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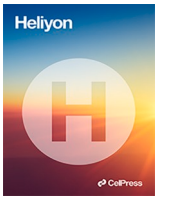

Research article

Analyzing the differences and correlations between key metabolites and dominant microorganisms in different regions of Daqu based on off-target metabolomics and high-throughput sequencing

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ABSTRACT

Daqu is usually produced in an open environment, which makes its quality unstable. The microbial community of Daqu largely determines its quality. Therefore, in order to improve the fermentation characteristics of Daqu, samples were collected from Jinsha County (MT1), Xishui County (MT2), and Maotai Town (MT3) in Guizhou Province to explore the microbial diversity of Daqu and its impact on Daqu's metabolites.Off-target metabolomics was used to detect metabolites, and high-throughput sequencing was used to detect microorganisms. Metabolomics results revealed that MT1 and MT2 had the highest relative fatty acid content, whereas MT3 had the highest organooxygen compound content. Principal component analysis and partial least squares discriminant analysis revealed significant differences in the metabolites among the three groups, followed by the identification of 33 differential metabolites (key metabolites) filtered using the criteria of variable importance in projection >1 and $p < 0.001$. According to the microbiological results, *Proteobacteria* was the dominant bacteria phylum in three samples. *Gammaproteobacteria* was the dominant class in MT1(26.84 %) and MT2(36.54 %), MT3 is *Alphaproteobacteria*(25.66 %). And *Caulobacteraceae* was the dominant family per the abundance analysis, MTI was 24.32 %, MT2 and MT3 were 33.71 % and 24.40 % respectively. Three samples dominant fungi phylum were *Ascomycota*, and dominant fungi family were *Thermoascaceae*. *Pseudomonas* showed a significant positive connection with various fatty acyls, according to correlation analyses between dominant microorganisms (genus level) and key metabolites. Fatty acyls and *Thermomyces* showed a positive correlation, but *Thermoascus* had the reverse relation. These findings offer a theoretical framework for future studies on the impact of metabolites on Baijiu quality during fermentation.

1. Introduction

One of the four most well-known distilled spirits in the world, Chinese liquor (termed baijiu), is the most popular, accounting for

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one-third of global consumption [1]. Baijiu fermentation involves complex fungi and bacteria, such as *Saccharomyces cerevisiae*, non-*Saccharomyces cerevisiae*, *Lactobacillus*, and *Bacillus* [2]. These microorganisms are mainly derived from jiuqu, which is the most basic and important component of the fermentation process. There are three primary categories of Jiuqu in Chinese liquor: Daqu, Xiaoqu, and Fuqu [3]. Among these, Daqu is utilized more frequently in the brewing of alcohols and is mostly employed to manufacture soy sauce, liquors with strong flavors, and liquors with mild flavors [4]. Daqu is typically used as a fermentation agent for the production of sauce-flavored liquor. It is made from wheat as the major raw material and is fermented in an open atmosphere, which requires one month of fermentation and three to six months of aging. During fermentation, many enzymes, microorganisms, and metabolites are produced, which are crucial for the development of the Baijiu flavor [5–7].

Microbial diversity was initially mainly based on traditional fermentation cultivation [8]. However, cultivable microbial technologies have limitations, which restrict their application in studying microbial diversity [9]. With the development of molecular biology, high-throughput sequencing (HTS) has been routinely used to detect the microbial structure of Daqu [10]. Research has shown that metagenomics methods that do not rely on cultivation reveal higher microbial diversity and improve our understanding of unique and diverse microbial communities [11]. Microorganisms can adapt to fluctuating environments through a series of cellular and molecular systems when facing complex heterogeneous environments or ecological disturbances [12]. Therefore, During the production process, different geographical locations can have an impact on the structure of microorganisms. For example, the aroma-producing functional microbiota in the Guizhou, Shandong, and Hubei regions was dominated by *Kroppenstedtia, Bacillus, Thermoascus, Virgibacillus,* and *Thermomyces;* Shanxi flavor Daqu was enriched with the microbiota of *S. cerevisiae* and *Lactobacillus* through fermentation; Henan Tao flavor Daqu was dominated by *Bifidobacterium* and *Saccharomycopsis*; and Sichuan Daqu was dominated by *Thermoascus, Lactobacillus,* and *Thermoactinomyces* [13].

In addition, most of the current studies on Daqu focus only on the microbial community structure; studies on the metabolites of Daqu from various geographic locations are rare, and correlation analyses between the two are rarely conducted. With the development of molecular biology, a more comprehensive and wider range of metabolite information can be obtained at the molecular level [14]. Through qualitative and quantitative analyses of low molecular weight metabolites in various samples, metabolomics can link differentially expressed metabolites to phenotypic changes. This method can identify metabolites that play important roles and investigate the causes of changes and has been used in an increasing number of studies to explore potential biomarkers [15–17]. It was discovered that the brewing process has an impact on the quantities of five of the recognized sake metabolites (aspartic acid, glutamic acid, methionine, cysteine, and -ethylglucoside), which are linked to the flavor and aroma of sake [18,19]. Nuclear magnetic resonance spectroscopy and mass spectrometry (MS) are the two main instrumental platforms used to evaluate metabolomics [20]. Nuclear magnetic resonance spectroscopy has a far lower sensitivity than that of MS, but it can provide extremely detailed structural information on compounds to help with the accurate identification of unidentified substances [21–23]. MS is frequently used in conjunction with several separation methods, including liquid chromatography (LC), gas chromatography, and capillary electrophoresis [24,25]. The most commonly used method in metabolomics, LC-MS, covers polar to nonpolar metabolites with the best coverage while avoiding chemical derivatization [26,27].

Zunyi, Guizhou, was chosen as one of the "Top 10 Spirits Producing Regions in the World" in 2018, making it one of China's leading manufacturers of alcoholic beverages [13]. To demonstrate the influence of various environments on the metabolites of Daqu and to improve the quality of Daqu in various regions, we measured the metabolites of Daqu in Jinsha County, Xishui County, and Maotai Town in Guizhou Province, as well as the dominant microorganisms in the three areas. The relations between dominant microorganisms and key metabolites were further studied by our team. Here, we provide an important basis for the further utilization of the microbial resources of Daqu in the Chishui River beach area.

Fig. 1. Geographic location of the three Daqu and their production process. MT1: Jinsha County, Guizhou Province; MT2: Xishui County, Guizhou Province; MT3: Moutai Town, Guizhou Province.

2. Materials and methods

2.1. Sample preparation of Daqu

The three kinds of Daqu were collected from Jinsha County, Guizhou Province (E105°47′–106°44′ and N27°07′–27°46′), Xishui County, Guizhou Province (E105°50′–106°44′ and N25°06′–28°50′), and Maotai Town, Guizhou Province (E106°22′ and N27°51′) (as shown in Fig. 1A). The three kinds of Daqu were randomly sampled from different parts, crushed, and mixed, then placed in sterile self-sealing bags, labeled as MT1, MT2, and MT3, and then stored at −40 °C for subsequent experimental measurements. The production processes of the three varieties of Daqu were essentially the same: solid organic wheat batches free of mold or insect damage were chosen, crushed, and mixed with 40 % water and muqu. The mixture was then stepped back into the shape of a tortoise and placed in a warehouse for stacking and fermentation, which took 40 d to complete. At this time, the temperature of the Daqu dropped, the hardness increased owing to its water reduction, and it showed an ochre-yellow color. After fermentation was completed, the stacks of Daqu were dismantled and put into a repository for storage, after which they were ground and used for the production of Baijiu (Please refer to Fig. S1 for the specific process).

2.2. Metabolomics experiments

The experimental process and parameter settings are based on the literature [28], as follows: 100 mg of MT1, MT2, and MT3 samples were weighed accurately in a 2 mL centrifuge tube, added with glass beads and 1000 μL of 50 % methanol in water, and vortexed for 30 s. The tube was then placed in an adapter, immersed in liquid nitrogen for 5 min, removed, thawed at about 25 °C, replaced in the adapter, mounted on a tissue grinder, and ground at 55 Hz for 2 min. After repeating this procedure three times, the tubes were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was concentrated and dried. The dried sample was reconstituted by adding a 2-phenylalanine (4 ppm) solution, and then the supernatant was filtered through a 0.22 μm membrane and the filtrate was used for LC-MS detection.

The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA).

Chromatography was carried out with an ACQUITY UPLC® HSS T3 (150 × 2.1 mm, 1.8 μm) (Waters, Milford, MA, USA). The column was maintained at 40 °C. The flow rate and injection volume were set at 0.25 mL/min and 2 μL, respectively. For LC-ESI (+)-MS analysis, the mobile phase consisted of (C) 0.1 % formic acid in acetonitrile (v/v) and (D) 0.1 % formic acid in water (v/v). Separation was conducted under the following gradient: 0–1 min, 2 % C; 1–9 min, 2–50 % C; 9–12 min, 50–98 % C; 12–13.5 min, 98 % C; 13.5–14 min, 98–2% C; and 14–20 min, 2 % C. For LC-ESI (−)-MS analysis, the analytes were carried out with (A) acetonitrile and (B) ammonium formate (5 mM). Separation was conducted under the following gradient: 0–1 min, 2 % A; 1–9 min, 2–50 % A; 9–12 min, 50%–98 % A; 12–13.5 min, 98 % A; 13.5–14 min, 98%–2% A; 14–17 min, 2 % A.

Mass spectrometric detection of metabolites was performed on a Q Exactive (Thermo Fisher.

Scientific, USA) equipped with an ESI ionization source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisitions were performed. The parameters were as follows: sheath gas pressure, 30 arb; aux gas flow, 10 arb; spray voltage, 3.50 kV and −2.50 kV for ESI(+) and ESI(−), respectively; capillary temperature, 325 °C; MS1 range, m/z 81–1000; MS1 resolving power, 70,000 FWHM; number of data-dependent scans per cycle, 10; MS/MS resolving power, 17,500 FWHM.

After the raw data were obtained, they were converted into mzXML format using Proteowizard software. The XCMS program package of R was used to perform peak identification, peak filtration, and peak alignment, and a data matrix was obtained, including mass-to-charge ratio (m/z), retention time, and intensity.

2.3. DNA extraction and sequencing

Using the Zymo Research BIOMICS DNA Microprep Purification Kit (Cat #D4301) for gDNA purification, gDNA integrity was detected using 0.8 % agarose electrophoresis (see Fig. S2 for gel electrophoresis), followed by nucleic acid concentration detection using Tecan F200 (PicoGreen dye method.

The V4 hypervariable region of the bacterial 16S rRNA gene and the ITS2 region of the fungus were analyzed by the Applied Biosystems® PCR System 9700 Thermal Cycler using primer pairs 515 F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′) as well as ITS3 (5′-GATGAAGAACGYAGYRAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATATGC-3′). PCR amplification was performed as follows: pre-denaturation 94 °C for 1 min, one cycle; denaturation 94 °C for 20 s, annealing 54 °C for 30 s and extension 72 °C for 30 s, 25–30 cycles; 72 °C for 5 min, one cycle; and holding time at 4 °C. Three technical replicates were performed for each sample and equal amounts of linear-phase PCR products were mixed for subsequent library construction. The PCR products were mixed with six times the sampling buffer, followed by electrophoretic detection of the target fragments using a 2 % agarose gel. Samples that passed the assay were used as destination strips for recovery using the Zymoclean Gel Recovery Kit (D4008) and quantified using a Qubit 2.0 Fluorometer (Thermo Scientific). Equimolar amounts were mixed. After library construction, high-throughput sequencing was performed using the PE250, HiSeq Rapid SBS Kit v2 (FC-402-4023,500 cycle).

Each sample sequence was split from the raw reads based on the barcode using Sabre, and the barcode sequence was truncated. Subsequently, we used QIIME2(QIIME2 version information: 2020.2) for quality control with the following standards [29]: sequences with an average quality <30 bp were filtered out, sequences with a length <200 bp were removed; sequences with more than zero fuzzy bases (N) were removed. Next, QIIME2 was used to perform sequence denoising and chimerism removal on the sequence based on the deblur algorithm, generating an ASV feature table and a feature sequence. A species classification dataset for the SILVA database

was constructed using a classifier based on the Naive Bayes algorithm, and we used this dataset to annotate species in ASV feature sequences. QIIME2 was used for multiple alignments of the feature sequences, and an evolutionary tree was constructed using the built-in FastTree plugin.

Both community composition and Alpha diversity analyses were conducted using R. Alpha diversity index was analyzed by the Vegan package for numbers.The Wilcoxon rank sum test uses the Wilcox.test function of the stats package, and the Kruskal.test function is used for the Kruaksl Wallis rank sum test of two groups. Multiple comparisons using Agricola package.

2.4. Statistical analysis

All tests were repeated at least three times and the data were analyzed using SPSS (Version 8.0; Origin Lab, USA). Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and variable importance in projection (VIP) between the chemical constituents of the samples were performed using the SIMCA-P software (Version 14.1; Umetrics, Umea, Sweden). A heat map was drawn online using Chiplot.

3. Results and discussion

3.1. Metabolite identification

We utilized an LC-MS non-targeted approach for the three kinds of Daqu, and the data obtained were analyzed using bioinformatics, resulting in the identification of a total of 230 metabolites. The metabolite types included 55 carboxylic acids and derivatives, 37 organooxygen compounds, 28 fatty acids, and 28 benzene and substituted derivatives (Fig. 2A). As shown in Fig. 2B, the metabolites with high relative abundances in MT1 were fatty acyls, benzene and its substituted derivatives, and organooxygen compounds, accounting for 51.62 %, 10.97 %, and 11.70 %, respectively. Similarly, the metabolites with the highest relative abundance in MT2 were fatty acids (50.25 %), followed by carboxylic acids and derivatives (16.67 %) and organooxygen compounds (8.93 %). The highest relative content in MT3 was organooxygen compounds (30.81 %), followed by carboxylic acids and derivatives (18.74 %) and others (11.83 %), including 2-naphthylamine, epsilon-caprolactam, gabapentin, and enoxacin. In conclusion, it can be tentatively concluded that there are differences in the metabolite composition of Daqu from the three regions.

3.2. Marker compounds

PCA can visualize the overall distribution of samples and is widely used as an unsupervised pattern-recognition method. To further investigate the differences in metabolites among the three groups, we performed PCA (Fig. 3A). Principal components 1 and 2 accounted for 70.1 % and 22.6 % of the total variance (92.8 %), respectively. The PCA clearly distinguished the three groups, indicating that the metabolites in the different regions of Daqu differed significantly. The dots representing parallel samples within the group were not clearly dispersed, indicating that the experiment was reproducible without any obvious bias. However, PCA may lead to misclassification, and data must be further analyzed using supervised PLS-DA [30]. PLS-DA is a multivariate statistical method that

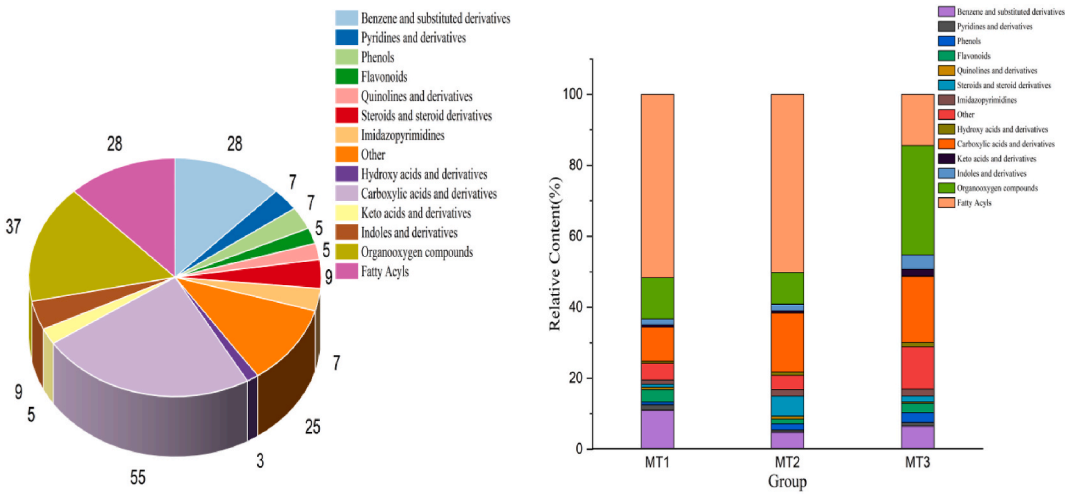

Fig. 2. Pie chart of the amount of each type of metabolite (A). Histogram of the relative content of each type of metabolite (B). MT1: Jinsha County, Guizhou Province; MT2: Xishui County, Guizhou Province; MT3: Moutai Town, Guizhou Province.

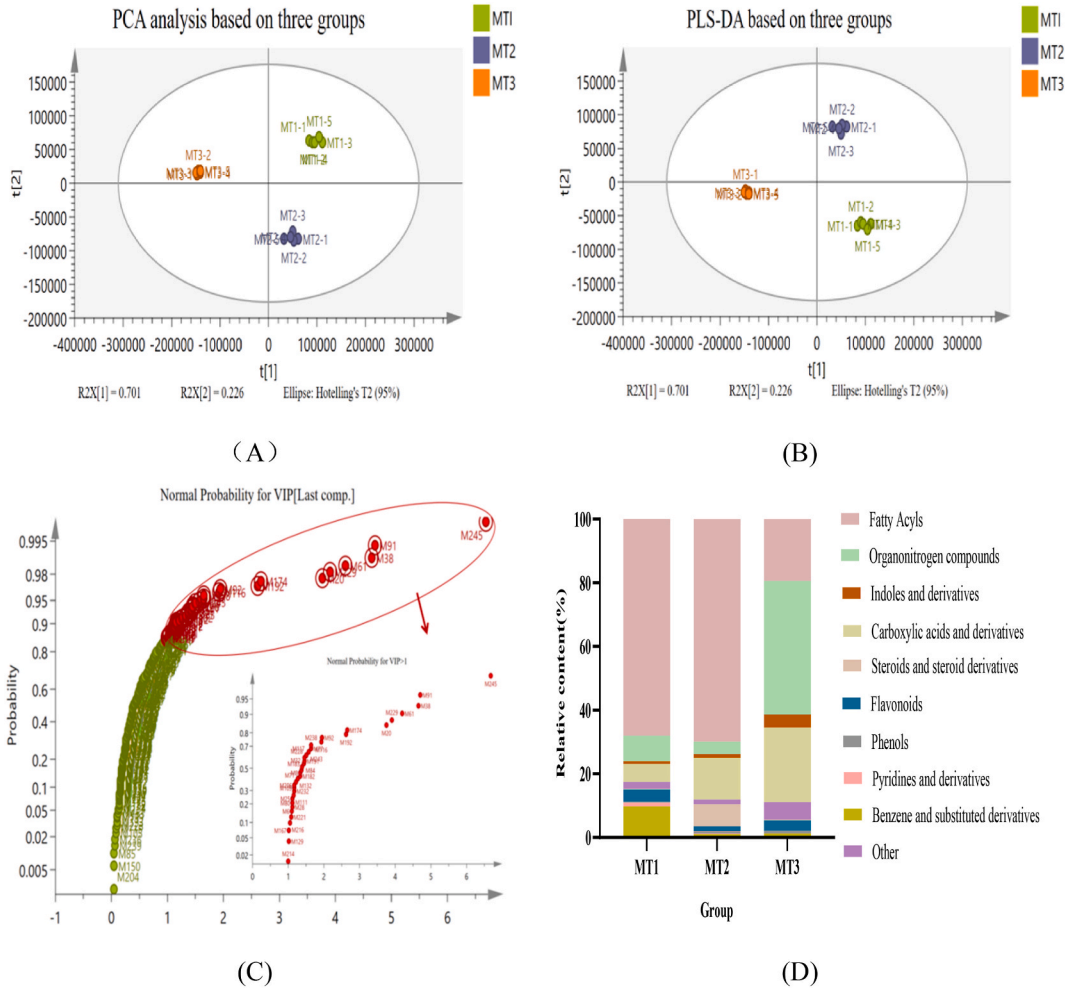

Fig. 3. Principal component analysis (PCA) of metabolic data (A). Partial least squares analysis of metabolic data (B). The partial least squares discriminant analysis (PLS-DA) diagram of all compounds. Red dots represent key metabolites, with greater variable importance in projection (VIP) indicated by greater distance from the origin (C). Key metabolites classification chart (D). MT1: Jinsha County, Guizhou Province; MT2: Xishui County, Guizhou Province; MT3: Moutai Town, Guizhou Province. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

allows for the regression modeling of multiple independent variables and can accurately identify the key variables that affect a group and further screen for key metabolic products [31]. As shown in Fig. 3B, MT1, MT2, and MT3 had completely separated score plots, which was consistent with the PCA results. The model had excellent fitting parameters ($R^2X = 0.941$, $R^2Y = 0.998$, and $Q^2 = 0.995$), which proved that the developed model conformed to the real sample data. The VIP and *p*-values were analyzed to identify the key metabolites responsible for the metabolic changes between the different samples (Fig. 3C). Compounds with VIP >1.0 and $p < 0.05$ were considered to be significantly different [32]. As shown in Fig. 3C and Table 1, 33 key metabolites that are important for recognizing Daqu from different regions were screened. The key metabolites included benzene and its substituted derivatives, pyridines and derivatives, phenols, steroids and steroid derivatives, carboxylic acids and derivatives, indoles and derivatives, and organooxygen compounds. The relative abundance of each metabolite in different types of Daqu is shown in Fig. 3D.

3.3. Heatmap analysis

Heatmaps were used to visualize the high and low levels of each key metabolite in the three samples, where each column represents a parallel sample of Daqu and each row represents a key metabolite (Fig. 4). The d-arabitol, 2-naphthylamine, pyridoxamine, 2-isopropylmalic acid, palmitoleic acid, and arabitol contents were high in MT1. Among these, 2-isopropylmalic acid is a precursor for leucine biosynthesis in *S. cerevisiae*, which is isomerized in the cytoplasmic lysate to 3-isopropylmalic acid, which is further converted to leucine [33]. Leucine is a taste-active substance that significantly contributes to wine [34]. Palmitoleic acid is a monounsaturated fatty acid that can be converted into volatile esters to improve wine [35]. Arabitol is a sweetener that imparts mild sweetness to food

Table 1
Critical VIP compounds of three samples.

NO.	Compounds	MZ	RT	CAS	formula	Precursor_type	VIP	*P*
M38	2′,4′-Dihydroxyacetophenone	153.1013	338.2	89-84-9	$C_8H_8O_3$	[M+H]+	4.65	<0.001
M111	Procaine	237.1594	328.9	59-46-1	$C_{13}H_{20}N_2O_2$	[M+H]+	1.15	<0.001
M77	Pyridoxamine	169.0973	232.6	85-87-0	$C_8H_{12}N_2O_2$	[M+H]+	1.64	<0.001
M6	m-Cresol	109.0229	938.7	108-39-4	C_7H_8O	[M+H]+	1.10	<0.001
M174	Lycopene	536.4366	866.3	502-65-8	$C_{40}H_{56}$	[M]+	2.66	<0.001
M61	Dehydroepiandrosterone	288.2887	681.1	53-43-0	$C_{19}H_{28}O_2$	[M]+	4.19	<0.001
M216	17a-Estradiol	271.2259	810.9	57-91-0	$C_{18}H_{24}O_2$	[M − H]-	1.05	<0.001
M80	Loratadine	152.1435	895.4	79794-75-5	$C_{10}H_{17}N$	[M+H]+	1.12	<0.001
M109	beta-Alanyl-L-lysine	270.2	270.2	90970-40-4	$C_9H_{19}N_3O_3$	[M]+	1.17	<0.001
M117	2-Naphthylamine	144.0815	356.8	91-59-8	$C_{10}H_9N$	[M+H]+	1.50	<0.001
M20	Pipecolic acid	130.087	175.9	535-75-1	$C_6H_{11}NO_2$	[M+H]+	3.76	<0.001
M25	L-Isoleucine	132.1019	511.4	73-32-5	$C_6H_{13}NO_2$	[M+H]+	1.12	<0.001
M71	D-Pyroglutamic Acid	130.0488	678.7	4042-36-8	$C_5H_7NO_3$	[M+H]+	1.21	<0.001
M84	L-Tyrosine	182.08	187.4	60-18-4	$C_9H_{11}NO_3$	[M+H]+	1.43	<0.001
M132	Beta-Leucine	132.101	608	5699-54-7	$C_6H_{13}NO_2$	[M+H]+	1.27	<0.001
M243	N2-gamma-Glutamylglutamine	256.0938	100.7	10148-81-9	$C_{10}H_{17}N_3O_6$	[M-H2O-H]-	1.58	<0.001
M232	1H-Indole-3-acetamide	174.0753	98	879-37-8	$C_{10}H_{10}N_2O$	[M]-	1.16	<0.001
M28	D-Arabitol	153.0756	97.6	488-82-4	$C_5H_{12}O_5$	[M+H]+	1.11	<0.001
M116	Phenylethanolamine	138.091	274.2	7568-93-6	$C_8H_{11}NO$	[M+H]+	1.93	<0.001
M183	trans-1,2-Cyclohexanediol	115.9191	843.1	1460-57-7	$C_6H_{12}O_2$	[M]-	1.37	<0.001
M191	Arabitol	151.0588	203.7	488-81-3	$C_5H_{12}O_5$	[M − H]-	1.45	<0.001
M192	Phenyl acetate	134.894	857.1	122-79-2	$C_8H_8O_2$	[M − H]-	2.61	<0.001
M206	D-Galactose	179.0564	87.4	59-23-4	$C_6H_{12}O_6$	[M − H]-	1.17	<0.001
M221	Fructose 1,6-bisphosphate	339.1997	767.2	488-69-7	$C_6H_{14}O_{12}P_2$	[M − H]-	1.09	<0.001
M229	L-Iditol	181.0676	526.5	488-45-9	$C_6H_{14}O_6$	[M − H]-	3.91	<0.001
M32	3-Hydroxymethylglutaric acid	144.9825	921.4	503-49-1	$C_6H_{10}O_5$	[M + H-H2O]+	1.38	<0.001
M91	Oleamide	282.2784	912.2	301-02-0	$C_{18}H_{35}NO$	[M+H]+	4.70	<0.001
M92	13-L-Hydroperoxylinoleic acid	295.2262	550.2	33964-75-9	$C_{18}H_{32}O_4$	[M + H-H2O]+	1.95	<0.001
M167	Prostaglandin C1	337.2345	744	35687-86-6	$C_{20}H_{32}O_4$	[M+H]+	1.03	<0.001
M214	Palmitic acid	255.2326	930.7	57-10-3	$C_{16}H_{32}O_2$	[M − H]-	1.00	<0.001
M228	2-Isopropylmalic acid	175.0589	102.1	3237-44-3	$C_7H_{12}O_5$	[M − H]-	1.46	<0.001
M238	Palmitoleic acid	253.217	886.5	373-49-9	$C_{16}H_{30}O_2$	[M − H]-	1.65	<0.001
M245	12,13-DHOME	313.2382	706.3	263399-35-5	$C_{18}H_{34}O_4$	[M − H]-	6.68	<0.001

VIP, variable importance in projection. MT1: Jinsha County, Guizhou Province; MT2: Xishui County, Guizhou Province; MT3: Moutai Town, Guizhou Province.

products and is currently produced by microorganisms, but research on its production metabolism is limited [36].

Metabolites with a high MT2 content are mainly steroids, including dehydroepiandrosterone and 17a-estradiol, which are mostly characterized by their prebiotic functions (such as antioxidant), and wine is one of the main sources of these compounds [37]. However, their changes during the fermentation process and their effects on the quality of the finished wine are yet to be investigated. In addition, 13-l-hydroperoxylinoleic acid and palmitic acid were high in MT2, which is a precursor of palmitoleic acid.

3.4. Heatmap analysis

Key metabolites with high MT3 levels included trans-1,2-cyclohexanediol, 1h-indole-3-acetamide, fructose 1,6-bisphosphate, l-tyrosine, l-isoleucine, and d-pyroglutamic acid. d-Amino acids are found in high relative contents in fermented foods, where they are formed mainly in response to specific enzymes secreted by microorganisms, such as amino acid racemase and d-amino acid transaminase [38]. They have been found to be positively correlated with both the strong flavor and sweetness of sake [39]. L-amino acids are often regarded as indicators of the improved nutritional value of processed foods, such as grain sorghum [40]. Among them, L-tyrosine, an aromatic amino acid, is a key precursor of aromatic compounds in white wine and is hydrolyzed to form a series of intermediate metabolites, such as cinnamic acid, caffeic acid, ferulic acid, and erucic acid, which affect the taste and flavor of wine [41].

3.5. Microbial diversity

The sequences of all the samples were clustered according to a 97 % similarity level, and 195 bacterial and 228 fungal OTUs were obtained. Shannon, Simpson, Chao1, and ACE indices were used to represent the α-diversity of microbial communities in Daqu (Table 2). The larger the Shannon index/smaller the Simpson index, the higher the microbiota diversity. Chao1 and ACE indices represent the abundance of microbiota, and the larger the value, the richer the microflora [42]. The results showed that MT1 samples had the highest bacterial abundance and diversity, whereas MT2 had the lowest. For fungi, the highest abundance and diversity were also found in the MT1 samples, whereas the lowest were found in MT3. This indicates that the abundance and diversity of bacterial and fungal communities showed significant variability among the different types of Daqu. It has been suggested that slightly lower

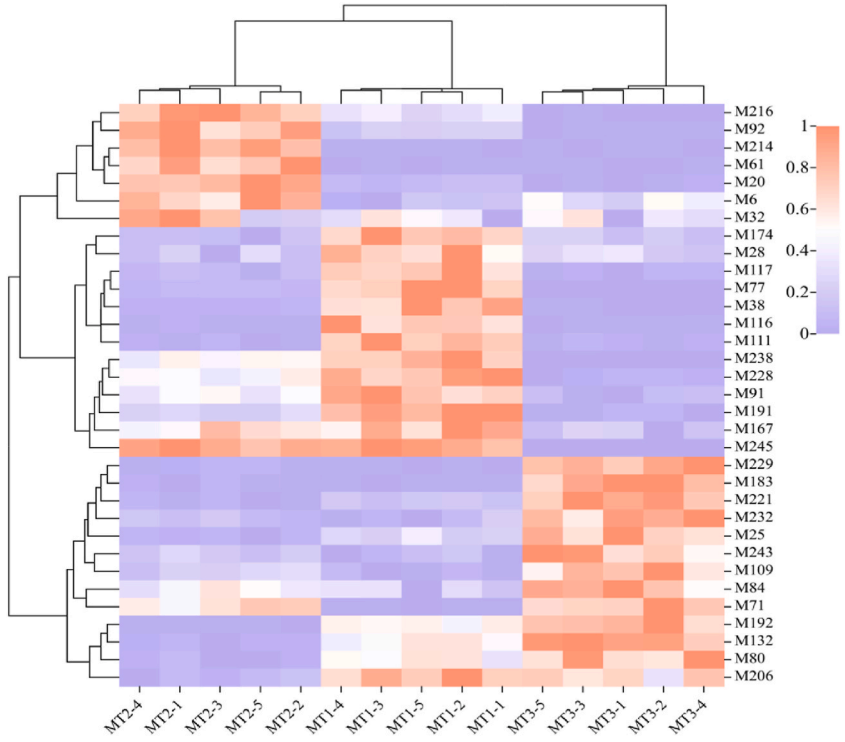

Fig. 4. Heatmap analysis of critical metabolites in three samples. A color-coded scale grading from blue to red corresponds to the content of critical metabolites shifting from low to high. MT1: Jinsha County, Guizhou Province; MT2: Xishui County, Guizhou Province; MT3: Moutai Town, Guizhou Province. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Alpha of the bacteria and fungus community of three samples.

Sample ID	Shannon		Simpson		ACE		Chao1		Good's Coverage	
	Bacteria	Fungus	Bacteria	Fungus	Bacteria	Fungus	Bacteria	Fungus	Bacteria	Fungus
MT1	3.37	2.63	0.83	0.74	149.44	186.78	149.14	185.62	0.99	0.99
MT2	2.46	2.23	0.91	0.80	130.32	110.04	132	110.11	0.99	0.99
MT3	2.86	1.78	0.90	0.83	96.92	60.71	95.67	61.00	0.99	0.99

MT1: Jinsha County, Guizhou Province; MT2: Xishui County, Guizhou Province; MT3: Moutai Town, Guizhou Province.

microbial community diversity may improve the performance of Daqu in some aspects, but too low a diversity may in turn lead to weaker enzyme activity and aroma production capacity of Daqu [43,44].

3.6. Community composition

This research employed high-throughput sequencing technology to investigate the microbial diversity in three varieties of Daqu. Including the diversity of bacteria (Fig. 5A–C, E) and fungi (Fig. 5B–D, F). The taxonomic composition reveals that MTI and MT2 are dominated by *Proteobacteria* and *Firmicutes*, respectively, whereas MT3 is dominated by *Proteobacteria* and *Bacteroidetes*. *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* have been frequently reported from the other Jiuqu, but their relative abundance varies among the three types of Daqu in this study [45]. Xie et al. studied the bacterial community and dynamic succession of sesame flavor Baijiu Daqu, and their findings showed that the most important flora was *Firmicute* [46]. *Proteobacteria* are thermophilic, correlating positively with increased temperatures. The *Proteobacteria* with relative abundances of 53.84 %, 74.23 %, and 48.25 % in MT1, MT2, and MT3, which indicates that the microbes in Daqu, Xishui County, are more tolerant to temperature and suitable for producing Baijiu under high temperature conditions.

Daqu is usually operated in a relatively high temperature range during the Daqu making process. However, the suitable growth temperature range for most fungi is usually 20–30 °C, and the lethal temperature range is usually 50–60 °C. Therefore, only a small number of heat-resistant and thermophilic fungi show dominance in Daqu samples. As shown in Fig. 5B, the fungal communities at the phylum level across all three Daqu varieties are remarkably similar, with *Ascomycota* as the predominant group, comprising nearly 99 % of the total. According to the distribution of microbial communities, there are significant differences in the bacterial communities of

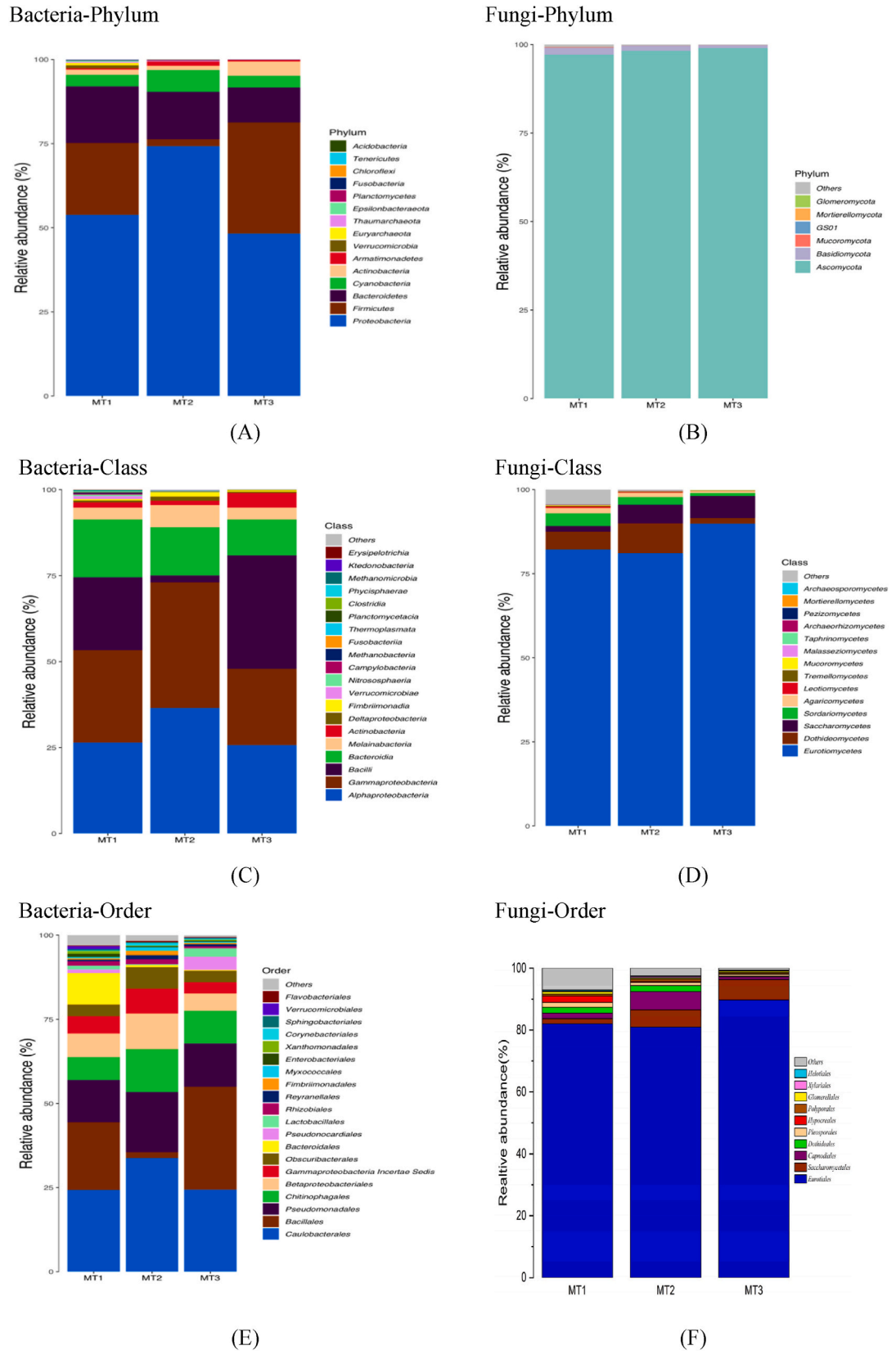

Fig. 5. Phyla, class, and order in Daqu on the basis of 16s and ITS amplicon sequencing.

Daqu in the three regions, while the fungal communities are relatively similar. In the dominant phyla MT1 and MT3, the most abundant bacterial families are *Caulobacteraceae*, *Pseudomonasdaceae*, and *Bacillaceae*. In MT2, the predominant bacterial families are *Caulobacteraceae*, *Pseudomonasdaceae*, and *Chitinophagaceae*, with the former two having the largest proportion in MT3. Consequently, future research should primarily aim to maintain the stability of the Daqu bacterial community.

To visually display the differences in the three groups, species with higher abundance were selected at both the family and genus levels to generate cumulative histograms in this study. The three families are dominated by *Caulobacteraceae* and *Thermoascaceae*, but their proportions are different (Fig. 6A and B). At the genus level, *Pseudomonas* was dominant in all three samples, but its relative abundance varied, with 12.03 % in MT1, 17.02 % in MT2, and 12.24 % in MT3 (Fig. 6C and D). The presence of *Pseudomonas* as a marker bacterium was found in half-young Daqu and demonstrated its association with the L-lysine metabolic pathway (L-lysine biosynthesis I), according to a previous report [47]. In addition, *Kroppenstedtia*, *Janthinobacterium*, and *Acidibacter* were the dominant genera in MT1. The relative abundance of *Janthinobacterium* increased from 4.84 % in MT1 to 8.84 % in MT2, whereas *Acidibacter* increased from 5.14 % to 7.41 %. *Kroppenstedtia* increased to 10.46 % in MT3, making it the second most dominant genus after *Pseudomonas*. Although *Kroppenstedtia* has been found in several regions of Jiuqu, it has been poorly studied. The year 2011 saw the establishment of the classification of *Kroppenstedtia*, which is currently recorded in the database as having four active species, one of which, *Kroppenstedtia eburnea*, is known to have the ability to degrade starch [48,49]. According to a previous study, it may be positively associated with the synthesis of terpene flavor compounds and various amino acids [13]. In addition, *Vibrionimonas* and *Bacillus* were the dominant bacteria in MT3, with relative abundances of 6.77 % and 7.66 %, respectively. *Bacillus* also produces

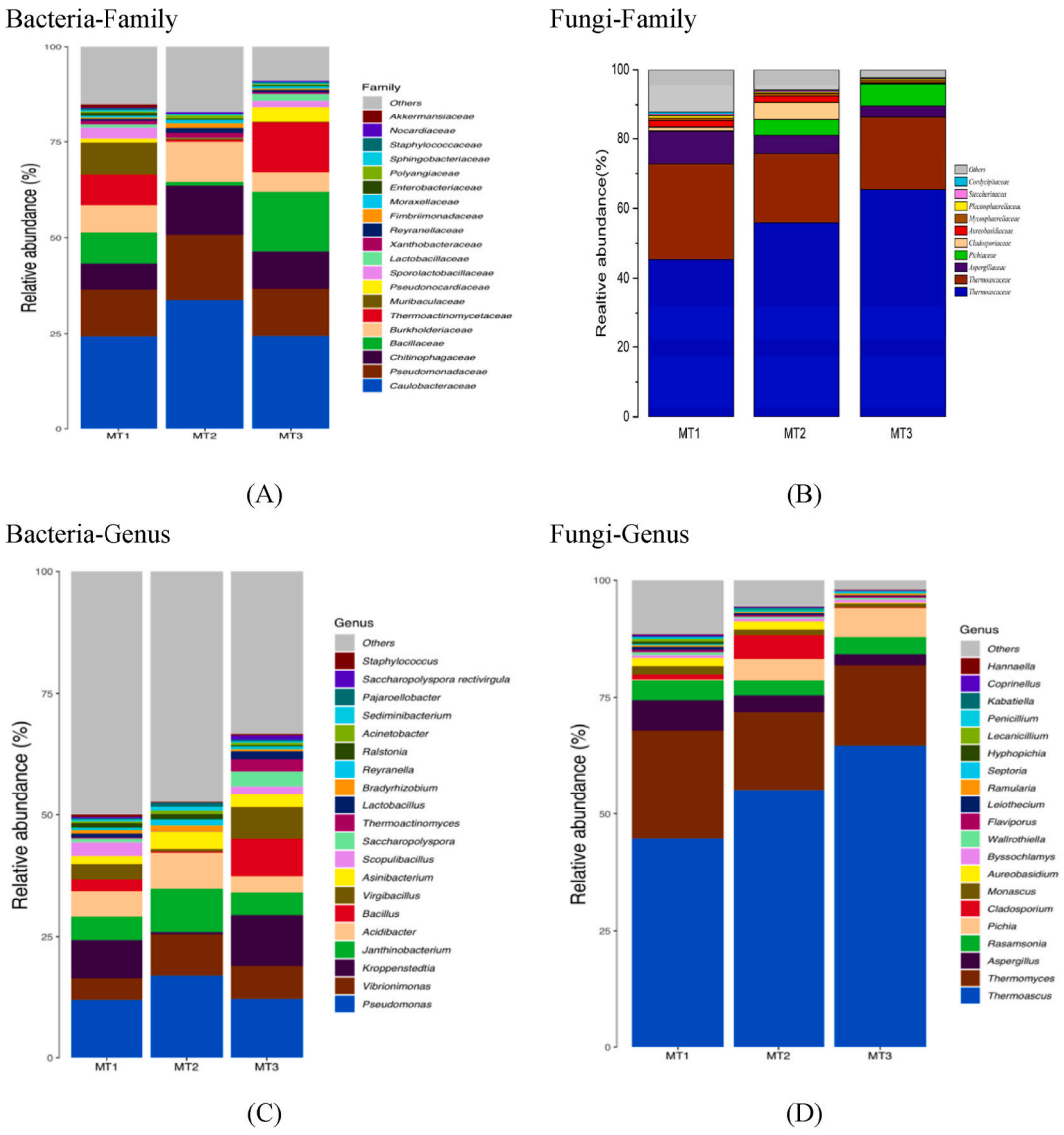

Fig. 6. Dominant family, genus, and species in Daqu on the basis of 16s and ITS amplicon sequencing.

enzymes that degrade starch, such as α-amylase, glucoamylase, and α-glucosidase, and is one of the most representative bacteria in Daqu [50]. It can also synthesize ethyl hexanoate and vanillin, which are important for the synthesis of flavor compounds in Baijiu [51,52]. Meanwhile, it has been claimed that *Virgibacillus* can promote the production of pyrazines [53]. Pyrazines are important flavor compounds with roasted and nutty aromas and are found at higher concentrations in sauce liquor than in other flavor types [54,55].

The sum of the relative abundances of *Thermoascus* and *Thermomyces* exceeded 70 % in the three groups, and both were the dominant fungi in the three samples. However, their flora structures were different, with *Thermoascus* having a higher relative abundance in MT3 than in the other two samples and *Thermomyces* having the highest relative content in MT1. *Thermoascus* and *Thermomyces* are fungi commonly found in high-temperature Daqu. *Thermomyces* produces large amounts of lipase, which in turn catalyzes triglyceride hydrolysis, producing glycerol and free fatty acids, which are important contributors to wine flavor [56,57]. Similarly, *Thermoascus* can synthesize amylases and xylanases to break down raw materials and synthesize esters [13,58]. *Aspergillus* and *Rasamsonia* were also highly abundant in MT1 (6.49 % and 4.19 %, respectively), and *Rasamsonia* is also among the dominant genera in MT2 and MT3. Both *Aspergillus* and *Rasamsonia* play important roles in the production of lytic enzymes in Daqu, and *Aspergillus* has a strong potential to synthesize the flavor substance 4-vinylguaiacol [59,60]. In addition, MT2 and MT3 shared the dominant genus *Pichia*, and *Cladosporium* was the dominant genus specific to MT2. *Pichia* is the main fermentation-functional fungus in Daqu and can utilize glucose to produce ethyl acetate [61].

Overall, the dominant fungal species were more similar in the three groups than the bacteria but differed in abundance. Various genera with enzyme-producing abilities had a higher relative abundance in MT3; therefore, it is hypothesized that the enzyme activity of MT3 is higher than that of MT1 and MT2.

3.7. Correlation analysis of the microbial community diversity and metabolites

The 33 key metabolites screened were correlated with microorganisms whose relative abundances were in the top 20 (at the genus level). The correlation results are shown in Fig. 7A and B. As the genus with the highest relative abundance among the three types of Daqu, *Pseudomonas* showed a significant positive correlation ($r > 0.7$) with various fatty acids. These include 13-l-hydroperoxylinoleic acid, palmitic acid, and 2-isopropylmalic acid. *Virgibacillus* is widely present in natural environments and can utilize most carbohydrates as carbon and energy sources. In addition, Virgibacillus can also produce extracellular enzymes such as amylase, protease, inulinase, and gelatinase [62]. In this study, *Virgibacillus* was the most abundant in MT2 and was significantly positively correlated with both of the only two flavonoids (dehydroepiandrosterone and 17a-estradiol). Li et al. found that *Virgibacillus* is also positively correlated with phenylacetaldehyde and phenylethyl alcohol in Daqu, which is beneficial for the production of flavor compounds in Daqu [45]. *Kroppenstedtia* was significantly positively correlated with a number of substances in organic oxides, including phenyl acetate, trans-1,2-cyclohexanediol, l-iditol, and d-galactose. In addition, *Kroppenstedtia* and *Bacillus* spp. were positively correlated with l-isoleucine, pyrrolidonecarboxylic acid, and l-tyrosine. Previous studies have suggested that *Kroppenstedtia* may contribute to multiple amino acid synthesis pathways. According to reports, *Kroppenstedtia* is also an advantageous group of high-temperature

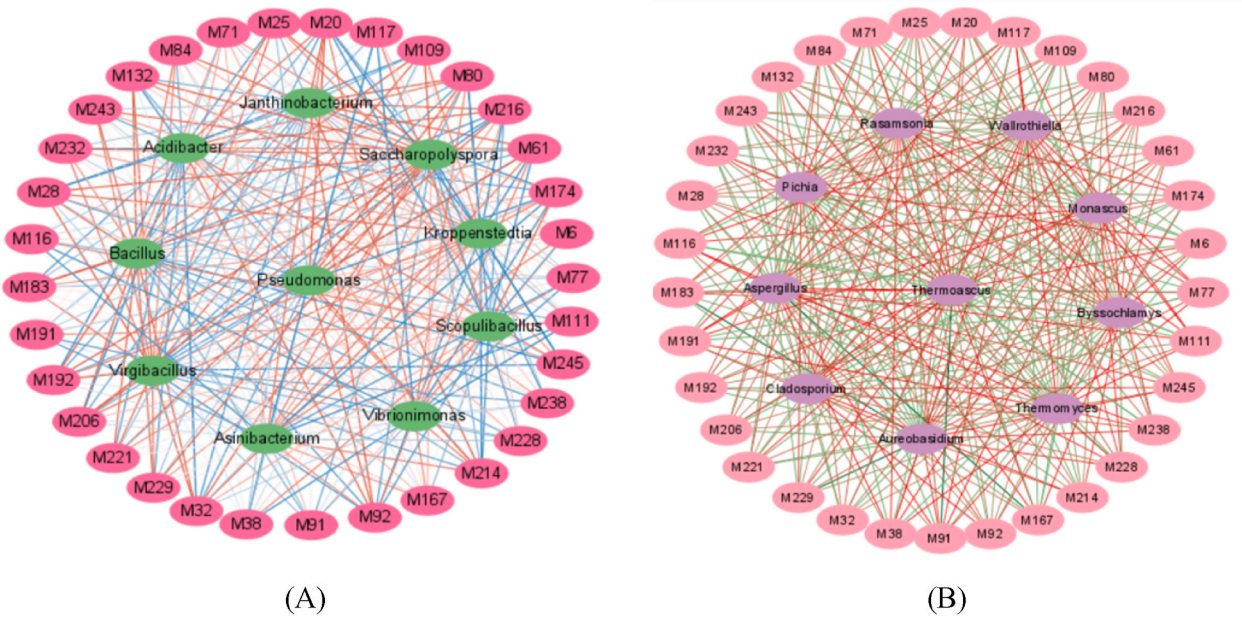

Fig. 7. The correlation network diagram of key metabolites with dominant microorganisms. Pink dots represent key metabolites, green dots represent dominant bacteria, and purple dots represent dominant fungi. Red lines indicate positive correlations, blue lines indicate negative correlations, and thicker lines indicate stronger correlations. Key metabolites and dominant bacterial genera (A). Key metabolites and dominant fungal genera (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sesame flavored Daqu, with a strong ability to secrete cellulase and the sugars produced by degradation can be further converted to provide carbon skeletons for amino acid synthesis [63,64].

Similarly, *Thermoascus* was positively correlated with amino acids, alcohols, esters, and phenols and negatively correlated with fatty acids. By contrast, *Thermomyces* was significantly positively correlated with oleamide, 2-isopropylmalic acid, and palmitoleic acid. This may be due to the close correlation between *Thermomyces* and FAO-PWY, a pathway associated with fatty acid metabolism [13,65]. Yeast is an important fungal genus in Daqu, and *Pichia* was the most abundant genus in this study. Correlation analysis showed that it was also significantly positively correlated with alcohol esters, such as phenyl acetate, trans-1,2-cyclohexanediol, and l-iditol. *Pichia*, a non-brewer's yeast, secretes higher glucosidase activity than that of brewer's yeasts, is more tolerant to the fermentation environment, and plays an important role in the production of branched-chain aldehydes, alcohols, and other substances involved in the production of food substances [66].

4. Conclusions

This study explored the differences in the metabolites and microbial flora of Daqu from three regions, analyzed the key metabolites and dominant microorganisms (genus level) in the three types of Daqu, and explored the potential relations among them. There were differences in the metabolites of the three types of Daqu, with fatty acyls dominating Daqu from Jinsha County and Xishui County, and organic oxides dominating Daqu from Maotai Town. Bacterial and fungal abundance and diversity were the highest in Daqu of Jinsha County, and the dominant genera were *Pseudomonas*, *Kroppenstedtia*, *Thermoascus*, and *Thermomyces*. *Pseudomonas* showed a significant positive correlation with various fatty acids, and *Kroppenstedtia* showed a significant positive correlation with various organo-oxygen compounds. *Thermomyces* and *Thermoascus* are thermophilic bacteria found in Daqu, with *Thermomyces* being positively correlated with fatty acids and *Thermoascus* with the opposite. In conclusion, this study provides a reference for understanding the microbial communities and metabolic profiles of Daqu of different origins, but the analysis of dominant microorganisms has not been precise to the species level, and the impact of dominant microbial communities on the physicochemical properties of Daqu has not been explored, which is needed further research in the future. It is also necessary to track the impact of key metabolites on the quality of Baijiu during fermentation.

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Additional information

No additional information is available for this acticle.

Data availability statement

The article already contains all the data.

CRediT authorship contribution statement

Yijie Dai: Writing – original draft. **Lei Yu:** Software, Formal analysis. **Jintao Ao:** Methodology, Formal analysis. **Rui Wang:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Yijie Dai reports financial support was provided by The start-up fund research project for introducing talents to Guiyang University, China. Yijie Dai reports financial support was provided by The Key Laboratory for Critical Degradation Technologies of Pesticide Residues in Superior Agricultural Products in Guizhou Ecological Environment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36944.

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