and volatile compounds in these pit mud samples is also conducted, and the correlation heat map could clearly present the correlation between microbes and compounds. Moreover, *Lentimicrobium*, *Syner-01* and *Blvii28_wastewater-sludge group* are found in the pit mud used for manufacturing Taorong-type Baijiu for the first time, which provides resources for establishing the microbial information database of Taorong-type Baijiu. Furthermore, the findings of this study also provide theoretical support for improving the quality of pit mud and enhancing the flavor and quality of Taorong-type Baijiu.

Declarations

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References

1. Xie, M. *et al.* High throughput sequencing of the bacterial composition and dynamic succession in Daqu for Chinese sesame flavour liquor[J]. *Journal of the Institute of Brewing*, **126** (1), 98–104 (2020).
2. Hu, X. *et al.* Profiling the composition and metabolic activities of microbial community in fermented grain for the Chinese strong-flavor Baijiu production by using the metatranscriptome, high-throughput 16S rRNA and ITS gene sequencings[J]. *Food Res. Int*, **138**, 109765 (2020).
3. Chai, L. J. *et al.* Mining the Factors Driving the Evolution of the Pit Mud Microbiome under the Impact of Long-Term Production of Strong-Flavor Baijiu[J].*Applied and Environmental Microbiology*, 2021, 87(17)
4. Tao, Y. *et al.* The functional potential and active populations of the pit mud microbiome for the production of Chinese strong-flavour liquor[J]. *Microb Biotechnol*, **10**, 1603–1615 (2017).
5. Liang, H. *et al.* 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]. *Journal of the Science of Food and Agriculture*, **95** (13), 2729–2735 (2015).
6. Chen, B. *et al.* A preliminary study of the calcification reason of pit mud in Chinese Strong-flavor liquor[J]. *BioRxiv*, **6** (3), 68–74 (2018).
7. Qian, W. *et al.* Cooperation within the microbial consortia of fermented grains and pit mud drives organic acid synthesis in strong-flavor Baijiu production[J]. *Food Res. Int*, **147**, 110449 (2021).
8. Liu, M. K. *et al.* Deep sequencing reveals high bacterial diversity and phylogenetic novelty in pit mud from Luzhou Laojiao cellars for Chinese strong-flavor Baijiu[J]. *Food Res. Int*, **12** (5), 326–330 (2017).
9. Hu, X. *et al.* The Effect of Spatial Location of Fermentation Pit on Prokaryotic Community Diversity in Pit Mud for Chinese Strong-Flavor Baijiu Production[J]. 2021
10. Xu, J. L. *et al.* Culturing Bacteria From Fermentation Pit Muds of Baijiu With Culturomics and Amplicon-Based Metagenomic Approaches[J]. *Frontiers in Microbiology*, **11** (8), 1223–1227 (2020).
11. Zhang, M. *et al.* Profiling the effects of physicochemical indexes on the microbial diversity and its aroma substances in pit mud[J]. *Letters in Applied Microbiology*, **71** (6), 667–678 (2020).

12. Wang, B. *et al.* Synergistic effect of multiple saccharifying enzymes on alcoholic fermentation for Chinese baijiu production[J]. *Applied and environmental microbiology*, **86** (8), e00013–20 (2020).
13. Hu, Y. *et al.* Microbial community changes during the mechanized production of light aroma Xiaoqu Baijiu[J]. *Biotechnology & Biotechnological Equipment*, **35** (1), 487–495 (2021).
14. Gao, J. *et al.* Domination of pit mud microbes in the formation of diverse flavour compounds during Chinese strong aroma-type Baijiu fermentation[J]. *LWT*, **137**, 110442 (2021).
15. Song, Z. *et al.* Unraveling core functional microbiota in traditional solid-state fermentation by high-throughput amplicons and metatranscriptomics sequencing[J]. *Frontiers in microbiology*, **8**, 1294 (2017).
16. Liu, P. & Miao, L. Multiple batches of fermentation promote the formation of functional microbiota in Chinese miscellaneous-flavor baijiu fermentation[J]. *Frontiers in microbiology*, **11**, 75 (2020).
17. Wang, X. *et al.* Bacterial diversity of Moutai-flavour Daqu based on high-throughput sequencing method[J]. *Journal of the Institute of Brewing*, **123** (1), 138–143 (2017).
18. Chen, L. *et al.* Analyzing bacterial community in pit mud of Yibin Baijiu in China using high throughput sequencing[J]. *PeerJ*, **8**, e9122 (2020).
19. Tan, G. *et al.* Analysis of bacterial communities in pit mud from Zhijiang Baijiu distillery using denaturing gradient gel electrophoresis and high-throughput sequencing[J]. *Journal of the Institute of Brewing*, **126** (1), 90–97 (2020).
20. Li, H. *et al.* Bacterial diversity in traditional Jiaozi and sourdough revealed by high-throughput sequencing of 16S rRNA amplicons[J]. *LWT-Food Science and Technology*, **81**, 319–325 (2017).
21. Georgiou, G. *et al.* The promise and challenge of high-throughput sequencing of the antibody repertoire[J]. *Nature biotechnology*, **32** (2), 158–168 (2014).
22. Chen, Q. *et al.* Characterization of volatile profile from ten different varieties of Chinese jujubes by HS-SPME/GC–MS coupled with E-nose[J]. *Food Res. Int*, **105**, 605–615 (2018).
23. da Silva, G. C. *et al.* Method development by GC–ECD and HS-SPME–GC–MS for beer volatile analysis[J]. *Food chemistry*, **167**, 71–77 (2015).
24. Sánchez-Palomo, E., Diaz-Maroto, M. C. & Perez-Coello, M. S. Rapid determination of volatile compounds in grapes by HS-SPME coupled with GC–MS[J]., **66** (5), 1152–1157 (2005).
25. Garcia, A. & Barbas, C. *Gas chromatography-mass spectrometry (GC-MS)-based metabolomics[M]//Metabolic Profiling* 191–204(Humana Press, 2011).
26. Gao, Z., Wu, Z. & Zhang, W. Effect of pit mud on bacterial community and aroma components in yellow water and their changes during the fermentation of Chinese strong-flavor liquor[J]. *Foods*, **9** (3), 372 (2020).
27. Liu, B., Chen, Y. L. & Yu, Z. L. Study on enzymatic preparation technology of Huang Shui esterification liquid. *J Chinese Ins Food Sci Technol*, **9**, 116–121 (2009).
28. Tan, G. *et al.* Analysis of bacterial communities in pit mud from Zhijiang Baijiu distillery using denaturing gradient gel electrophoresis and high-throughput sequencing[J]. *Journal of the Institute*

- of Brewing*, **126** (1), 90–97 (2020).
29. Deng, Y. *et al.* Mechanisms of the regulation of ester balance between oxidation and esterification in aged Baijiu[J]. *Scientific reports*, **10** (1), 1–7 (2020).
 30. Pang, X. N. *et al.* Influence of indigenous lactic acid bacteria on the volatile flavor profile of light-flavor Baijiu[J]. *LWT*, **147**, 111540 (2021).
 31. Sun, Z. *et al.* Prokaryotic diversity and biochemical properties in aging artificial pit mud used for the production of Chinese strong flavor liquor[J]. *3 Biotech*, **7** (5), 1–9 (2017).
 32. Deng, J. *et al.* Analysis of bacterial community structure in pits of Luzhou-flavor liquor based on high-throughput sequencing[J]. *Modern food technology*, **31** (7), 50–55 (2015).
 33. Zou, W., Ye, G. & Zhang, K. Diversity, function, and application of Clostridium in Chinese strong flavor baijiu ecosystem: a review[J]. *Journal of food science*, **83** (5), 1193–1199 (2018).
 34. Luo, Q. *et al.* Comparison between bacterial diversity of aged and aging pit mud from Luzhou-flavor liquor distillery[J]. *Food Science and Technology Research*, **20** (4), 867–873 (2014).
 35. Gao, J. *et al.* Domination of pit mud microbes in the formation of diverse flavour compounds during Chinese strong aroma-type Baijiu fermentation[J]. *LWT*, **137**, 110442 (2021).
 36. Chai, L. J. *et al.* Profiling the Clostridia with butyrate-producing potential in the mud of Chinese liquor fermentation cellar[J]. *International journal of food microbiology*, **297**, 41–50 (2019).
 37. Lu, M. *et al.* Profiling prokaryotic community in pit mud of Chinese strong-aroma type liquor by using oligotrophic culturing[J]. *International Journal of Food Microbiology*, **337**, 108951 (2021).
 38. Tao, Y. *et al.* Prokaryotic communities in pit mud from different-aged cellars used for the production of Chinese strong-flavored liquor[J]. *Applied and environmental microbiology*, **80** (7), 2254–2260 (2014).
 39. Zhang, H. *et al.* Prokaryotic communities in multidimensional bottom-pit-mud from old and young pits used for the production of Chinese Strong-Flavor Baijiu[J]. *Food chemistry*, **312**, 126084 (2020).
 40. Hu, X. *et al.* Illuminating anaerobic microbial community and cooccurrence patterns across a quality gradient in Chinese liquor fermentation pit muds[J]. *Appl Environ Microb*, **82** (8), 2506–2515 (2016).
 41. Deng, L. *et al.* Comparative Analysis of Physicochemical Properties and Microbial Composition in High-Temperature Daqu With Different Colors[J]. *Frontiers in microbiology*, **11**, 3010 (2020).
 42. Wang, H., Huang, Y. & Huang, Y. Microbiome diversity and evolution in stacking fermentation during different rounds of Jiang-flavoured Baijiu brewing[J]. *LWT*, **143**, 111119 (2021).
 43. Wang, B. W. *et al.* Specific Volumetric Weight-Driven Shift in Microbiota Compositions With Saccharifying Activity Change in Starter for Chinese Baijiu Fermentation[J]. *Frontiers in Microbiology*, **51** (24), 67–71 (2018).
 44. Aleem, B. *et al.* Random mutagenesis of super Koji (*Aspergillus oryzae*): improvement in production and thermal stability of α -amylases for maltose syrup production[J]. *BMC microbiology*, **18** (1), 1–13 (2018).

Figures

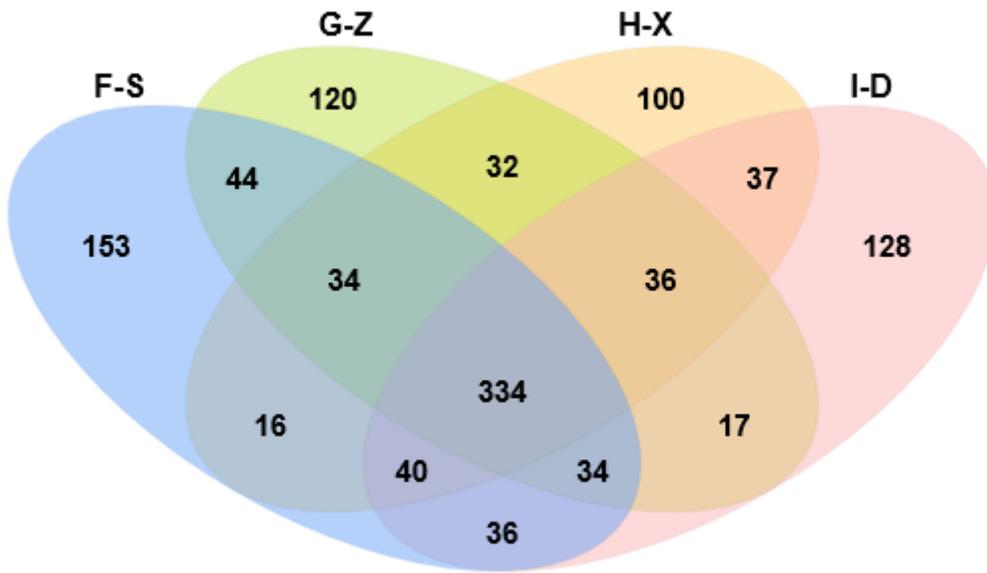


Figure 1

Venn Diagram of the Pit Mud from F-S, G-Z, H-X and I-D

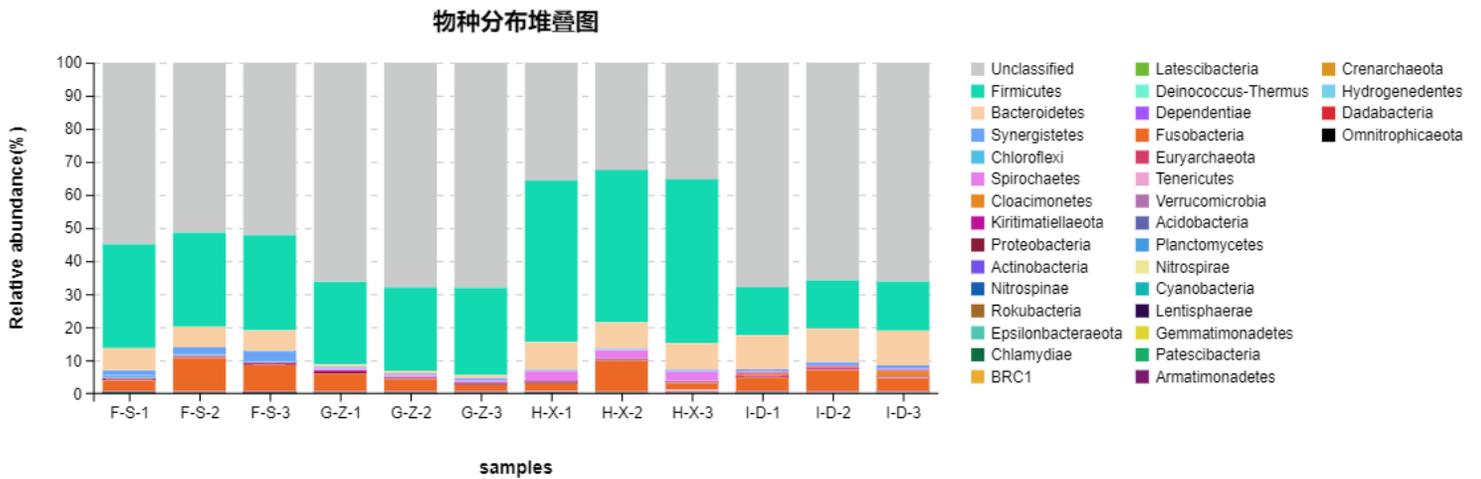


Figure 2

Stack Diagram of Species Distribution of Bacteria in the Pit Mud from F-S, G-Z, H-X and I-D at the Phylum Level

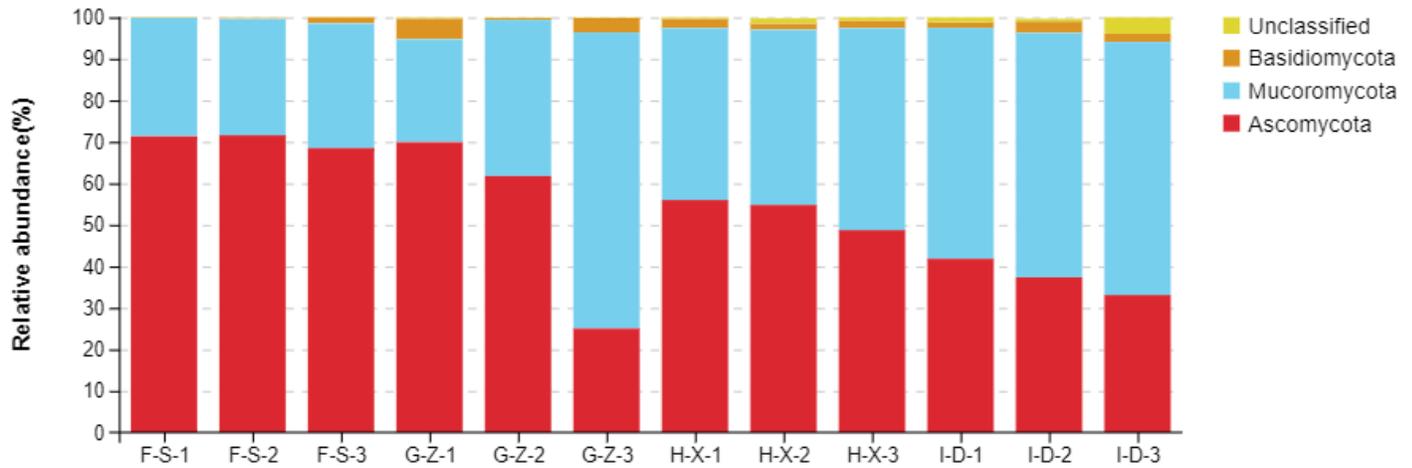


Figure 3

Stack Diagram of Species Distribution of Fungi in the Pit Mud from F-S, G-Z, H-X and I-D at the Phylum Level

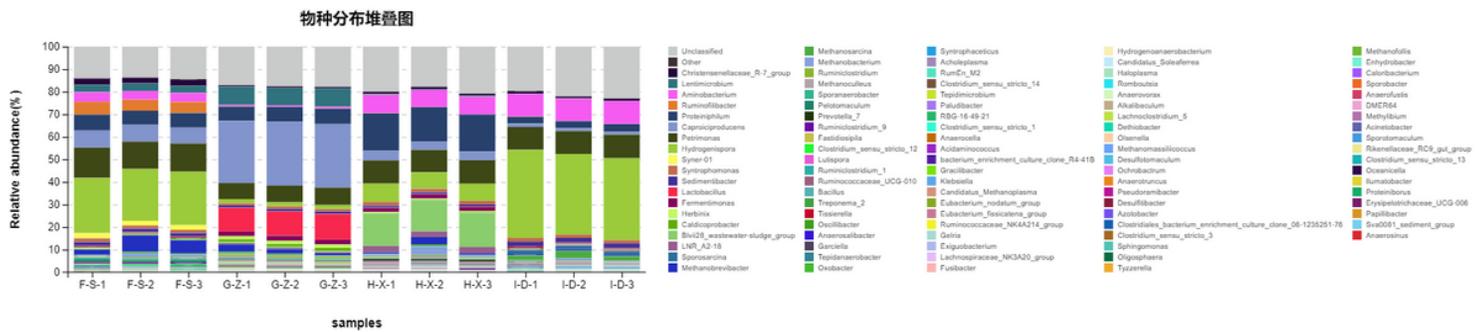


Figure 4

Stack Diagram of Species Distribution of Bacteria in the Pit Mud from F-S, G-Z, H-X and I-D at the Genus Level

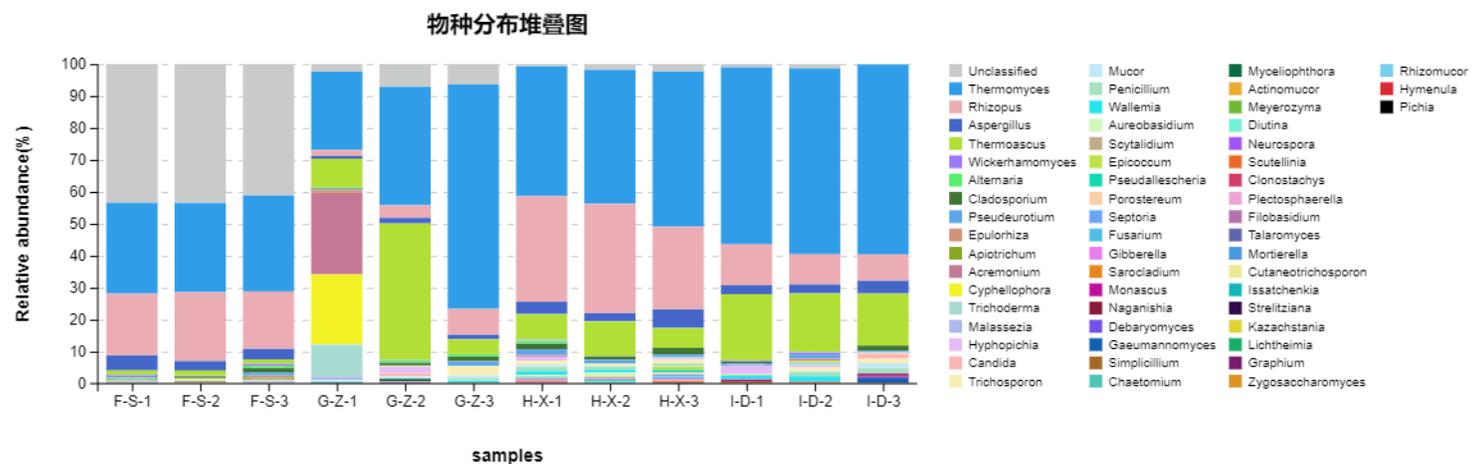


Figure 5

Stack Diagram of Species Distribution of Fungi in the Pit Mud from F-S, G-Z, H-X and I-D at the Genus Level



Figure 6

Heat Map of the Correlation between Bacteria and Main Volatile Compounds in Pit Mud

Figure 7

Heat Map of the Correlation between Fungi and Main Volatile Compounds in Pit Mud