

Fungal Community Compositional Diversity and Fragrant Components in Medium- and High-Temperature Taorong-Type Daqu

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Abstract

The microbial communities and fragrant components of Taorong-typed were analyzed using high-throughput sequencing (HTS) and headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS). At the phylum and genus levels with abundance $\geq 0.01\%$ as the threshold, 3 phyla including Mucoromycota, Ascomycota, Basidiomycota were found in both medium- and high-temperature Daqu, but the abundances differed. At the genus level, 15 and 13 genera were recognized. *Rhizopus* (72.40%) and *Thermomyces* (53.32%) accounted for the largest proportions in medium- and high-temperature Daqu respectively. The medium-temperature Daqu and high-temperature Daqu were found with 40 and 29 fragrant components respectively, and contained the highest proportions of pyrazines (53.12%) and acids (32.68%) respectively. Correlation analyses between microbes and fragrant components showed *Aspergillus*, *Hyphopichia*, *Trichosporon* and *Alternaria* were all highly and positively correlated with pyrazines, but the dominant fungal communities were highly correlated only with a few single acid compounds, but not with acid compounds on the whole.

Introduction

Liquors, as treasures of the Chinese nation, are characterized by extraordinary fermentation characteristics, diverse styles and unique flavors after their long development history (Xie et al., 2020; Liu et al., 2018). Taorong-flavored liquors are among the 13 flavors of fragrant liquors in China and innovatively integrate Luzhou, Maotai, Fen, and sesame flavors. The Taorong flavor caters to numerous customers because of its fragrant, elegant and exquisite liquor, mellow and plump mouthfeel, and clean and bright aftertaste (Hu et al., 2020). Taorong-type Daqu is a microecological product enriched with microbial communities, bacteria and a complex Daqu fragrance and is capable of saccharification, fermentation and fragrance formation (Du et al., 2019; Huang et al., 2021). High-temperature Daqu (maximum starter temperature controlled above 60 °C) and medium-temperature Daqu (maximum starter temperature controlled at 50-60 °C) varieties exist (di Cologna et al., 2018; Deng et al., 2020; Yang et al., 2018). Medium- and high-temperature Daqu is mainly produced from wheat through natural inoculation, raw-material starter production, low-temperature fungal culture, high-temperature conversion, and storeroom storage (Fan et al., 2018; Fan et al., 2020). Medium- and high-temperature Daqu is pivotal for microbe inoculation and creates a fermentation environment for liquor-making (Shi et al., 2018). Because of differences in Daqu production processes, Daqu from different production temperatures differs in its microbial community composition, which directly affects the compositions of the fermented mash zymophyte system, enzymatic system, and substance system (Gabriel et al., 2020). Hence, the compositional analysis of microbial communities in medium- and high-temperature Daqu is urgently needed.

Bacteria, fungi and actinomycetes play important roles in Daqu, and fungal communities are pivotal in producing alcohols, enzymes and fragrances (Yi et al., 2019). Fungi consist of molds and saccharomycetes. Molds can secrete saccharifying enzymes, lipases, proteases and hydrolases, decompose macromolecular substances in fermented mashes, and produce flavor substances and their precursors in liquors; thus, they are closely related to the formation of all flavor substances in liquors (Zuo et al., 2020). Moreover, saccharomycetes are a critical group of flora that can produce esters, fragrances and alcohols and thereby provide power for fermentation in liquors (Li et al., 2020).

Headspace-solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) is a practical technique for characterizing volatile components in complex systems (Wang et al., 2021; Xue et al., 2021). Compared with conventional flavor substance extraction, SPME is more convenient, faster, economically safer, solventless, highly selective, and more sensitive (Jin et al., 2019). SPME can be directly combined with GC-MS, high-performance liquid chromatography, or capillary electrophoresis, which largely accelerates analysis and detection (Li et al., 2020). Compared with the traditional culture isolation method, high-throughput sequencing (HTS) can be used for both culturable and unculturable microbes (Fan et al., 2021). It has the outstanding characteristics of accurate quantification, high sensitivity, a low workload, and low costs in comparison with fluorescence in situ hybridization, terminal restriction fragment length polymorphism analysis, and 18S rDNA cloning library analysis (Fan et al., 2021; Yang et al., 2018; Yang et al., 2012). Hence, HTS can better determine the compositions of flora communities in samples.

Currently, there has been little research on fungal community compositions and fragrance compositions in medium- and high-temperature Daqu. Herein, HTS was combined with HS-SPME-GC-MS to study Yangshao Taorong-type medium- and high-temperature Daqu. Specifically, the main fungal compositions and fragrance compositions of Taorong-type medium- and high-temperature Daqu were analyzed. The findings will theoretically underlie the fungal flora library establishment and quality identification of Taorong-flavored Daqu.

Materials And Methods

Materials and reagents

The Daqu used herein was Taorong-flavored Daqu liquor (Henan Yangshao Liquor Co. Ltd.) that had been stored for 5 months. The medium-temperature Daqu and high-temperature Daqu were marked D-Z and E-G, respectively. Daqu was crushed, immediately placed in ice boxes, transported to the laboratory and stored in a refrigerator at -20 °C. The reagents used herein included anhydrous ethanol (Guangzhou Chemical Reagent Factory); Goldview agarose (Beijing Mengyimei Commercial Center); PCR reagents (Biolion Technology Co. Ltd.); and soil DNA extraction kits (Guangzhou Magen Technology Co. Ltd.). AMPure XP magnetic beads were purchased from Beckman Instruments (USA).

Instruments

The instruments used included a -80 °C refrigerator (Zhongke Meiling Cryogenics Co. Ltd.), a 4 °C centrifuge and pipettors (Eppendorf AG, Germany), an ultrapure water apparatus (Shanghai RephiLe Bioscience), a gel-imaging system (Shanghai Biotanon Co. Ltd.), a PCR apparatus (Dongsheng Xingye Science Equipment Co. Ltd.), an agarose gel electrophoresis analyzer (Beijing Liuyi Biotechnology Co. Ltd.), a vortex oscillator (Guangzhou Wego Instrument Co. Ltd.), a GC-MS meter (Shimadzu, Japan), and a solid-phase microextraction device (Merck, USA).

Experimental methods

Detection and identification of flavor components

Pretreatment of Daqu samples: At each time point, Daqu (1 g) was placed into a headspace bottle, and 2 g of NaCl and 5 mL of distilled water were added. Then, the bottle was plugged and shaken.

HS-SPME conditions: Headspace bottles containing pit mud samples were placed into a water bath at 50 °C for 10 min of preheating, and then solid-phase CAR/PDMS (75 µm CAR/PDMS, carbon molecular sieve/polydimethyl silane) extraction fiber heads were inserted into the silica gel plugs for 30 min of headspace adsorption.

GC conditions: An HP-FFAP column (30×0.32 mm²×0.25 µm) was used without diffusion at a flow rate of 1.21 mL/min, an inlet temperature of 250 °C, and a temperature increase to 40 °C over 3 min, followed by an increase at 5 °C/min to 60 °C, no heating, and then a temperature increase at 8 °C/min to 230 °C and holding for 7 min.

MS conditions: An interface temperature of 220 °C, electron ionization source, electron energy of 70 eV, and ion source temperature of 200 °C were used (Hong et al., 2020).

Total DNA extraction and quantitative PCR of Daqu microbes

Crushed samples of medium-temperature Daqu or high-temperature Daqu were mixed well (each sample measured 0.25-0.5 g), and total metagenomics were extracted from the microbes using HiPure soil DNA kits. Then, the total DNA quantity and integrity were detected by agarose electrophoresis, and the DNA was stored at -20 °C.

ITS1_{plant} was amplified by PCR with the primers ITS1_F-KY02 (5'-TAGAGGAAGTAAAAGTCGTAA-3') and ITS86R (5'-TTCAAAGATTGATGATTAC-3').

The first-round amplification procedure was as follows: 1.5 μL of primer R (10 μM), 1.5 μL of primer F (10 μM), 3 μL of 25 mM MgSO_4 , 1 μL of KOD enzyme, 5 μL of 2 mM dNTPs, 5 μL of 10 \times Buffer KOD, and X μL of the template (100 ng) were mixed, and the volume was brought up to 50 μL with H_2O . The PCR procedure was 94 $^\circ\text{C}$ for 2 min; 98 $^\circ\text{C}$ for 10 s, 62-66 $^\circ\text{C}$ for 30 s, and 68 $^\circ\text{C}$ for 30 s (30 cycles); and 68 $^\circ\text{C}$ for 5 min. The PCR products were purified using AMPure XP beads and then quantified with Qubit 3.0. The second-round amplification procedure was as follows: 1 μL of universal PCR primer (10 μM), 1 μL of index primer (10 μM), 3 μL of 25 mM MgSO_4 , 1 μL of KOD enzyme, 5 μL of 2 mM dNTPs, 5 μL of 10 \times Buffer KOD, and X μL of template (100 ng) were mixed, and the volume was brought up to 50 μL with H_2O . The PCR procedure was as follows: 94 $^\circ\text{C}$ for 2 min; 98 $^\circ\text{C}$ for 10 s, 65 $^\circ\text{C}$ for 30 s, and 68 $^\circ\text{C}$ for 30 s (12 cycles); and 68 $^\circ\text{C}$ for 5 min.

Data processing

After raw reads were acquired from sequencing, the low-quality reads were filtered using FASTP, and double-end reads were spliced into tags using FLASH. Then, the tags were filtered to form clean tags. The clean tags were clustered, and chimeric tags identified during this process were removed using UCHIME from USEARCH, which left the effective tags remaining. Then, operational taxonomic units (OTUs) were obtained and clustered using UPARSE from USEARCH. The abundance of OTUs was statistically analyzed on the basis of the effective tags.

Results

Rationality analysis of sequencing data

Table 1 Basic sequencing data of medium-temperature Daqu and high-temperature Daqu

Sample	Raw PE	Clean PE	Effective Tags	Effective Ratio
D-Z-1	135860	135780	128618	94.67%
D-Z-2	131580	131502	123692	94.01%
D-Z-3	132312	132228	125387	94.77%
E-G-1	131114	131015	123134	93.91%
E-G-2	129348	129258	121116	93.64%
E-G-3	134324	134220	126666	94.30%

The average numbers of series in the original data of medium-temperature Daqu and high-temperature Daqu were 133251 and 131595, respectively (Table 1). After quality control and filtering, the average numbers of high-quality series were 133170 and 131498, respectively. After the removal of chimeras, the average numbers of effective series were 125899 and 123639, respectively, and the average proportions of effective series were 94.48% and 93.95%, respectively.

Venn diagram analysis

The numbers of OTUs in medium-temperature Daqu and high-temperature Daqu were 139 and 213, respectively, with 84 shared OTUs. Specifically, medium-temperature Daqu and high-temperature Daqu shared 84 fungal species and possessed 55 and 129 exclusive OTUs, respectively.

Alpha diversity analysis

Table 2 Medium-temperature Daqu and high-temperature Daqu fungal diversity index table

Sample	Shannon	Simpson	Goods-Coverage	Chao1	ACE
D-Z-1	2.02	0.58	99.97%	194.1	205.8
D-Z-2	1.99	0.57	99.97%	190.7	195.6
D-Z-3	2.01	0.58	99.99%	241.1	234.9
E-G-1	2.21	0.67	99.95%	266.4	270.8
E-G-2	2.17	0.66	99.95%	254.2	263.0
E-G-3	2.27	0.66	99.95%	284.2	289.0

The average Shannon index of the high-temperature Daqu was larger than that of the medium-temperature Daqu (2.21 vs. 2.01, Table 2), suggesting that the microbial species diversity in the samples of high-temperature Daqu was richer and significantly more complex. The average Chao1 and ACE values of the high-temperature Daqu were both larger than those of the medium-temperature Daqu (268.3 vs. 208.7 and 274.3 vs. 212.1), indicating that the microbial diversity in high-temperature Daqu was higher. In addition, the sample coverage rates of both types of Daqu were larger than 99.9%, indicating that the sequencing results were highly reliable and sufficiently reflected the real situations of the samples.

Fungal community composition of Daqu

Based on the species annotations of OTUs, the fungal community compositions of each sample at the phylum and genus taxonomic levels were analyzed (Figs. 2).

At the phylum level, with an abundance $\geq 0.01\%$ as the threshold, 3 phyla, Mucoromycota (72.41%), Ascomycota (27.41%) and Basidiomycota (0.18%), were identified in medium-temperature Daqu (Fig. 2(A)). The three phyla Ascomycota (75.51%), Mucoromycota (24.44%) and Basidiomycota (0.04%) were identified in high-temperature Daqu.

At the genus level with an abundance $\geq 0.01\%$ as the threshold, 15 and 13 genera were recognized in medium- and high-temperature Daqu, respectively. The genera in medium-temperature Daqu included *Rhizopus* (72.40%), *Aspergillus* (13.65%), *Hyphopichia* (3.97%), *Thermoascus* (3.49%), *Thermomyces* (1.30%), *Alternaria* (0.27%), *Trichosporon* (0.15%), *Wickerhamomyces* (0.07%), *Monascus* (0.07%), *Issatchenkia* (0.02%), *Cutaneotrichosporon* (0.01%), *Geosmithia* (0.01%), *Mucor* (0.01%) and *Fusarium* (0.01%). The genera in high-temperature Daqu included *Thermomyces* (53.32%), *Rhizopus* (24.44%), Unclassified (11.91%), *Thermoascus* (7.14%), *Aspergillus* (2.52%), *Monascus* (0.31%), *Hyphopichia* (0.09%), *Alternaria* (0.05%), *Wickerhamomyces* (0.04%), *Cladosporium* (0.03%), *Cyphellophora* (0.02%), *Pseudeurotium* (0.01%), and *Papiliotrema* (0.01%).

The proportions of *Thermomyces*, *Thermoascus* and *Monascus* in high-temperature Daqu were all larger than those in medium-temperature Daqu, indicating that these three microbial genera are highly heat-resistant and adapted to hot conditions. The proportions of *Rhizopus*, *Aspergillus*, *Hyphopichia*, *Alternaria*, *Trichosporon*, *Wickerhamomyces* and *Mucor* all decreased with increasing starter temperatures, indicating that these microbial genera are adapted to medium-temperature environments. In addition, three genera, *Cyphellophora*, *Pseudeurotium* and *Papiliotrema*, were only identified in high-temperature Daqu, suggesting that these three genera are thermally resistant and can survive hot conditions. *Thermomyces* can thrive at up to 60 °C but cannot survive below 20 °C (Singh et al.,2003;Tang et al.,2019). They can secrete thermophilic enzymes, improve the catalytic efficiency of reactions owing to their high thermal stability, and facilitate binding between enzymes and substrates owing to their low viscosity in substrates under hot conditions, which are all advantages for catalysis by thermophilic mannanase(Yi et al., 2019). *Thermoascus* can produce heat- and acid-resistant xylanase, which adapts to 70 °C and pH 4.8 (Ai et al.,2019). During the growth process, *Monascus* can produce various enzymes, and their high enzymatic esterifying activity can catalyze the synthesis of acids and alcohols that are major sources of fragrances in liquors(Gabriel et al.,2020). Moreover, *Rhizopus* can generate abundant highly active amylases, proteases and lipases and

can produce citric acid, gluconic acid, lactic acid, succinic acid and other organic acids (Londoño-Hernández et al.,2017). *Aspergillus*, which is pivotal in liquor-making, can secrete saccharifying enzymes and amylases and facilitate the fermentation of Daqu, which is favorable for liquor production by fermentation (di Cologna et al.,2018).

Fragrance component analysis of medium- and high-temperature Daqu

Table 3 Fragrance components

Type	Medium-temperature Daqu (D-Z)					High-temperature Daqu (E-G)			
	No.	Compound name	Relative percentage (%)			Compound name	Relative percentage (%)		
Alcohols	R-A	phenylethanol	3.47	3.51	3.52	phenylethanol	3.80	3.81	3.82
	R-B	cineole	0.69	0.71	0.72	cineole	1.70	1.73	1.73
	R-C	benzyl alcohol	0.53	0.54	0.55	benzyl alcohol	0.85	0.84	0.86
	R-D	3,6,9,12-tetradecane-1-methanol	0.66	0.69	0.66	—	—	—	—
	R-E	—	—	—	—	tetrahydro-2,5-dimethyl-2hydro-pyran methanol	1.06	1.08	1.07
	R-F	—	—	—	—	4-methylene-6-hepten-2-methanol	0.66	0.69	0.69
Pyrazines	S-A	tetramethylpyrazine	46.88	46.83	46.84	tetramethylpyrazine	22.60	22.59	22.58
	S-B	trimethylpyrazine	5.40	5.38	5.39	trimethylpyrazine	1.80	1.80	1.82
	S-C	2,3-dimethylpyrazine	0.52	0.49	0.49	—	—	—	—
	S-D	2,3,5-trimethyl-6-ethylpyrazine	0.42	0.38	0.37	—	—	—	—
Acids	T-A	—	—	—	—	3-methyl butyric acid	13.30	13.28	13.29
	T-B	valeric acid	11.78	11.80	11.79	valeric acid	0.25	0.26	0.24
	T-C	hexanoic acid	5.29	5.31	5.27	hexanoic acid	12.47	12.45	12.46
	T-D	acetic acid	2.65	2.63	2.67	acetic acid	2.39	2.37	2.35
	T-E	oleic acid	2.60	2.56	2.55	—	—	—	—
	T-F	stearic acid	1.44	1.44	1.47	—	—	—	—
	T-G	butyric acid	1.10	1.06	1.05	butyric acid	1.07	1.08	1.09
	T-H	n-palmitic acid	0.68	0.65	0.65	n-palmitic acid	0.84	0.82	0.83
	T-I	octanoic acid	0.45	0.42	0.42	octanoic acid	1.04	1.01	1.01
	T-J	4-methylvaleric acid	0.36	0.33	0.36	4-methylvaleric acid	0.90	0.92	0.91
	T-K	heptanoic acid	0.24	0.22	0.23	heptanoic acid	0.46	0.49	0.46
	T-L	(R)-(-)-4-methylhexanoic acid	0.21	0.23	0.22	—	—	—	—
	T-M	—	0.18	0.19	0.17	—	—	—	—
	T-N	nonanoic acid	0.17	0.16	0.12	—	—	—	—
	T-O	3-methyl-2-crotonic acid	0.06	0.08	0.10	—	—	—	—
		2-methyl-1-methyl propyl-butyric acid	—	—	—	—	—	—	—
Esters	U-A	2-isobutoxy ethyl butyrate	0.51	0.48	0.48	—	—	—	—
	U-B	—	0.34	0.37	0.37	—	—	—	—
	U-C	2,4-dimethyl-3-isobutyl carbonic ester	0.30	0.27	0.30	—	—	—	—
		—	0.27	0.28	0.29	—	—	—	—

	U-D	ethyl phenylacetate	0.27	0.25	0.26		0.46	0.43	0.46
	U-E	2-valeric-ethoxyethyl	0.24	0.24	0.21	ethyl 3-phenylpropionate	—	—	—
	U-F	ethyl 3-phenylpropionate	0.13	0.10	0.13	—	—	—	—
	U-G	1-methoxyl-2-propyl acetate	0.07	0.09	0.08	—	—	—	—
	U-H	2-ethoxy ethyl 2-methyl butyrate	—	—	—	—	1.81	1.82	1.80
	U-I	—	—	—	—	ethyl caproate	0.43	0.42	0.41
	U-J	butyric-2-methyl-1-methyl propyl	—	—	—	ethyl palmitic	0.37	0.39	0.41
	U-K	—	—	—	—	ethyl phenylacetate	—	—	—
		—	—	—	—				
		—	—	—	—				
Phenols	V-A	2,4-bi(1,1-dimethyl ethyl)-phenol	0.62	0.67	0.66	2,4-bi(1,1-dimethyl ethyl)-phenol	1.76	1.75	1.74
	V-B	—	0.22	0.19	0.19	—	—	—	—
	V-C	2-methoxyl-4-vinyl phenol	0.17	0.19	0.21	—	1.16	1.17	1.18
		p-cresol				p-cresol			
Aromatics	W-A	p-xylene	1.71	1.72	1.73	p-xylene	2.65	2.66	2.64
	W-B	1,4-diethylbenzene	0.26	0.27	0.28	1,4-diethylbenzene	0.50	0.53	0.50
Aldehydes	X-A	1H-pyrrole-2-formaldehyde	0.34	0.36	0.35	1H-pyrrole-2-formaldehyde	0.87	0.88	0.86
	X-B	benzaldehyde	0.19	0.15	0.17	benzaldehyde	0.96	0.94	0.95
Alkenes	Y-A	D-limonene	2.0	1.9	2.1	D-limonene	4.05	4.08	4.05
Ketones	Z-A	1-(1H-pyrrole-2-acetyl)-ethyl ketone	0.46	0.46	0.49	1-(1H-pyrrole-2-acetyl)-ethyl ketone	1.93	1.93	1.90
Aromatic hydrocarbons	A-A	o-isopropyl phenylmethane	0.65	0.67	0.66	o-isopropyl phenylmethane	1.37	1.38	1.39

In total, 40 and 29 fragrance components were identified in medium- and high-temperature Yangshao Daqu, respectively (Table 3). Medium-temperature Daqu contained 14 acids, 8 esters, 4 pyrazines, 4 alcohols, 3 phenols, 2 aromatics, 2 aldehydes, 1 alkene, 1 ketone and 1 aromatic hydrocarbon, with average relative concentrations of 27.1%, 2.11%, 53.12%, 5.42%, 1.04%, 1.99%, 0.52%, 2%, 0.47% and 0.66%, respectively. High-temperature Daqu contained 9 acids, 5 alcohols, 4 esters, 2 pyrazines, 2 aromatics, 2 phenols, 2 aldehydes, 1 alkene, 1 ketone, and 1 aromatic hydrocarbon, with average relative concentrations of 32.68%, 8.12%, 3.07%, 24.4%, 3.17%, 2.91%, 1.81%, 4.06%, 1.92% and 1.38%, respectively.

These results indicate that alcohols, pyrazines, esters, acids and phenols are diverse and abundant in Daqu. Alcohols are a major class of flavor substances in liquors and are the precursors of esters. Alcohols can highlight the fragrances of esters and make liquors taste mellow, which together contribute to long-lasting fragrances (He et al.,2020). Pyrazines are capable of expanding blood vessels, improving blood circulation and protecting the liver (preventing alcohols from injuring the gastric mucosa and liver) (Chen et al.,2020a). Esters mostly have aromatic smells that endow liquors with fruit fragrances and are

the key substances that determine the styles and quality of liquors (Suren et al., 2010). Acids at appropriate concentrations can increase the brightness of liquors and remove dry and spicy tastes, making liquors smoother (Yang et al., 2018). Phenols endow liquors with unique sooty, burnt, milky and pit mud tastes and are major components of the dominant flavors of liquors (Wang et al., 2019).

Heatmap of the species distribution at the genus level and correlation analysis of microbes and fragrance compositions

Correlation analysis of microbes and fragrance compositions

The Spearman correlation coefficients of microbes and flavor components were calculated, and thereby, the correlations of the qualified data were plotted (Fig. 3(B)).

A redder color indicates a higher proportion of a given species in Fig. 3(A). In medium-temperature Daqu, the relatively abundant genera included *Rhizopus*, *Aspergillus*, *Hyphopichia*, *Thermoascus* and *Thermomyces*. In high-temperature Daqu, the relatively abundant genera were *Thermomyces*, *Thermoascus* and *Monascus*.

Rhizopus was largely and positively correlated with 3,6,9,12-tetradecane-1-ethanol, valeric acid, and (R)-(-)-4-methylhexanoic acid (Fig. 3(B)). *Aspergillus* was positively correlated with pyrazines, 2-isobutoxy ethyl butyrate, and ethyl phenylacetate, showing the largest correlation with trimethyl pyrazine. *Hyphopichia* was positively correlated with pyrazines, showing the largest correlations with 2,3-dimethyl pyrazine and 2,3,5-trimethyl-6-ethyl pyrazine, which are two compounds that were only found in medium-temperature Daqu. *Thermoascus* was negatively but not highly correlated with pyrazines and positively correlated with acids and was most positively correlated with 2-ethoxy ethyl-2-methyl butyrate. *Thermomyces* was negatively correlated with pyrazines, and except for a negative correlation with 3,6,9,12-tetradecane-1-ethanol, it was highly and positively correlated with other types of acids, although the correlation with overall acids was low. *Alternaria* was positively correlated with pyrazines and most highly and positively correlated with 3,6,9,12-tetradecane-1-ethanol and 1-methoxyl-2-propyl acetate. *Trichosporon* was positively correlated with pyrazines and very positively correlated with 3,6,9,12-tetradecane-1-ethanol, oleic acid, 2-isobutoxy ethyl butyrate, and 1-methoxyl-2-propyl acetate.

Conclusion

The fungal community compositions and fragrance components in Yangshao medium- and high-temperature Daqu were studied. At the threshold of $\geq 0.01\%$ abundance, three phyla were identified in both medium- and high-temperature Daqu: Mucoromycota, Ascomycota and Basidiomycota. At the genus level, 15 and 13 genera were recognized in medium- and high-temperature Daqu, respectively. *Rhizopus* (72.40%) and *Thermomyces* (53.32%) accounted for the largest proportions in medium- and high-temperature Daqu, respectively. In addition, *Cyphellophora*, *Pseudeurotium* and *Papiliotrema* were identified in high-temperature Daqu but were not detected in medium-temperature Daqu. GC identified 40 and 29 fragrance components in Yangshao medium- and high-temperature Daqu, respectively, which contained the highest proportions of pyrazines (53.12%) and acids (32.68%), respectively. Correlation analysis of microbes and fragrance compositions showed that pyrazines were highly correlated with *Aspergillus*, *Trichosporon*, *Hyphopichia* and *Alternaria*. Acids were generally not highly correlated with the dominant flora, but high correlations were found for oleic acid with *Trichosporon* and *Hyphopichia* and for valeric acid and (R)-(-)-4-methylhexanoic acid with *Rhizopus*.

Xue et al. (2020) studied the microbial diversity in Daqu from Xiangyang Shihua liquors using HTS. The dominant fungal phyla in the Daqu (abundance $> 0.1\%$) were Ascomycota (99.70%), Basidiomycota (0.17%) and Mucoromycota (0.12%), and the dominant fungal genera ($> 1\%$) included *Saccharomycopsis* (57.08%), *Monascus* (17.58%), *Thermoascus* (5.38%), *Penicillium* (4.76%), *Aspergillus* (1.78%) and *Leiothecium* (1.22%). Their findings are consistent with our results regarding phyla and genera. Zhou et al. (2021) investigated the microbial community compositions in medium-high temperature Daqu and the Daqu-making environment by HTS and detected 31 genera in the Daqu and after fermentation. In particular, *Aspergillus* and *Rhizopus* were the most abundant in the Daqu, and upon fermentation, the dominant genera in the Daqu included *Aspergillus* ($39.14 \pm 0.21\%$), *Candida* ($6.39 \pm 0.31\%$) and *Rhizopus* ($0.3739 \pm 0.01\%$). After fermentation,

Thermoascus gradually became another dominant genus (23.96±0.01%). Hence, the fungal genera found in Daqu and during fermentation are consistent with our results. Chen et al.(2020b) analyzed the microbial community compositions in Taorong-type Daqu by HTS and found that except for the unclassified genera in 4 Daqu samples, other dominant fungal genera were *Thermoascus* (44.07%), *Aspergillus* (10.04%), *Thermomyces* (3.79%), *Alternaria* (1.78%), *Emericella* (1.02%), *Wickerhamomyces* (0.78%) and *Rhizomucor* (0.42%). Their findings on fungal genera are consistent with our results. The results of the above studies are similar to our HTS results. Nevertheless, we identified more fungal communities and found *Pseudeurotium* and *Papiliotrema* for the first time in the medium- and high-temperature Daqu of Taorong-flavored liquors.

The unique starter production process and flora environment are the fundamental causes of the diversity of fungal flora in Yangshao Taorong-type Daqu, and fragrance components are an important standard for the evaluation of Daqu and finished liquor products. Thus, HTS and HS–SPME–GC–MS were combined to analyze the major fungal compositions and fragrance components in medium- and high-temperature Taorong-type Daqu. The findings of this study will theoretically underlie the fungal flora library establishment and quality identification of Taorong-type Daqu.

Declarations

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Authors' contributions

YL and CP conceived the study. HL collected the samples. XL, HZ, XS, and LZ performed the experiments and analyzed the data. YH wrote the first version of the manuscript. SH and CP supported the research. All authors critically revised the manuscript and have approved its content.

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Availability of data and materials

The datasets used or analyzed during the preparation of the manuscript are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All of the authors consent to the publication of this manuscript in Annals of Microbiology.

Competing interests

The authors declare that they have no competing interests.

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References

1. Ai M, Qiu X, Huang J, Wu C, Jin Y, Zhou R (2019) Characterizing the microbial diversity and major metabolites of Sichuan bran vinegar augmented by *Monascus purpureus*. *Int J Food Microbiol* 292: 83-90.
2. Chen, M., Qin, A., Lam, J. W., & Tang, B. Z. (2020a). Multifaceted functionalities constructed from pyrazine-based AI Egen system. *Coordin Chem Rev* 422, 213472.
3. Chen M, Hou J, Zhang Z (2020b) Correlation between microbial diversity and volatile flavor components of Taorong-type Daqu. *China Brewing* 39(10): 54-60.
4. di Cologna NDM, Gómez-Mendoza DP, Zanoelo FF, Giannesi GC, de Alencar Guimarães NC, de Souza Moreira LR, Ricart CAO (2018) Exploring *Trichoderma* and *Aspergillus* secretomes: proteomics approaches for the identification of enzymes of biotechnological interest. *Enzyme Microb Tech* 109: 1-10.
5. Deng L, Mao X, Liu D, Ning XQ, Shen Y, Chen B, Luo HB (2020) Comparative analysis of physicochemical properties and microbial composition in high-temperature Daqu with different colors. *Front Microbiol* 3010.
6. Du, H., Wang, X., Zhang, Y., Xu, Y. (2019). Exploring the impacts of raw materials and environments on the microbiota in Chinese Daqu starter. *Int J Food Microbiol* 297, 32-40.
7. Fan, G., Sun, B., Fu, Z., Xia, Y., Huang, M., Xu, C., & Li, X. (2018). Analysis of physicochemical indices, volatile flavor components, and microbial community of a light-flavor Daqu. *J Am Soc Brew Chem* 76(3), 209-218.
8. Fan, G., Fu, Z., Teng, C., Liu, P., Wu, Q., Rahman, M. K. R., & Li, X. (2020). Effects of aging on the quality of roasted sesame-like flavor Daqu. *Bmc Microbiol* 20(1), 1-16.
9. Fan, W., Zhao, X., Du, G., Chen, J., Li, J., Zheng, J., Zhao, D. (2021). Metaproteomic analysis of enzymatic composition in Baobaoqu fermentation starter for Wuliangye baijiu. *Int J Food Sci Tech* 56(8), 4170-4181.
10. Gabriel, R., Prinz, J., Jecmenica, M., Romero-Vazquez, C., Chou, P., Harth, S., Singer, S. W. (2020). Development of genetic tools for the thermophilic filamentous fungus *Thermoascus aurantiacus*. *Bionotechnol Biofuels* 13(1), 1-15.
11. Hu, X., Wang, K., Chen, M., Fan, J., Han, S., Hou, J., Chi, L., Liu, Y., Wei, T., (2020). 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. *Food Res Int* 138, 109765.
12. Huang, X., Fan, Y., Meng, J., Sun, S., Wang, X., Chen, J., Han, B. Z. (2021). Laboratory-scale fermentation and multidimensional screening of lactic acid bacteria from Daqu. *Food Biosci*, 40, 100853.
13. Hong, J., Tian, W., & Zhao, D. (2020). Research progress of trace components in sesame-aroma type of baijiu. *Food Res Int* 137, 109695.
14. He, N. X., Bayen, S. (2020). An overview of chemical contaminants and other undesirable chemicals in alcoholic beverages and strategies for analysis. *Compr Rev Food Sci F.*, 19(6), 3916-3950.
15. Jin, Y., Li, D., Ai, M., Tang, Q., Huang, J., Ding, X., Zhou, R. (2019). Correlation between volatile profiles and microbial communities: a metabonomic approach to study Jiang-flavor liquor Daqu. *Food Res Int*, 121, 422-432.
16. Londoño-Hernández, L., Ramírez-Toro, C., Ruiz, H. A., Ascacio-Valdés, J. A., Aguilar-Gonzalez, M. A., Rodríguez-Herrera, R., Aguilar, C. N. (2017). *Rhizopus oryzae*-Ancient microbial resource with importance in modern food industry. *Int J Food*

Microbiol257, 110-127.

17. Liu, J., Chen, J., Fan, Y., Huang, X., Han, B. (2018). Biochemical characterisation and dominance of different hydrolases in different types of Daqu-a Chinese industrial fermentation starter. *J Sci Food Agr* 98(1), 113-121.
18. Li, W., Fan, G., Fu, Z., Wang, W., Xu, Y., Teng, C., Li, X. (2020). Effects of fortification of Daqu with various yeasts on microbial community structure and flavor metabolism. *Food Res Int* 129, 108837.
19. Singh, S., Madlala, A. M., Prior, B. A. (2003). *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *Fems Microbiol Rev* 27(1), 3-16.
20. Shi, F., Zhou, X., Zhou, Q., Tan, Z., Yao, M. M., Wei, B. D., Ji, S. J. (2018). Membrane lipid metabolism changes and aroma ester loss in low-temperature stored Nanguo pears. *Food Chem* 245, 446-453.
21. Suren S, Madlala A, Prior A.(2010). *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *Fems Microbiol Rev* 11(1): 3-16
22. Tang, H., Liang, H., Song, J., Lin, W., Luo, L. (2019). Comparison of microbial community and metabolites in spontaneous fermentation of two types Daqu starter for traditional Chinese vinegar production. *J Biosci Bioeng* 128(3), 307-315.
23. Wang, J., Wu, Z., Zhang, T., Wang, Y., Yang, B. (2019). High-level expression of *Thermomyces dupontii* thermophilic lipase in *Pichia pastoris* via combined strategies. *3 Biotech* 9(2), 1-9.
24. Wang, H., Su, W., Mu, Y., Zhao, C. (2021). Correlation Between Microbial Diversity and Volatile Flavor Compounds of Suan zuo rou, a Fermented Meat Product From Guizhou, China. *Front Microbiol* 12.
25. Xie, M., Lv, F., Ma, G., Farooq, A., Li, H., Du, Y., Liu, Y. (2020). High throughput sequencing of the bacterial composition and dynamic succession in Daqu for Chinese sesame flavour liquor. *J I Brewing*,126(1), 98-104.
26. Xue, Y., Tang, F., Cai, W., Zhao, X., Song, W., Zhong, J., ... & Shan, C. (2021). Bacterial Diversity, Organic Acid, and Flavor Analysis of Dachu and Ercha Fermented Grains of Fen Flavor Baijiu. *Front Microbiol* 12, 769290-769290.
27. Xue Y, Guo Z, Zhao H. (2020). Analysis of microbial diversity in Shihua Baijiu Daqu based on MiSeq technology. *China Brewing* 39(7):163-168.
28. Yi, Z., Jin, Y., Xiao, Y., Chen, L., Tan, L., Du, A., Zhao, H. (2019). Unraveling the contribution of high temperature stage to Jiang-flavor Daqu, a liquor starter for production of Chinese Jiang-flavor Baijiu, with special reference to metatranscriptomics. *Front Microbiol* 10, 472.
29. Yang, J. G., Dou, X., Ma, Y. Y. (2018). Diversity and dynamic succession of microorganisms during Daqu preparation for Luzhou-flavour liquor using second-generation sequencing technology. *J I Brewing*, 124(4), 498-507.
30. Yang, F., Zeng, X., Ning, K., Liu, K. L., Lo, C. C., Wang, W., Xu, J. (2012). Saliva microbiomes distinguish caries-active from healthy human populations. *ISME J*, 6(1), 1-10.
31. Zuo, Q., & Huang, Y. (2020). Evaluation of bacterial diversity during fermentation process: a comparison between handmade and machine-made high-temperature Daqu of Maotai-flavor liquor. *Ann Microbiol* 70(1), 1-10.
32. Zhou Tianci, He Hongki, Zhou Qingwu, Cao Rujie, Ma Yesheng, Duhai, & Xu Yan (2021) Source of high temperature large song microorganisms based on high-throughput amplicon sequencing technology. *Food and fermentation industry*, 47 (16), 66-71.

Figures

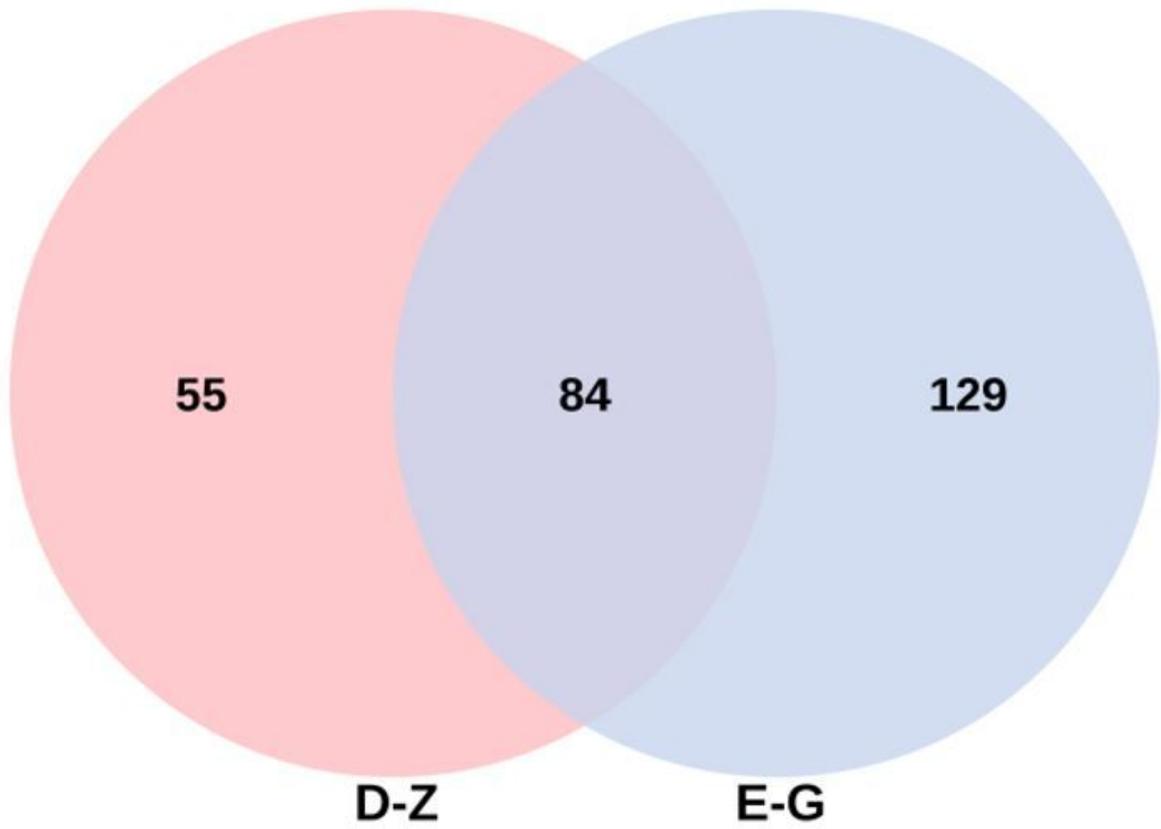


Figure 1

Venn diagrams of OTUs from medium-temperature Daqu and high-temperature Daqu

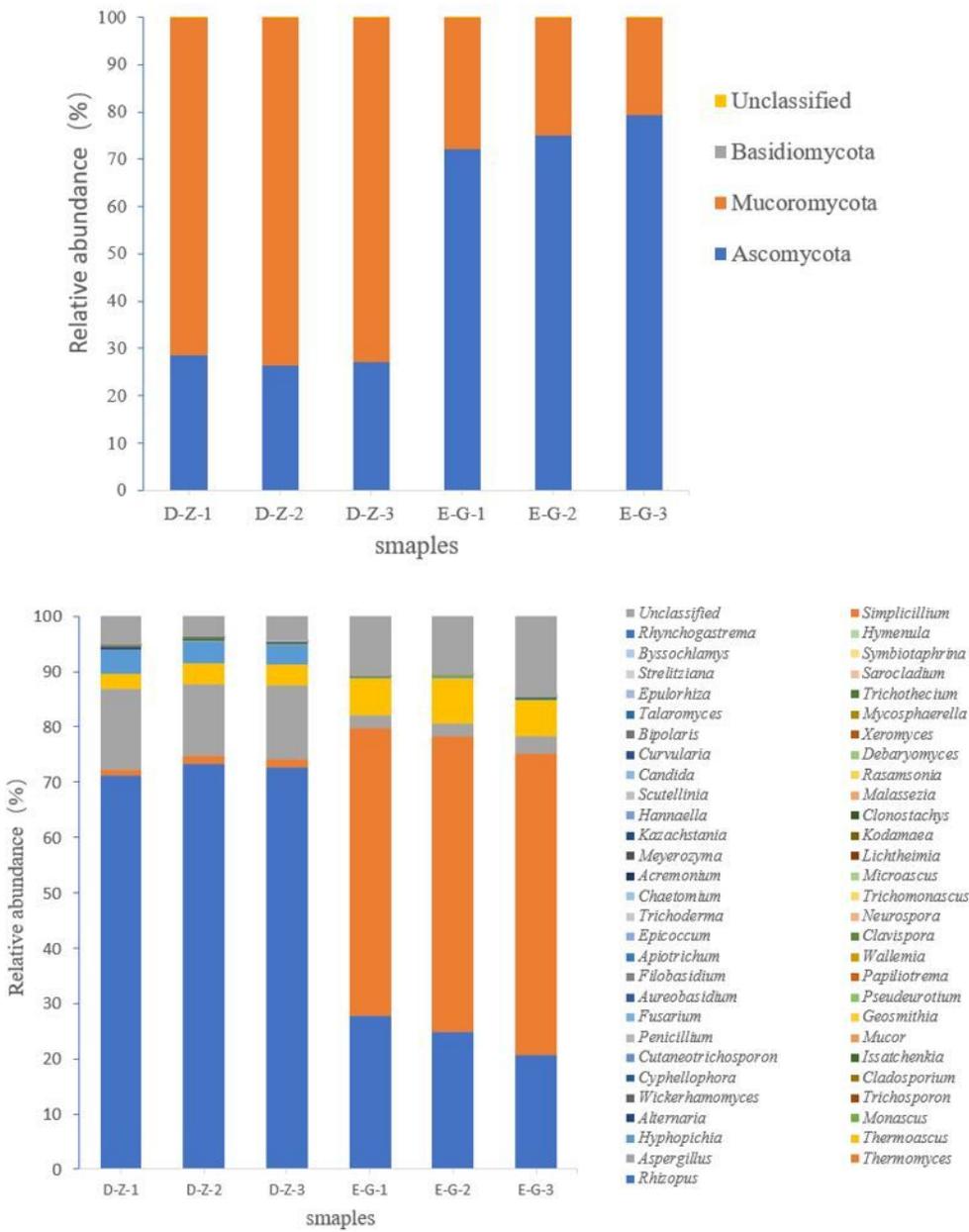


Figure 2

(A) Fungal taxonomy of medium- and high-temperature Daqu at the phylum level

(B) Fungal taxonomy of medium- and high-temperature Daqu at the genus level

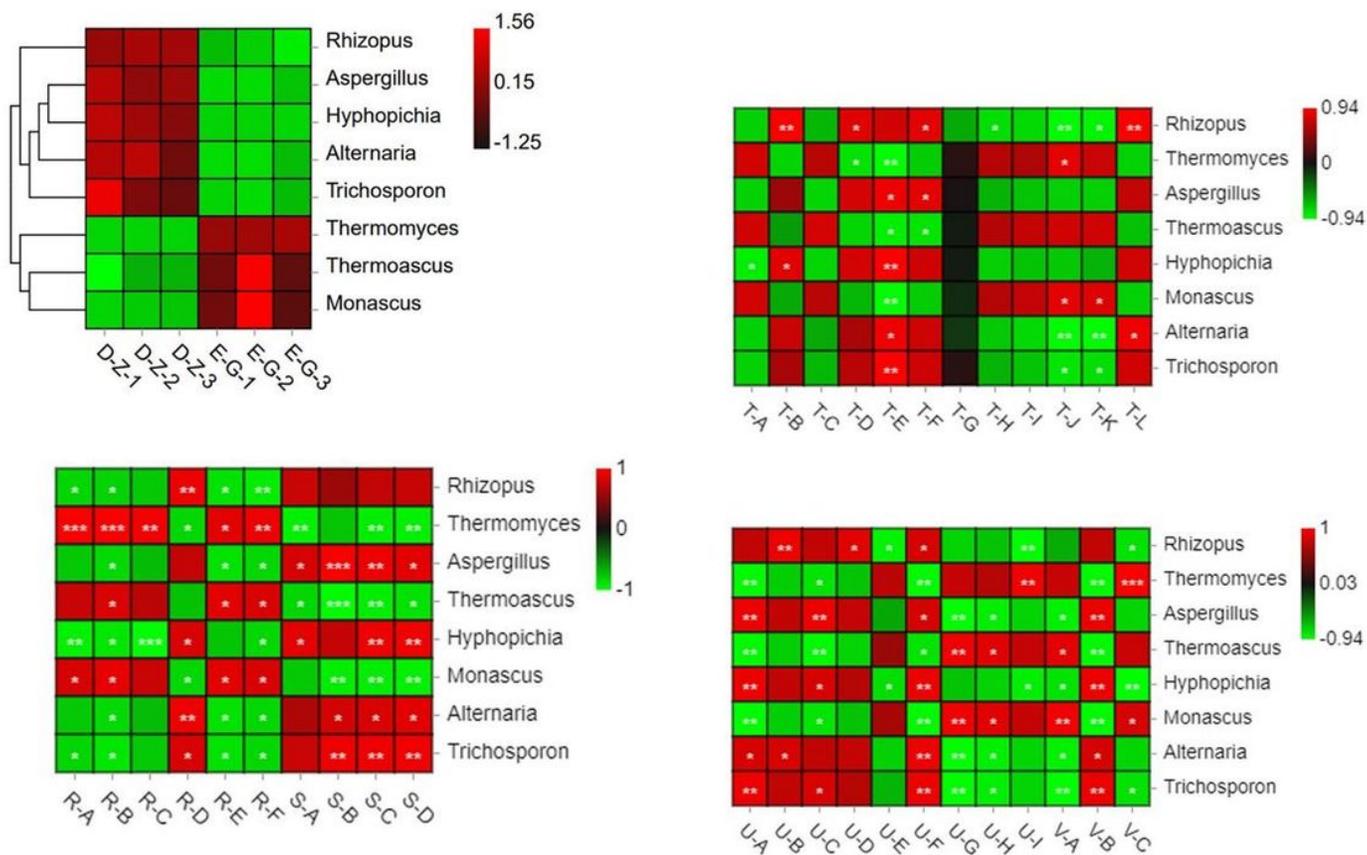


Figure 3

(A) Heatmap of the species distribution at the genus level

(B) Calculated Spearman correlation coefficients of microbes and flavor components and the corresponding plotted correlations of the qualified data