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ORIGINAL ARTICLE

Bacterial diversity in the central black component of Maotai Daqu and its flavor analysis

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Abstract Maotai Daqu (MTDQ) is a kind of microflora starter for making the distilled liquor Maotai. It mainly contains bacteria, yeasts and moulds. When it comes to its central black component, it is nearly all bacteria. This study aims to clarify the bacterial diversity of the central black component of MTDQ, to analyze the role bacteria plays in Maotai liquor brewing, and to study the flavor components produced during the mixed solid-fermentation of three of the predominant flavor-producing bacteria so as to develop their practical application in other industries. Bacterial diversity was studied using amplified ribosomal DNA restriction analysis; *Actinobacteria* and *Firmicutes* were the predominant bacteria. The three flavor-producing strains were identified as *Bacillus subtilis*, *B. licheniformis* and *B. amyloliquefaciens*, which are the predominant bacteria. Their fermentation products include propanoic acid, 1,3-butanediol, acetic acid, methyl ester, etc. Most of which are analogous to the main flavor components of Maotai liquor. The results imply that the sample has diverse bacterial communities. Some bacteria play a very important role in the formation of the unique wine body composition of the Maotai liquor through their biotransformation, and some could be the main origins of the flavor of Maotai-type liquor.

Keywords Central black component of Maotai Daqu · Bacterial diversity · Culturable flavor-producing bacteria · Flavor components · Mixed solid-fermentation

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Introduction

Maotai-flavor liquor is often called the national drink of China. It is as symbolic as whisky is in Scotland, and brandy in France. It is described as highly complex-flavored, sweet and refreshing (Wang et al. 2008). The unique location of the town of Maotai itself, the regional environment and the high-temperature fermentation processes involved are prerequisites for production of Maotai liquor. Since the 1980s, many liquor manufacturers have visited Maotai factory to learn the Maotai production techniques but none have achieved their intended purpose. Namely, if one leaves Maotai, one can't produce Maotai-flavored liquor (Chen et al. 2013). Offsite tests have proved that only by closely integrating the unique brewing technology and unique natural environment can one brew the unique quality of Maotai liquor. In fact, Maotai-flavor liquor is distilled from the product of a special fermentation process using a wild microflora starter called Maotai Daqu (MTDQ)—a mixture of milled wheat and a complex microbial community. It mainly contains bacteria, yeasts and moulds (Wang et al. 2008; Liu et al. 2012). However, when it comes to its central black component, it is found to consist of nearly only bacteria (Jiang and Fan 2003). Furthermore, our preliminary experiments showed that the central black component of MTDQ usually possesses a bacterial community and better sauce flavor because of the higher temperature and

lower air permeability used. Though the central black component is very important for Maotai liquor production, studies about its composition, as well as the bacterial effects in this particular part, are rare.

Fan et al. (2006) separated 85 species of microorganisms from Maotai fermented grains using culturable methods. He speculated that at least 10 of the 85 species isolated were from MTDQ. Jiang (2004) and Liu et al. (2012) analyzed the microbial succession and their function in the fermentation processes, and proposed that MTDQ is the main saccharifying agent in Maotai liquor production. The substances and energy produced by the bacteria's physiological and biochemical metabolic activity were the main driving power behind fermentation; it was the key to forming the flavoring substances, and some of the bacteria were able to produce a variety of metabolic enzymes to make the flavors (Jiang 2004; Wu and Xu 2012). The results of the above studies showed that the bacterial populations in MTDQ play a very important role in the flavor formation and Maotai liquor production. Therefore, a comprehensive understanding of the bacterial diversity in MTDQ, especially the particular central black component and main functions in the fermentation, will help demystify the uniqueness of Maotai liquor. However, former studies have mainly analyzed the major microbial groups and their general succession in different production processes of Maotai liquor on a macroscopic scale using traditional methods. In-depth studies which specifically focus on the bacterial populations of MTDQ using molecular biology methods are, so far, few in number.

The microbial community in MTDQ is very diverse and largely non-cultivable. The traditional cultivation method can only reveal a fraction of the microbial species in MTDQ. Molecular biology methods can avoid the limitations of the traditional culture methods, and reveal the true microbial diversity. Among the molecular methods, 16S rRNA gene sequence analysis has been widely used in the literature (Liu et al. 2008). As microbial 16S rRNA gene sequences accumulate in public databases such as GenBank, the technology is becoming a powerful tool for bacterial identification, classification and community diversity analysis for environmental samples (Liu et al. 2008). Amplified ribosomal DNA restriction analysis (ARDRA) is a modern bioassay technology based on conservation of a prokaryote's rRNA sequences. Its principle is to digest amplified rRNA using restriction endonuclease followed by analysis of the bacterial diversity according to restriction maps (Vaneehoutte et al. 1992). As this method is fast, efficient, and has specific characteristics, it is extensively used to study microbial polymorphism.

The MTDQ used in Maotai brewing is often composed of white, yellow and black parts, each accounting for a certain proportion. The central black component of MTDQ is the product of high-temperature fermentation. It has almost no yeasts and moulds—only bacteria, adapted to the unique high-temperature, oxygen-deficient environment, are able to survive (Jiang and Fan 2003). Thus, a study of the bacterial diversity of the central black component of MTDQ could help to understand better the unique high-temperature production processes of Maotai liquor. In addition, screening the culturable flavor-producing bacteria and performing mixed solid-fermentation may help us to acquire an in-depth understanding of the role that the bacteria in the central black component of MTDQ play in the production of Maotai liquor. A bacterial diversity study individually targeting the central black component of MTDQ using molecular biological methods has not been reported at present.

In the present study, we aim to study comprehensively the bacterial diversity in the central black component of MTDQ. We analyze the connection between the flavor components of the Maotai-flavor liquor and the dominant flavor-producing bacteria (isolated from the central black component of MTDQ). Using the study, we hope to lay a theoretical foundation for further clarification of the role of the various types of bacteria in the central black component of MTDQ, to investigate the flavor-producing mechanism in Maotai liquor, and to demystify the uniqueness of Maotai liquor.

Materials and methods

Sampling

A few pieces of mature MTDQ were collected in November 2011 from the starter-making plant belonging to Guizhou Moutai Co. Ltd. (Maotai Town, Guizhou Province, China). They were placed in sterile bags, sealed, and stored in a refrigerator at 4 °C before analysis. Then, three pieces of MTDQ were chosen and put into the clean bench. The central black component was then sampled respectively. These three samples were used in the ensuing experiments.

Extraction of genomic DNA

Total genomic DNA was separately extracted from the three samples using an UltraClean soil DNA isolation kit (MoBio, USA) in accordance with the instructions in the kit (0.5 g MTDQ). Extracted DNA was mixed and stored in 50 μL of 10^4 pmol/μL Tris buffer at −20 °C.

PCR amplification and gel purification of bacterial 16S rDNA fragments

The universal bacterial PCR primers used for amplification were 16S-fD1: 5′-AGAGTTTGATCCTGGCTCAG-3′, and 16S-rD1: 5′-ACGGTTACCTTGTTACGACTT-3′ (Weisburg et al. 1991). The position of the primers were 8–27 and 1492–1512, respectively, and the length of the amplified fragments

were about 1500 bp. PCR was carried out in 50 μL reactions containing 5 μL of 10× buffer (10 pmol/μL of Tris–HCl pH 8.8; 50 pmol/μL of KCl), 2×10^3 pmol/μL of Mg^{2+}, 200 pmol/μL of dNTPs, 400 pmol/μL of upstream and downstream primers, 1 μL of template DNA (total DNA), 0.2 U/μL of TaqDNA polymerase, and 30 μL of sterile ddH_2O. The conditions used were as follows: first denaturation at 94 °C for 5 min, and then 10 cycles at 94 °C for 1 min, 65 °C down to 55 °C, each cycle decreased 1 °C for 1 min, 72 °C for 2 min; another 20 cycles at 94 °C for 1 min; 55 °C for 1 min, 72 °C for 2 min; 72 °C for another 10 min. PCR products were detected by electrophoresis in 1.0 % agarose gel.

Ligation, transformation, and colony PCR amplification

Purified PCR products were ligated into pGEM-Teasy vector (TaKaRa Inc.). The ratio of the concentration of target DNA and pGEM-Teasy vector was 2:1. The ligation reactions (10 μL) containing 5 μL of 2× rapid ligation buffer, 2 μL of the purified PCR product, 1 μL of pGEM-Teasy vector, 1 μL of T4DNA ligase, and 1 μL of ddH_2O, were incubated at 16 °C overnight. The plasmid carrying the target 16S rDNA fragment was cloned into *Escherichia coli* DH5α competent cells. After cloning, the recombinant transformants were screened using blue-white screening LB medium containing X-Gal, IPTG, and 0.8×10^3 pL/μL ampicillin, cultured at 37 °C for 12–16 h. Two hundred white colonies were picked and grown in LB liquid medium containing 0.8×10^3 pL/μL ampicillin, at 37 °C and 220 rpm overnight. Colony PCR was conducted using the cultured *E. coli* cells as template with universal primer T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ACGATTTAGGTGACACTATAG-3′) (Liu et al. 2008), under the same conditions as described above.

*Msp*I digestion and ARDRA analysis

Since different organisms have different nucleotide sequences, the numbers and the distances of the restriction sites of the sequences are different among different organisms. DNA fragments will generate polymorphisms after digestion using a specific kind of restriction endonuclease. The polymorphism in the DNA is shown by different banding patterns (which are also referred to as restriction maps or ARDRA patterns). The bands will be visualized by staining with ethidium bromide and using UV illumination during agarose gel electrophoresis. The clones that produce the same restriction maps (DNA fragments of the same size) after digestion using a specific restriction endonuclease are grouped together and counted as the same genotype. They are then classified as an operational taxonomic unit (OTU) (Díez et al. 2001; Liu et al. 2011).

*Msp*I restriction endonuclease has high specificity, high enzyme digestion frequency and produces extensive restriction fragmentation. Its recognition site is C↓CGG. The colony PCR products were digested by *Msp*I restriction endonuclease (TaKaRa Co.). *Msp*I enzyme digestion system, containing 1 μL of 10× T buffer, 5 μL of plasmid DNA, 5 U of *Msp*I enzyme, 1 μL of 1 % BSA, ddH_2O up to 10 μL, was incubated at 37 °C for 4 h, 65 °C for 20 min. The digested products were separated by electrophoresis in a 2 % agarose gel (Biowest, Spain), and visualized by a gel imaging system (LELDOC-II, UVP Inc.) to identify the various restriction patterns. All positive clones were analyzed and classified into a number of OTUs. The number of OTUs for sample and number of positive clones belonging to each OTU type were calculated, and one representative clone from each OTU was chosen for partial 16S rRNA gene sequencing.

DNA nucleotide sequence analysis

Nucleotide sequences of 16S rRNA gene clones were compared with those in the NCBI GenBank database by using the BLASTn searching tool. One to three gene sequences with the highest sequence similarity to the query sequence were retrieved from GenBank. The target 16S rRNA gene sequence and the retrieved sequences from the database were compared using the ClustalX program (Larkin et al. 2007). The sequences were identified with Chimera_Check (Cole et al. 2005) and removed if discovered. Some un-related sequences were discarded based on the preliminary phylogenetic analysis results. Finally, a streamlined group of sequences were again aligned by the ClustalX algorithm according to the default settings. Sequences were edited and the fuzzy sites were removed or corrected by reference to the secondary structure of 16S rRNA gene. Multiple sequence alignment was carried out and a neighbor joining (N-J) phylogenetic tree with 1000 bootstrap was then constructed using the Tamura-Nei model by Mega 4.0 (Tamura et al. 2007) package.

Statistical analysis

Coverage (*C*) of the constructed clone library was calculated using the formula $C = 1\text{-}nl/N$, where *nl* is the number of phylotypes that occurred only once in the clone library and *N* is the total number of clones analyzed (Jiang et al. 2009).

Isolation of flavor-producing strains and screening

Potato dextrose agar (PDA) medium and wheat medium (which contains half wheat flour, a total of 100 g, plus 50 % H_2O) were used to isolate culturable flavor-producing bacteria. The central black component of MTDQ was inoculated into sterilized PDA plating medium and the cultures were incubated at 50 °C for 24 h. A single colony from about 17

colonies was picked to do purification cultivation. This purified strain was added to the wheat medium and incubated in accordance with a temperature gradient of 37-46-55 °C for 6 days total (each gradient lasted for 2 days). The bacteria which produce flavor in this medium were selected as the flavor-producing strains (Xie et al. 1992; Lian 1995).

Species identification

A morphological observation was carried out. The screening strains were incubated at 37 °C and 120 rpm in liquid PDA medium for 2 days. The precipitate was collected and fixed using 2.5 % glutaraldehyde. These samples were used to do morphological observation using a scanning electron microscope (SEM).

Then, PCR amplification of the 16S rRNA genes and sequence analysis were performed. Single colonies were placed into 20 μL ddH_2O and mixed. The mixed liquid was taken as the PCR template directly. The universal bacterial PCR primers used for amplification were 16S-fD1 and 16S-rD1. PCR was carried out in 50 μL reactions containing 5 μL of 10× buffer (10pmol/μL of Tris–HCl pH 8.8; 50 pmol/μL of KCl), 2×10^3 pmol/μL of Mg^{2+}, 200 pmol/μL of dNTPs, 400 pmol/μL of upstream and downstream primers, 1 μL of template DNA (total DNA), 0.2 U/μL of TaqDNA polymerase, and 30 μL of sterile ddH_2O with the following conditions: first, denaturation at 94 °C for 5 min, and then 30 cycles at 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min; 72 °C for another 10 min. PCR products were detected by electrophoresis in 1.0 % agarose gel and used for nucleotide sequencing. The nucleotide sequences of the 16S rRNA genes were submitted to GenBank and were compared with those in the NCBI GenBank database using the BLASTn search tool. Then, the location of the strains in taxonomy was initially identified.

Gas chromatography–mass spectrometry (GC-MS) of the fermentation products

The three screened, flavor-producing strains were inoculated into the wheat medium at 10 % (each strain accounts for one-third of the total inoculation amount) and incubated using temperature gradients of 37-46-55 °C (2 days at each gradient). After two cycles, the flavoring substances were extracted. At the same time, samples without inoculating bacteria were taken as control blanks.

The flavoring substances were extracted using anhydrous ethanol. Anhydrous ethanol was added to submerge the fermentation medium and the sample shocked for 3 h. This operation was repeated three times and then all the extracts were combined together. Finally, the extracts were concentrated using a rotary evaporator and dried over anhydrous sodium sulfate.

The extracted compounds were separated on a DM-FFAP capillary column (30 mm×0.25 mm×0.25 μm). Split injection was employed for both distillate and extract samples with a ratio of 10:1. The column oven temperature was programmed to rise from an initial temperature of 60 to 240 °C at 5 °C/min. The injection temperature was 230 °C. Helium was used as the carrier gas with a flow rate of 1.0×10^3 μL/min. All data were obtained by collecting the full-scan mass spectra within the scan range 10–800 amu. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library. Compounds were identified using the NIST05 mass spectral library and retention indices.

Results

16S rRNA gene clone libraries

Two hundred clones were randomly selected for sequencing from the 16S rRNA gene clone library. One hundred and forty-one clones from the sample were identified containing the target inserts. *Msp*I restriction map analysis revealed a total of 14 OTUs (which means that 14 different genotypes were found among the 141 clones after the clones were digested by the *Msp*I restriction endonuclease). Among these, five OTUs were dominant types (because they had a large number, i.e. more than 10 clones). The largest OTU contained 34 clones, while six OTUs had only a single clone. The coverage (Good 1953) of the clone library was 95.7 %. These results suggest that the clone library had a high degree of clone coverage. In addition, the rarefaction curve for the 16S rRNA gene sequences from the clone library was saturated, indicating that the number of clones analyzed was sufficient to reflect the diversity and to determine bacterial community structure (Fig. 1).

Bacterial community structure

Plasmid DNA from the clones representing different ARDRA banding patterns was sequenced, and the nucleotide sequences submitted to GenBank (KC907877-KC907889). The 16S rRNA nucleotide sequences were compared with sequences in the NCBI GenBank database. The BLASTn results showed that the difference between the majority of our clone sequences and the related sequences currently in the NCBI database was less than 5 %. Multiple sequences were aligned by ClustalX using Mega 4.0 software. The results showed that the C+G contents of our sequences were slightly higher than A+T contents. Therefore, both nucleotide conversion/tranversion and variation were taken into account when the neighbor-joining phylogenetic tree for the sample (Fig. 2) was

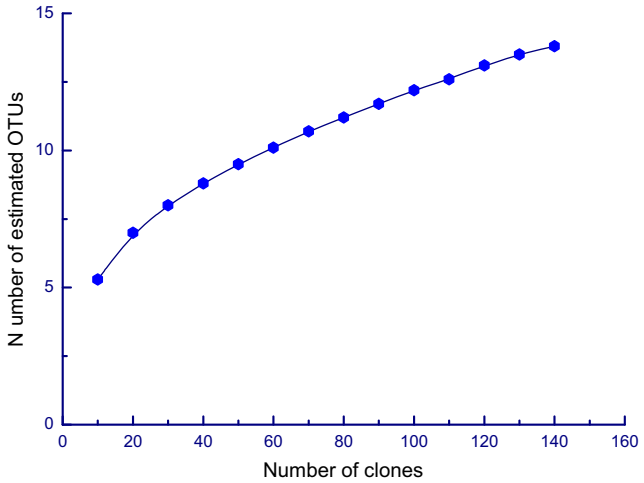

Fig. 1 Rarefaction curve for bacterial 16S rRNA gene clone sequences for the MTDQ sample showing that this curve is nearly saturated

constructed based on the OTUs using the Tamura-Nei model of Mega 4.0.

Figure 2 shows the N-J phylogenetic tree of the representative clones from the library and some reference bacterial 16S rRNA sequences retrieved from GenBank. It can be seen that the bacterial clones were classified into nine big clusters: *Thermoactinomyces*, *Saccharopolyspora*, *Bacillus*, *Staphylococcus*, *Lactobacillus*, *Micrococcus*, *Bacteroides*, *Flavobacterium*, and *Saccharomonospora*.

The number of clones is different among the nine clusters. *Bacillus* sp. carried the largest number of clones accounting for 45 % of the total. This is followed by *Thermoactinomyces* sp. at 24 %, *Saccharopolyspora* sp. at 21 %, and the remaining accounting for lesser proportions (Fig. 3).

Five OTUs, namely M3, M7, M4, M5 and M59 from the clone library, contain a large proportion of 16S rDNA clones, accounting for 24 %, 21 %, 21 %, 11 % and 10 % of the total clones, respectively. Based on the classification position in the N-J phylogenetic tree and the DNA sequence similarity, M3 belongs to *Thermoactinomyces vulgaris* of the class *Actinobacteria*, the phylum *Actinobacteria*; M7 is closely related to the genus *Saccharopolyspora* sp. of the class *Actinobacteria*, the phylum *Actinobacteria*; M4 can be assigned to the *Bacillus licheniformis* of the class *Bacillus*, the phylum *Firmicutes*; M5 is similar to *Bacillus amyloliquefaciens* of the class *Bacillus*, the *Phylum Firmicutes*; M59 belong to the *Bacillus subtilis* of the class *Bacillus*, the phylum *Firmicutes*.

Molecular identification and surface morphological observation of the culturable flavor-producing strains

Using a sensory evaluation, three of the strains isolated from the central black MTDQ were found to possess an obvious butter flavor, and hence were identified as the flavor-producing bacteria (Xie et al. 1992; Lian 1995). These three strains were identified as *B. licheniformis*, *B. amyloliquefaciens*, and *B. subtilis* by the 16S rRNA sequencing analysis. Their serial numbers are KC839816 (MT-1), KC839817 (MT-2) and KC839818 (MT-3), respectively. The three flavor-producing strains were also observed using SEM, as shown in Fig. 4.

Interpretation of the GC-MS results

Manual analysis and computer retrieval were used to determine the chemical structures of the corresponding spectra of each chromatographic peak. The compounds were thus identified and their corresponding relative contents were elucidated, as shown in Table 1.

Compared with the blank control group, Table 1 shows that the alcohol extracts from the experimental sample have a greater diversity of compounds. The experimental sample also had a higher percentage of many common compounds than the blank control, e.g., 2-butene, 2-methyl-; formic acid, 1-methylethyl ester; acetic acid, methyl ester; acetic acid; propanoic acid; 2,3-butanediol; acetic acid, diethyl-; 1,3-butanediol; pentanoic acid, 3-methyl-; acetic acid, diethyl-; phenylethyl alcohol; linoleic acid ethyl ester; propanedioic acid, phenyl-; tetradecanoic acid; pentadecanoic acid; isopropyl palmitate; heptadecanoic acid; octadecanoic acid; eicosanoic acid. The results show that the three strains produced novel compounds as well as increased the content of some common substances through their mixed solid-fermentation action. Most importantly, some of these compounds (acetic acid, methyl ester; acetic acid; propanoic acid; 2,3-butanediol; isopropyl palmitate; linoleic acid ethyl ester; phenylethyl alcohol) contribute to the key flavoring composition of Maotai liquor (Ji and Guo 2006).

Discussion

The analysis of the bacterial diversity in the sample showed that the dominant bacterial communities in the central black component of MTDQ were concentrated on the *Thermoactinomyces* sp. of the class *Actinobacteria*, the phylum *Actinobacteria*, *Saccharopolyspora* sp. of the class *Actinobacteria*, the phylum *Actinobacteria* and *Bacillus* sp. of the class *Bacillus*, the phylum *Firmicutes*.

T. vulgaris were predominant in the sample, accounting for 24 %. Many studies have shown that *T. vulgaris* can produce various enzymes, such as glucoamylase (Uotsu-Tomita et al. 2001), α-Amylase (Abe et al. 2005), carboxypeptidase T (Akparov et al. 2007), dehydrogenase, polyphenol oxidase, urease (Ke et al. 2010), and pullulanase (Shimura et al. 2011). Amylase can catalyze the hydrolysis of starch to produce dextrin, oligosaccharide, maltose and glucose (Wang et al.

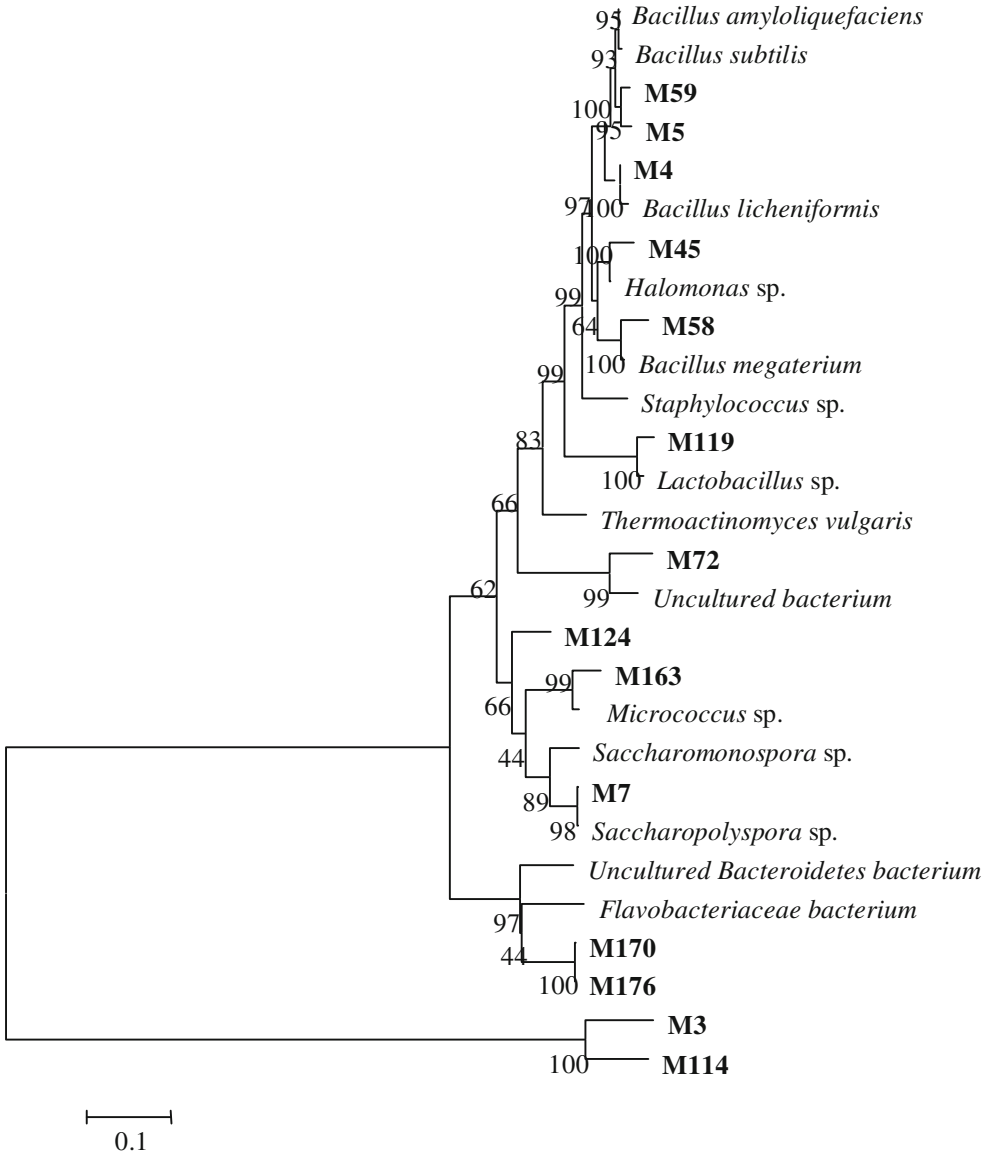

Fig. 2 A neighbor-joining phylogenetic tree showing the phylogenetic relationships of the bacterial 16S rRNA gene sequences cloned from the MTDQ sample to those related sequences from the GenBank database. The sequences obtained in this study are indicated in *bold*; M5, 58, . . ., 114 represent different clones of the sample. The reference sequences were from the GenBank database. Scale 0.1 represents the distance of evolution and 1,000 bootstrap replications were used

2010); pullulan-hydrolyzing amylase can hydrolyze the α-(1–6) glucosidic linkage of amylopectin, glycogen, and related macromolecular compounds to produce amylose, and also can make the starch completely amylolytic by synergistic reactions with other amylases (Ma et al. 2001); glucoamylase is a main starch-hydrolyzing enzyme and can produce glucose (Cao and Liu 2006); carboxypeptidase can hydrolyze protein to release free amino acids (Wu et al. 2012). The above analysis shows that decomposition of these enzymes can greatly reduce grain consumption, improve the utilization of macromolecules, meet the needs of their own growth and metabolism, and at the same time provide the precursor substances for the formation of a variety of flavoring substances, and consequently form the unique wine ingredients of Maotai.

Bacillus licheniformis made up 21 % of the sample. The extracellular products excreted by *B. licheniformis* show a strong enzymatic activity with respect to the proteases, lipase, thermostable α-amylase, cellulase, and hemicellulase. Cellulase and hemicellulase are mainly involved in the degradation of cellulose, starch, and wheat bran. Thermostable α-amylase enzyme is a novel liquefaction enzyme preparation, constituting the largest proportion of enzymes used in the fermentation industry, and widely used in beer brewing and alcohol industries. Application of thermostable α-amylase in the production of liquid yield can facilitate the separation of the waste mash steadily, which will help improve the quality of the wine base and improve the overall economic efficiency (Wang et al. 2010). In addition, it has also been reported that *B. licheniformis* has strong extracellular enzyme activity which degrades some complex plant carbohydrates, such as pectin, carboxymethylcellulose, and polygalacturonic acid (Sogarrd and Demark 1990). Yang et al. (2011) has preliminarily elucidated the contribution *B. licheniformis* makes to the Maotai flavor during the Maotai brewing process by

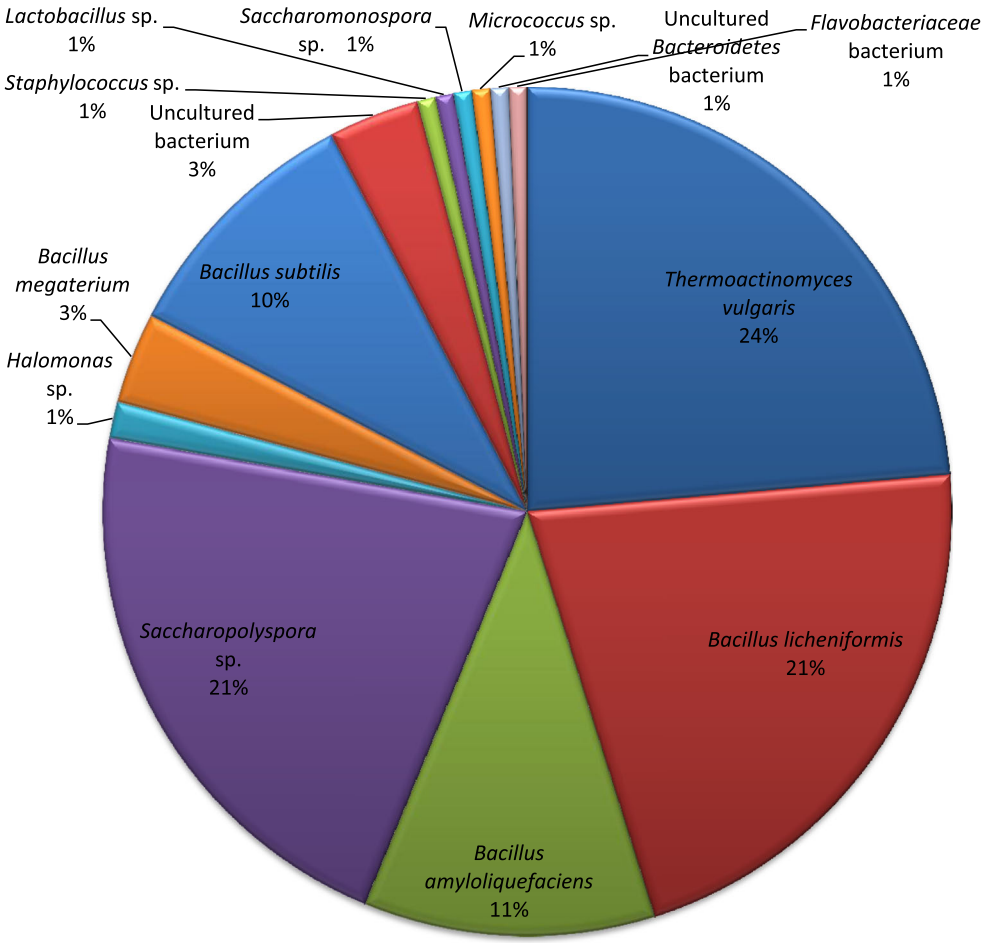

Fig. 3 The relative proportions of various bacterial groups in the MTDQ sample based on occurrence of clone sequences within that group

analyzing the solid-state fermentation metabolites. The results show that several of the substances produced by pure solid fermentation of *B. licheniformis* are flavors and precursor flavor substances in the food, which are also an integral part of the flavor-producing composition of Maotai (Yang et al. 2011).

Bacillus amyloliquefaciens is also a dominant group in the sample (accounting for 11 %). Shi et al. (1995) has pointed out that *B. amyloliquefaciens* can also produce α-amylase to make sugar ferment quickly and become fully decomposed into alcohols, esters, and various organic acids. Thus, the flavor substances can be refined and the flavor appeared to be much better.

Bacillus subtilis, accounting for 10 %, is another important group of bacteria in the sample. Former studies have reported that *B. subtilis* can produce dozens of enzymes (including proteases, α-amylase, cellulase, glucanase, phytase, pectinase and xylanase) to degrade the complex organic compounds in the raw MTDQ (Li 2009). For instance, cellulase can break down plant cellulose, which constitutes the walls of the plant's cells and is not easy to use, into readily absorbable glucose (Li 2009). In addition, major metabolites of *B. subtilis* contain 3-

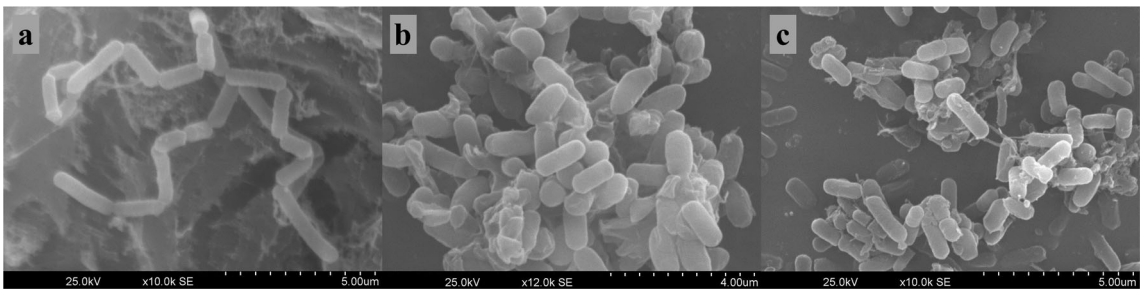

Fig. 4 SEM photographs of *Bacillus licheniformis* (**a**), *Bacillus amyloliquefaciens* (**b**), and *Bacillus subtilis* (**c**)

Table 1 Identified volatile compounds from the fermentation products

Compound	Peak area of the blank control	Relative content of the blank control	Peak area of the experimental sample	Relative content of the experimental sample
1,2-Benzenedicarboxylic acid, diisooctyl ester	24360	11.530 %	3277	0.245 %
1,2-Ethanediol	161	0.076 %		0.000 %
1,2-Ethanediol, monobenzoate	239	0.113 %	2767	0.207 %
1,3-Butanediol		0.000 %	144908	10.826 %
2,3-Butanediol	391	0.185 %	139295	10.406 %
2,5-Hexanedione, 3,4-dihydroxy-3,4-dimethyl-		0.000 %	36484	2.726 %
2(3H)-Furanone, dihydro-4-hydroxy-		0.000 %	5172	0.386 %
2-Butene, 2-methyl-	636	0.301 %	6821	0.510 %
2-Butenoic acid, 2-methyl-		0.000 %	1516	0.113 %
2-Cyclopentene-1,4-dione		0.000 %	3327	0.249 %
2-Furancarboxaldehyde, 5-(hydroxymethyl)-	17145	8.115 %		0.000 %
2-Furancarboxaldehyde, 5-methyl-	199	0.094 %		0.000 %
2-Furanmethanol, 5-methyl-		0.000 %	1680	0.126 %
2H-Pyran-2,6(3H)-dione		0.000 %	4155	0.310 %
2-Hydroxy-2-methylbutyric acid		0.000 %	160041	11.956 %
2-Methoxy-4-vinylphenol	1113	0.527 %	8602	0.643 %
3-[2-Diethylaminoethyl]-2,4-pentanedione		0.000 %	1479	0.110 %
3-Ethoxy-1,2-propanediol	7892	3.735 %	26508	1.980 %
3-Furancarboxylic acid		0.000 %	4848	0.362 %
3-Pyridinecarboxylic acid	892	0.422 %	5379	0.402 %
4-Ethoxyphenylhydrazine		0.000 %	451	0.034 %
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	983	0.465 %	16763	1.252 %
4H-Pyrido[1,2-a]pyrimidin-4-one, 3,6-dimethyl-		0.000 %	1729	0.129 %
4-Methoxycarbonyl-4-butanolide		0.000 %	3027	0.226 %
4-Pyridinecarboxamide		0.000 %	1929	0.144 %
5-Methyl-2-pyrrolidinone	206	0.098 %	694	0.052 %
5-Oxotetrahydrofuran-2-carboxylic acid, ethyl ester		0.000 %	1285	0.096 %
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-		0.000 %	11716	0.875 %
9,12-Octadecadienoic acid (Z,Z)-	64185	30.380 %	112224	8.384 %
9-Octadecenoic acid (Z)-, methyl ester	15445	7.310 %	79044	5.905 %
Acetamide, N-ethenyl-N-methyl-		0.000 %	494	0.037 %
Acetic acid, methyl ester		0.000 %	378	0.028 %
Acetic acid	6317	2.990 %	89747	6.705 %
Acetic acid, diethyl-		0.000 %	78557	5.869 %
Acetophenone	253	0.120 %	3044	0.227 %
Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	2097	0.993 %		0.000 %
Benzenamine, 4-methoxy-2-methyl-		0.000 %	1951	0.146 %
Benzene, 1,4-dimethoxy-2-methyl-		0.000 %	138	0.010 %
Benzene, 4-ethenyl-1,2-dimethoxy-	256	0.121 %	3053	0.228 %
Benzeneethanol, 3-methoxy-		0.000 %	3269	0.244 %
Butanamide, 3-methyl-		0.000 %	470	0.035 %
Carbonic acid, ethyl phenyl ester	177	0.084 %	4384	0.328 %
Dibutyl phthalate	3359	1.590 %	8661	0.647 %
Dimethyl trisulfide		0.000 %	631	0.047 %
DL-Alanine, N-acetyl-		0.000 %	5129	0.383 %
DL-Valine, N-acetyl-		0.000 %	1579	0.118 %
Eicosanoic acid		0.000 %	1896	0.142 %

Table 1 (continued)

Compound	Peak area of the blank control	Relative content of the blank control	Peak area of the experimental sample	Relative content of the experimental sample
Formic acid, 1-methylethyl ester	415	0.196 %	42965	3.210 %
Furan, 2-hexyl-	262	0.124 %		0.000 %
Guaiacol		0.000 %	383	0.029 %
Heptadecanoic acid		0.000 %	7200	0.538 %
Hexadecanoic acid, ethyl ester	275	0.130 %	534	0.040 %
Hexane-1,3,4-triol, 3,5-dimethyl-		0.000 %	40147	2.999 %
Hexanedioic acid, bis(2-ethylhexyl) ester	1872	0.886 %	5389	0.403 %
Indole		0.000 %	730	0.055 %
Isopropyl Palmitate		0.000 %	7635	0.570 %
Linoleic acid ethyl ester		0.000 %	1788	0.134 %
Maltol		0.000 %	5633	0.421 %
Methylenecyclopropanecarboxylic acid	61	0.029 %	408	0.030 %
n-Butyl myristate		0.000 %	4303	0.321 %
n-Hexadecanoic acid	48249	22.837 %	67446	5.039 %
Octadecanoic acid		0.000 %	4507	0.337 %
Pentadecanoic acid		0.000 %	9174	0.685 %
Pentanoic acid, 3-methyl-	82	0.039 %	32802	2.451 %
Pentanoic acid	167	0.079 %	333	0.025 %
Phenol, 3,5-dimethoxy-, acetate		0.000 %	3474	0.260 %
Phenylethyl alcohol	253	0.120 %	1686	0.126 %
Propanedioic acid, phenyl-	861	0.408 %	47639	3.559 %
Propanoic acid		0.000 %	1756	0.131 %
Propenylguaethol	5996	2.838 %		0.000 %
Pyrazine, 2,5-dimethyl-		0.000 %	431	0.032 %
Pyrazine, trimethyl-		0.000 %	942	0.070 %
Pyrazine, tetramethyl-	6477	3.066 %	32348	2.417 %
Pyridine, 1-acetyl-1,2,3,4-tetrahydro-		0.000 %	767	0.057 %
Pyridine, 3-phenyl-		0.000 %	1794	0.134 %
Tetradecanoic acid		0.000 %	3627	0.271 %

methyl-butyric acid, 2-methyl-propionic acid, 3-hydroxy-butanone, benzene, ethanol, 13-methyl-fourteen ethyl and hexadecyl ethyl (Yao et al. 2010). These substances are important flavor ingredients in Maotai.

Some species of *Saccharopolyspora* sp. are found to produce a number of enzymes involved in the catabolism of the macromolecular material found in MTDQ. For example, Chakraborty et al. (2011) isolated a strain of *Saccharopolyspora* sp. A9, which produces thermostable α-amylase to hydrolyze starch to glucose, maltose, and maltotriose. This function provides precursors for the formation of Maotai flavoring substances.

The results of our analysis indicate that the dominant bacteria in the central black component of MTDQ are mainly distributed in *Actinobacteria* and *Firmicutes*. These bacteria complete the complex metabolism of a variety of mixed microbial fermentation processes; enrich a large number of flavoring substances and their precursors of Maotai liquor; produce the unique wine ingredient and give Maotai liquor its unique style (sauce-flavor) and perfect quality through their mutual combination, repression and regulation of the different metabolic pathways in the production process. Furthermore, according to the literature, most of the compounds extracted from the experimental sample (Table 1) contribute to the main flavor components of Maotai liquor, and the total products extracted from the mixed solid-fermentation of the three flavor-producing strains cover almost all of the substances from the separate solid-fermentation of each single strain (Yang et al. 2011). This means that the three isolated strains are the dominant flavor-producing strains in MTDQ, and they produce a part of the flavor and also provide some precursors through their mixed solid-fermentation. However, the exact role of some of the rest of the flora is still not clear, and need to be further investigated in future studies.

Conclusions

Based on the above analysis and discussion, the main results are as follows:

The dominant bacteria of the central black component of MTDQ are mainly distributed in *Actinobacteria* and *Firmicutes*. Some bacteria play an important role in flavor-production and fermentation of Maotai liquor through metabolic enzymes, which contributes to the formation of the unique liquor body composition and unique style of Maotai, as well as its perfect quality.

Bacillus subtilis, *B. licheniformis*, and *B. amyloliquefaciens* belong to the dominant group of bacteria in the central black component of MTDQ. Most of the products excreted by the three strains through mixed solid-fermentation are the flavoring compounds in Maotai liquor. These three strains are the dominant culturable flavor-producing strains in the central black component of MTDQ.

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