Research Article

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The differences in main components, enzyme activity, and microbial composition between substandard and normal jiuyao

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Abstract

BACKGROUND: Jiuyao is a critical fermenting agent in traditional huangjiu brewing and it affects the quality of huangjiu. To assess and monitor the quality of jiuyao effectively we determined the differences between two common types of substandard jiuyao and normal jiuyao, with emphasis on the comparison of the main components, enzymatic activity, volatile substances, and microbial community structure.

RESULTS: The water and starch content, acid protease activity, and esterification capability of type I substandard jiuyao were significantly lower than those of the normal jiuyao, and the protein contents, liquefaction capability, glycation capability, and neutral protease activity were substantially higher than those of the normal jiuyao. Type II substandard jiuyao had significantly lower indices than the normal group except for the starch and free amino acid content, which were significantly higher than those of the normal jiuyao. Significant differences were observed between substandard and normal jiuyao in the content of 21 volatile compounds. 2-Pentylfuran could be used as a marker of substandard jiuyao. Type I substandard jiuyao contained a higher abundance of aerobic *Pediococcus* and *Marivita* in comparison with the normal jiuyao. Type II substandard jiuyao consisted of a greater abundance of anaerobic *Mucor* and *Staphylococcus.*

CONCLUSION: The quality of jiuyao was significantly affected by the water content. Due to the different abundances of aerobic and anaerobic bacteria in jiuyao, oxygen may also be an important parameter affecting the quality of jiuyao. We believe that the present study offers a theoretical basis for the evaluation and control of the quality of jiuyao.

Supporting information may be found in the online version of this article.

Keywords: jiuyao; main component; microbial community structure; volatile substance; moisture

INTRODUCTION

Huangjiu is a traditionally brewed Chinese wine, one of the longest-standing varieties worldwide, which is very popular in East and Southeast Asia.[1] Jiuyao is an active fermenting agent used in the brewing of traditional huangjiu and performs the dual role of fermentation and saccharification.[2] Huangjiu is made from indica rice flour mixed with polygonum hydropiper and aged jiuyao. Some microorganisms in the jiuyao can provide amylase and protease for huangjiu brewing, which hydrolyze proteins and starch from raw materials such as rice and wheat into peptides, amino acids, oligosaccharides, and other components.[3] Jiuyao confers a richer flavor to huangjiu than modern commercial ferments,[4] which may be because of the presence of microorganisms such as *Pediococcus* and *Weissella* in the jiuyao in addition to *Rhizopus* and *Saccharomyces*.[5] Xie *et al.*[6] suggested that the jiuyao plays a crucial role in developing the unique flavor of Shaoxing huangjiu.

Traditional jiuyao production is subject to variation in production processes, seasonal changes, and different processing environments, rendering the quality of huangjiu variable.[7] Substandard jiuyao often contains stray bacteria accompanied by off flavors and compositional changes, which can cause fluctuations in the quality of huangjiu if the substandard jiuyao is used to brew it.[2] Unfortunately, the identification of substandard jiuyao is mainly

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dependent on the experience of production personnel in terms of appearance and odor, and no objective evaluation method is available. The differences between substandard jiuyao and normal jiuyao have not been reported yet.

The main objective of this study was to compare the differences between substandard and normal jiuyao in terms of their major components, enzymatic activity, volatile substances, and microbial communities and to analyze the main factors affecting the quality of jiuyao with a view to offering data for the evaluation and control of the quality of jiuyao.

MATERIALS AND METHODS

Jiuyao samples

Five batches of newly produced normal jiuyao (group A) and three batches of two types of bad jiuyao (groups B and C) newly produced in 2021 were selected as samples. The samples were obtained from a huangjiu factory in Shaoxing, Zhejiang province, and refrigerated at 4 °C. Substandard jiuyao was selected by experienced workers based on color, odor, and other characteristics.

Determining the hardness of jiuyao

A total of 15 intact jiuyao from each group were selected for determining hardness using a TMS-PRO mass spectrometer (Food Technology Corporation, USA) with a speed of 60 mm min^{-1} before and after the test, a starting force of 5 g, a deformation rate of 30%, and a cylindrical probe.[8]

Analysis of basic indicators

The method described by Ouzouni *et al.* was used to determine the moisture content.[9] Free amino acid content was analyzed using high-performance liquid chromatography (HPLC) (Agilent 1260II, santa, clara, california, USA);[10] 0.1 g jiuyao powder was mixed with 0.8 mL10% trichloroacetic acid in a 2 mL Eppendorf (EP) tube, and then left for 2 h at 4 °C after ultrasound for 3–5 min. After centrifugation at 9600×*g* for 5 min, 1 mL of supernatant was transferred into a liquid-phase vial by 0.22 μL water-phase filtration membrane. The sample size was 1 μL. The diode array detector (DAD) signal A wavelength was 338 nm and the bandwidth was 10 nm. The reference wavelength was 390 nm and the reference broadband was 20 nm. The time was 10.80 min. The total protein content was determined by the Kjeldahl nitrogen determination method,[11] and starch and reductive sugar contents were determined using 3,5-dinitrosalicylic acid (DNS).[12]

Determination of enzyme activity

All samples were freeze-dried completely. Their liquefaction and saccharification capacity was analyzed and optimized following methods reported previously.[13] The amount of starch liquefied from 1.0 g of jiuyao per hour in a sodium acetate buffer (pH 4.6) at 30 °C was determined as 1 unit of liquefaction capacity. The amount of glucose produced per hour by 1.0 g of jiuyao in sodium acetate buffer (pH 4.6) at 40 °C was determined as the glycation capacity of 1 unit. Ethyl hexanoate synthesized from 5.0 g of jiuyao in a combination of hexanoic acid and ethanol at 35 °C every 7 days was determined as 1 unit of esterification capacity.[14] The amount of CO_2 produced from carbohydrates by 1.0 g of jiuyao per 72 h at 30 °C was used as a measure of fermentation capacity.[15] Protease activity was determined according to Kasana's method.[16]

Analysis of volatile compounds

The volatile compounds of jiuyao were extracted and identified using headspace solid-phase microextraction and gas chromatography–mass spectrometry (HS-SPME-GC–MS).[17] The volatiles were extracted using 10 mm/80 μm Divinylbenzene/Carbon WR/Polydimethylsiloxane (DVB/CWR/PDMS) fibers (Agilent). Jiuyao was ground and sieved through an 80 mesh sieve, and 1.0 g of ground jiuyao was taken into a 15 mL headspace vial each time, to which 6 mL of ultrapure water, 2 g of NaCl, and 10 μL of standard (0.00423 mg mL^{-1}, 2-octanol) were added. They were sonicated for 3 min and then equilibrated at 45 °C for 15 min.

After extraction at 45 °C for 40 min, the extraction head was inserted into the gas chromatography–mass spectrometry (GC–MS) system inlet. The gas chromatography (GC) was sampled at 250 °C for 5 min, and the extraction head was aged at 270 °C for 10 min between the two samples to prevent sample contamination. The volatile compounds of jiuyao obtained by extraction were detected using a triple quadrupole gas chromatograph (8890–7000D, Agilent) equipped with a VF-WAXms capillary column (30 m × 0.25 mm × 0.25 μm, Agilent). As described in the study performed by Chunlin,[18] the GC–MS condition was set as follows: high-purity helium was used as a carrier gas (1.0 mL min^{-1}) with no shunt. The initial column temperature was 40 °C, which was not maintained, and the temperature was increased to 220 °C with 3 °C min^{-1}, which was maintained for 7 min. The ionization mode was Electron Ionization (EI), the electron energy was 70 eV; the voltage of the electron multiplier was 350 V; the temperature of the ion source was 230 °C; the temperature of the transmission line was 250 °C; the scanning mass ranged from 33 to 350 m/z, and the scanning speed was 3.00 scans/s.

A qualitative comparison of the volatile substances was performed by using the NIST database, and the material content was semi-quantitatively calculated by using 2-octanol as the internal standard. The calculation formula was as follows:

$$C = \frac{Ac}{Ais} \times Cis \tag{1}$$

where *C* refers to the content of volatile aromatic substances of jiuyao, *Cis* refers to the content of the internal standard, *Ac* is the peak area of volatile aromatic substances of jiuyao, and *Ais* is the peak area of the internal standard.

To improve the accuracy of the qualification process, *n*-alkanes ($C_3 \sim C_{30}$) and volatile standards were sampled under the same warming conditions to obtain the experimental retention indices. The experimental retention indices were further compared with those recorded in the literature,[19] and compounds with 50 or fewer differences were retained, while the appearance times of the standards were determined.

The retention index of the chemical composition in the sample was calculated according to the retention time of the mixed standard of $C_3 \sim C_{30}$ *n*-alkanes.[20] The formula was as follows:

$$RI = 100n + \frac{T_{R(x)} - T_{R(n)}}{T_{R(n+1)} - T_{R(n)}} \times 100 \tag{2}$$

where *RI* is the temperature-programmed retention index; *n* is the number of carbon atoms of *n*-alkanes; TR_{n+1} and TR_n are the retention times of *n*-alkanes with carbon number *n* and naphth1/min; TR_x is retention time/min, and $TR_{n+1} > TR_x > TR_n$.

DNA extraction and high-throughput sequencing

Total genomic DNA was extracted from 1 g of fresh jiuyao samples using a kit (DNA1000, Agilent) by following the method described by Chen.[21] The v4 region of the bacterial 16 S rDNA gene was

amplified using 16 S primers 515F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACHVGGGTW-TCTAAT-3′). The fungal gene was amplified using ITS primers ITS1-1F-F (5′-CTTGGTCATTTA-GAGGAAGTAA-3′) and ITS1-1F-R (5′-GCTGCGTTCTTCATCGAT-GC-3′). These sequences were clustered into operational taxonomic units (OTUs) at a 97% threshold using UPARSE.[22] The samples were sequenced on the MiSeq platform (Novogene Bioinformatics, Beijing, China).

Statistical analysis

Data analysis was performed using Origin 2021, Canoco 5, and SPSS Statistics 25. Prior to redundancy analysis (RDA) and orthogonal partial least-squares discrimination analysis (OPLS-DA), weighted calculations were used to predict 16 S rDNA and ITS rDNA based on high-throughput sequencing data.[23] Shannon, Chao1,Simpson, and the abundance-based coverage estimator (ACE) indices were used to evaluate alpha diversity. A single-factor ANOVA was used to detect significant differences among the jiuyao samples. All the experiments were repeated three times, and the results were expressed as means ± standard deviations.

RESULTS

Physical and chemical indices of jiuyao

As shown in Fig. 1, no significant difference was observed in jiuyao hardness between the substandard and normal groups ($P > 0.05$). The water content in the substandard groups I and II was significantly lower than that in the normal group ($P < 0.05$), and the starch and free amino acid content was much lower in type I substandard jiuyao than that in the normal group, whereas the starch and free amino acid content of type II substandard jiuyao was significantly higher than that of the normal group ($P < 0.05$). The reducing sugar content in type I and II substandard jiuyao was significantly lower than that in the normal group ($P < 0.05$). The

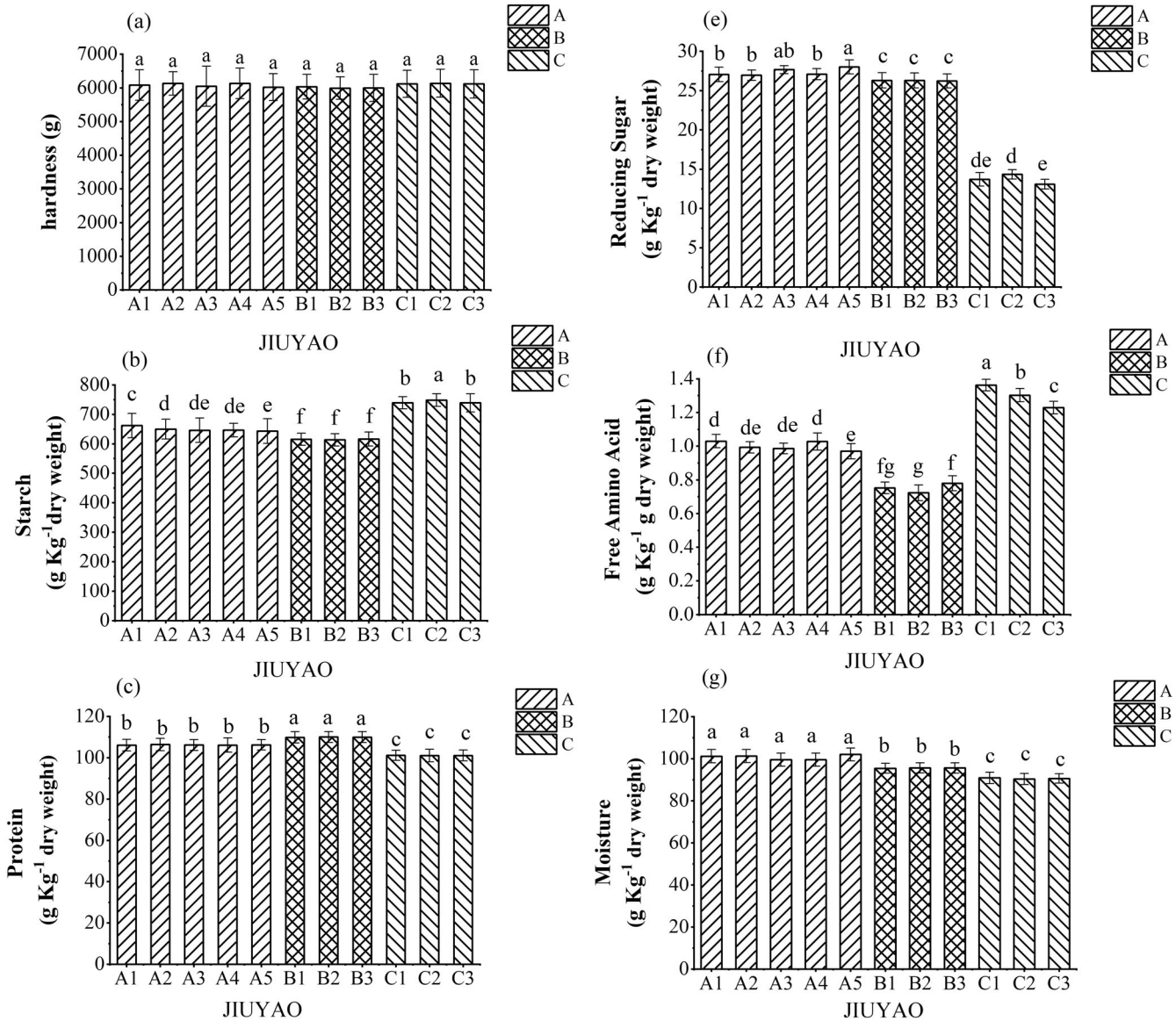

Figure 1. Physical and chemical indices of normal and substandard jiuyao. (a), (b), (c), (d), (e), and (f) indicate hardness, starch, protein, reducing sugar, free amino acid, and water content of the jiuyao, respectively. A, B, and C are normal jiuyao, type I substandard jiuyao, and type II substandard jiuyao, respectively. When the letters on the upper side of the error bars are different, it means there is a significant difference ($P < 0.05$), and when they are the same, it means there is no significant difference ($P > 0.05$).

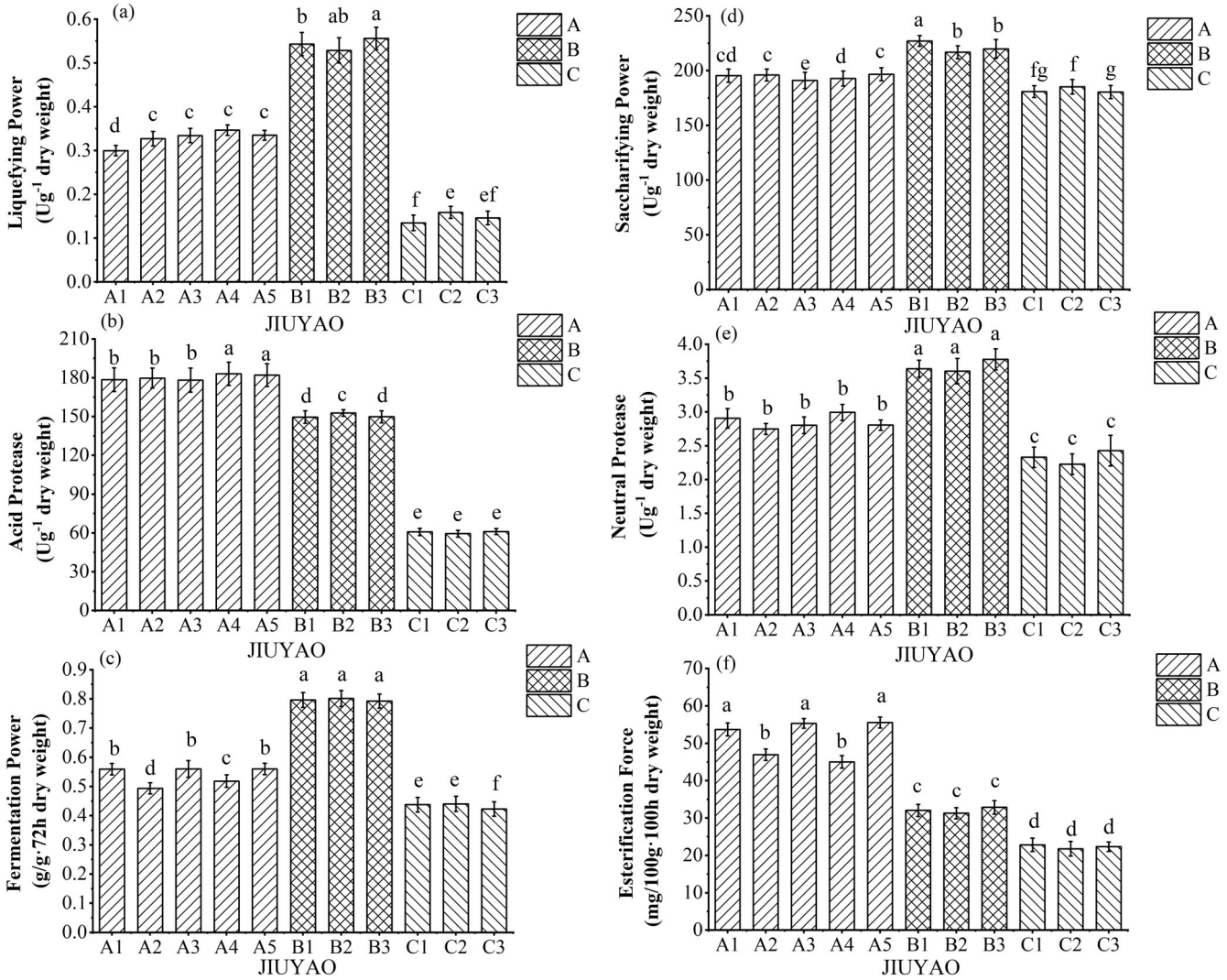

Figure 2. Enzyme activity of normal and substandard jiuyao. (a), (b), (c), (d), (e), and (f) are the liquefaction capacity, acid protease activity, fermentation capacity, saccharification capacity, neutral protease activity, and esterification capacity of the jiuyao, respectively. A, B, and C are normal jiuyao, type I substandard jiuyao, and type II substandard jiuyao, respectively. When the letters on the upper side of the error bars are different, it means there is a significant difference ($P < 0.05$), and when they are the same, it means there is no significant difference ($P > 0.05$).

protein content of the type I substandard group was significantly higher than that of the normal group, whereas the protein content of type II substandard group was lower than that of the normal group ($P < 0.05$).

Differences in the enzyme activity of different jiuyao

As shown in Fig. 2, compared with the normal jiuyao, the liquefaction capacity, fermentation capacity, saccharifying capacity, and neutral protease activity of type I substandard jiuyao were significantly higher than those in the normal group ($P < 0.05$), whereas the esterification capacity and acid protease activity were significantly lower than those in the normal group ($P < 0.05$). Compared with the normal group, all enzyme activity indices of type II substandard jiuyao were significantly lower than those in the normal group ($P < 0.05$).

Differences in volatile compounds in the jiuyao with varying quality

The results of the volatile compound compositions of different jiuyao are presented in Table 1.

The volatile components in different jiuyao samples were detected by HS-SPME-GC–MS. A total of 38 compounds were detected in 11 jiuyao samples, including two olefins, 11 alcohols, nine esters, eight aldehydes, four acids, one furan, two phenols, and one ketone. The content of six compounds such as hexanoic acid, ethyl ester, and cyclooctyl alcohol in type I substandard jiuyao were considerably higher than that of the normal group ($P < 0.05$), and the content of 19 compounds, including hexanal, heptanal, and nonanal, in type I substandard jiuyao was noticeably lower than in the normal group ($P < 0.05$). Type II substandard jiuyao contained 16 substances, including butanal, 3-methyl-, butanoic acid, ethyl ester, hexanoic acid, and ethyl ester, whose content was significantly higher than in the normal group ($P < 0.05$), and the content of 12 substances, including octanal and butanoic acid, 3-methyl-, in type II substandard jiuyao was significantly lower than in the normal group ($P < 0.05$). The content of hexadecanoic acid, ethyl ester, and phenethyl alcohol in the two groups of substandard jiuyao are significantly higher those in the normal group ($P < 0.05$).

Table 1. Volatiles detected in substandard and normal jiuyao

No.	Specimens RT/min	Name	Normal group (μg) kg^{-1} wet base	Substandard group 1 (μg kg^{-1}) wet base	Substandard group 2 (μg kg^{-1}) wet base
1	2.84	Ethyl acetate	7.60 ± 0.10^{a}	6.04 ± 0.28^{b}	4.99 ± 1.19^{c}
2	3.15	Butanal, 3-methyl-	3.86 ± 0.17^{b}	3.23 ± 0.12^{b}	12.01 ± 3.91^{a}
3	4.54	Isobutyl acetate	1.86 ± 0.57^{a}	not detected	not detected
4	5.00	Butanoic acid, ethyl ester	0.67 ± 0.02^{b}	not detected	1.56 ± 0.09^{a}
5	5.98	Hexanal	7.68 ± 3.38^{a}	1.66 ± 0.17^{c}	5.36 ± 1.15^{b}
6	6.44	2-Methyl-1-propanol	0.85 ± 0.03^{b}	not detected	2.95 ± 0.95^{a}
7	7.04	1-Butanol, 3-methyl-, acetate	5.76 ± 1.74^{a}	not detected	0.63 ± 1.10^{b}
8	9.00	Heptanal	1.58 ± 0.53^{a}	0.53 ± 0.30^{b}	0.47 ± 0.82^{b}
9	10.65	2-Pentylfuran	1.39 ± 0.49^{a}	not detected	not detected
10	10.74	Hexanoic acid, ethyl ester	1.26 ± 0.02^{c}	5.86 ± 1.66^{b}	10.56 ± 2.52^{a}
11	11.37	1-Pentanol	5.27 ± 0.61^{c}	7.00 ± 0.47^{b}	8.68 ± 0.83^{a}
12	12.78	Octanal	3.24 ± 1.04^{a}	1.57 ± 0.22^{b}	1.85 ± 1.27^{b}
13	15.39	Hexanol	52.25 ± 5.57^{b}	48.16 ± 7.37^{c}	89.42 ± 9.41^{a}
14	16.92	Nonanal	8.53 ± 0.94^{b}	4.94 ± 0.47^{c}	9.26 ± 9.05^{a}
15	17.38	3-Octen-2-one	5.22 ± 2.89^{a}	0.36 ± 0.06^{c}	2.08 ± 0.11^{b}
16	19.31	1-Octen-3-ol	16.41 ± 2.26^{a}	8.68 ± 1.83^{b}	11.31 ± 1.33^{b}
17	19.46	1-Heptanol	8.25 ± 0.25^{b}	11.29 ± 2.02^{b}	40.47 ± 4.11^{a}
18	19.64	Acetic acid	6.99 ± 1.17^{a}	15.30 ± 3.45^{a}	7.84 ± 5.40^{a}
19	21.86	1-Heptanol	8.25 ± 1.97^{b}	8.47 ± 1.40^{b}	52.29 ± 17.55^{a}
20	23.52	1-Octanol	20.81 ± 2.66^{a}	10.74 ± 2.67^{b}	20.56 ± 6.12^{a}
21	24.23	2,3-Butanediol	14.40 ± 2.49^{a}	3.88 ± 0.63^{a}	9.20 ± 8.67^{a}
22	25.63	Cyclooctyl alcohol	0.83 ± 0.15^{b}	2.40 ± 0.17^{a}	0.78 ± 0.66^{b}
23	26.54	Benzeneacetaldehyde	8.96 ± 0.21^{b}	10.31 ± 1.91^{b}	26.38 ± 5.97^{a}
24	27.43	1-Nonanol	8.18 ± 1.24^{b}	6.32 ± 1.39^{b}	12.27 ± 2.30^{a}
25	27.99	Butanoic acid, 3-methyl-	2.13 ± 0.24^{a}	0.71 ± 0.12^{b}	0.98 ± 0.19^{b}
26	29.57	α-Cedrene	1.04 ± 0.11^{ab}	1.10 ± 0.09^{b}	1.85 ± 0.57^{a}
27	30.49	Pentanoic acid	0.78 ± 0.06^{a}	1.18 ± 0.08^{a}	0.84 ± 0.44^{a}
28	30.76	(+)-DELTA-CADINENE	1.43 ± 0.06^{b}	1.01 ± 0.10^{b}	4.15 ± 2.00^{a}
29	31.87	Benzeneacetic acid, ethyl ester	3.43 ± 0.59^{a}	1.24 ± 0.31^{b}	1.24 ± 0.97^{b}
30	32.86	Acetic acid, 2-phenylethyl ester	1.83 ± 0.50^{a}	not detected	not detected
31	34.25	Hexanoic acid	8.35 ± 1.78^{a}	2.75 ± 0.42^{b}	2.90 ± 0.64^{b}
32	35.01	Benzyl alcohol	5.52 ± 0.93^{b}	15.11 ± 1.45^{a}	17.30 ± 6.63^{a}
33	36.13	Phenethyl alcohol	80.60 ± 11.34^{b}	175.56 ± 10.20^{a}	159.49 ± 8.78^{a}
34	36.41	Butylated hydroxytoluene	15.00 ± 2.49^{a}	6.36 ± 1.89^{b}	15.32 ± 1.48^{a}
35	39.77	gamma-Nonanolactone	2.04 ± 0.40^{a}	1.74 ± 0.33^{a}	1.98 ± 1.21^{a}
36	40.07	4-Ethyl-2-methoxypheno	2.14 ± 0.43^{a}	1.96 ± 0.43^{a}	2.73 ± 2.07^{a}
37	47.15	Hexadecanoic acid, ethyl ester	1.81 ± 0.21^{b}	6.13 ± 0.57^{a}	6.83 ± 1.22^{a}
38	48.74	(2,6,6-Trimethyl-2-hydroxycyclohexylidene) acetic acid lactone	0.95 ± 0.16^{b}	1.00 ± 0.08^{b}	2.21 ± 0.60^{a}

Different letters in the same row indicate significant differences ($P < 0.05$).

Microbial composition of different jiuyao

Bacterial and fungal community structures in jiuyao of different quality were analyzed by high-throughput sequencing and dilution curves, whereas Shannon indices were calculated to determine the reliability of the data, and microbial community structures in different quality samples of jiuyao were compared using species relative abundance maps.

As shown in Fig. **S1** in the supporting information, the increase in OTUs gradually decreased as the sequencing increased, with a tendency to level off, although it had not yet reached saturation. However, as the number of sequenced strips increased, the Shannon index constantly increased, and the curve flattened out, eventually reaching saturation, thus indicating that sequencing met the requirements.[24]

Analysis of microbial alpha diversity of jiuyao

The alpha diversity analysis of different jiuyao samples was performed using the QIIME 2 platform,[25] including the ACE, Chao1, Shannon, and Simpson indices. As shown in Fig. S2 in the supporting information, the ACE and Chao1 indices of bacterial alpha diversity in the type I substandard jiuyao group were the highest, whereas the Shannon and Simpson indices in the normal jiuyao group were higher than those in the substandard group. The results showed that type I substandard jiuyao had high microbial diversity, whereas the bacteria in the normal jiuyao group had high homogeneity. Regarding fungi, the four indices of the jiuyao samples in the normal group were higher than those in the substandard jiuyao samples, and the fungal community in the normal jiuyao group showed higher species evenness and alpha diversity.

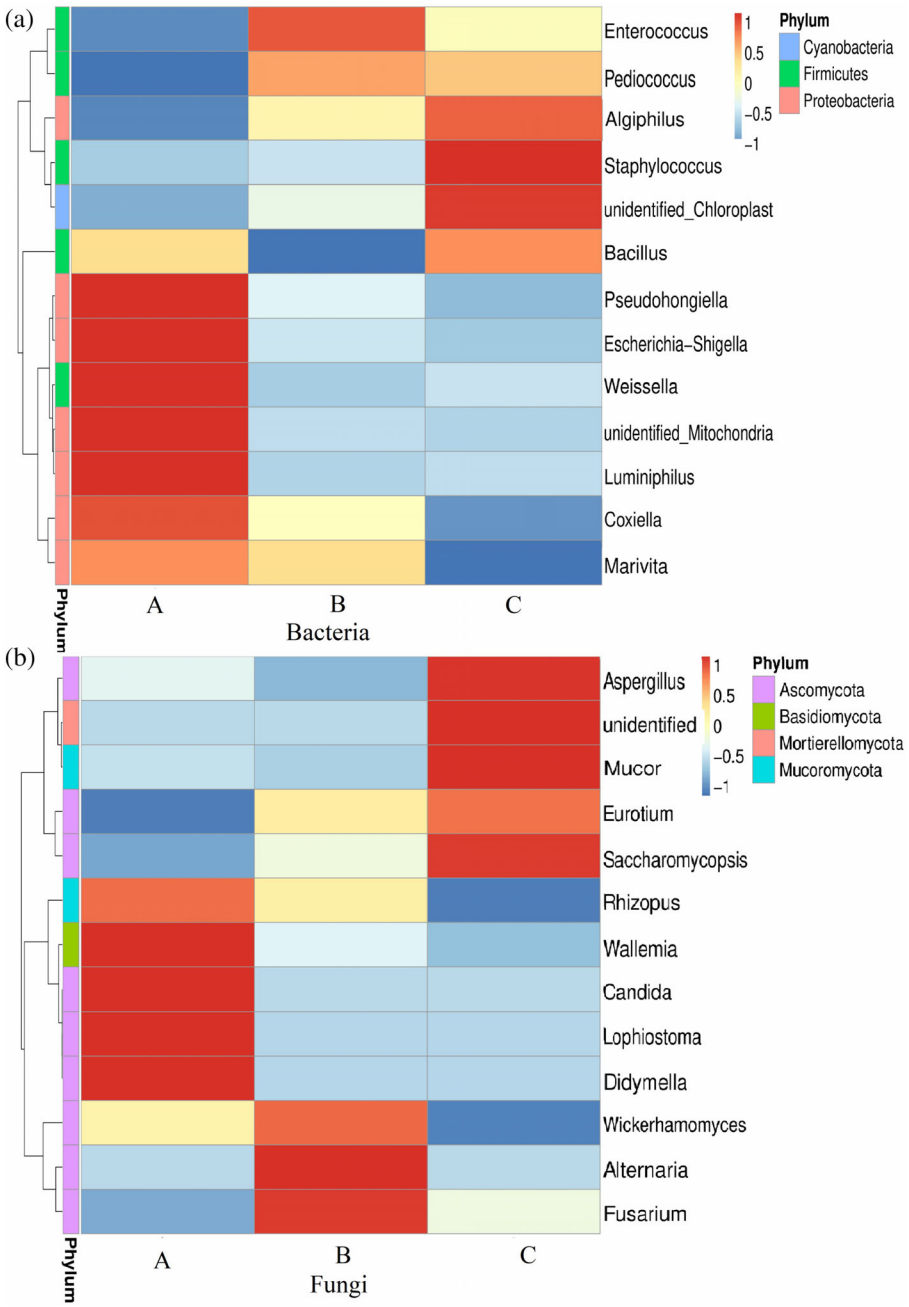

Figure 3. Relative abundance of microbial communities in normal and substandard jiuyao at the genus level.

Differences in the microbial compositions of different jiuyao

At the genus level, 220 bacterial genera were identified from 11 jiuyao samples. The bacterial species with higher relative abundance in the normal jiuyao samples were *Pediococcus* (6.272%), *Weissella* (0.401%), *Luminiphilus* (0.086%), *Staphylococcus* (0.093%), *Escherichia-Shigella* (0.143%), *Marivita* (0.067%), *Coxiella* (0.039%), *Enterococcus* (0.068%), *Bacillus* (0.051%), and *Algiphilus* (0.013%). Significant differences were observed in bacterial community distribution between the normal and substandard jiuyao samples. Compared with type I and type II substandard jiuyao groups, the normal jiuyao showed a higher abundance of *Weissella, Limnobacter*, and *Pseudohongiella*. A high abundance of *Enterococcus* was observed in type I substandard jiuyao, whereas type II substandard jiuyao showed a higher abundance of *Staphylococcus* (Fig. 3(a)).

A total of 87 fungal genera were identified, including *Saccharomycopsis* (68.411%), *Rhizopus* (31.527%), *Aspergillus* (0.023%), *Wallemia* (0.015%), *Mucor* (0.006%), *Pseudomycetes* (*Candida*) (0.004%), *Fusarium* (0.002%), *Wickerhamomyces* (0.001%), *Phaeosphaeria* (0.001%), and *Peridiomycetes* (*Mortierella*) (0.001%), which showed a high relative abundance in the normal samples. Significant differences in the distribution of fungal communities among different substandard groups of jiuyao were observed, and *Wallemia*, *Lophiostoma*, *Didymella*, and *Candida* were highly abundant in the normal group. *Alternaria* and *Fusarium* showed greater abundance in type I substandard jiuyao, whereas *Aspergillus*, *Mucor*, and

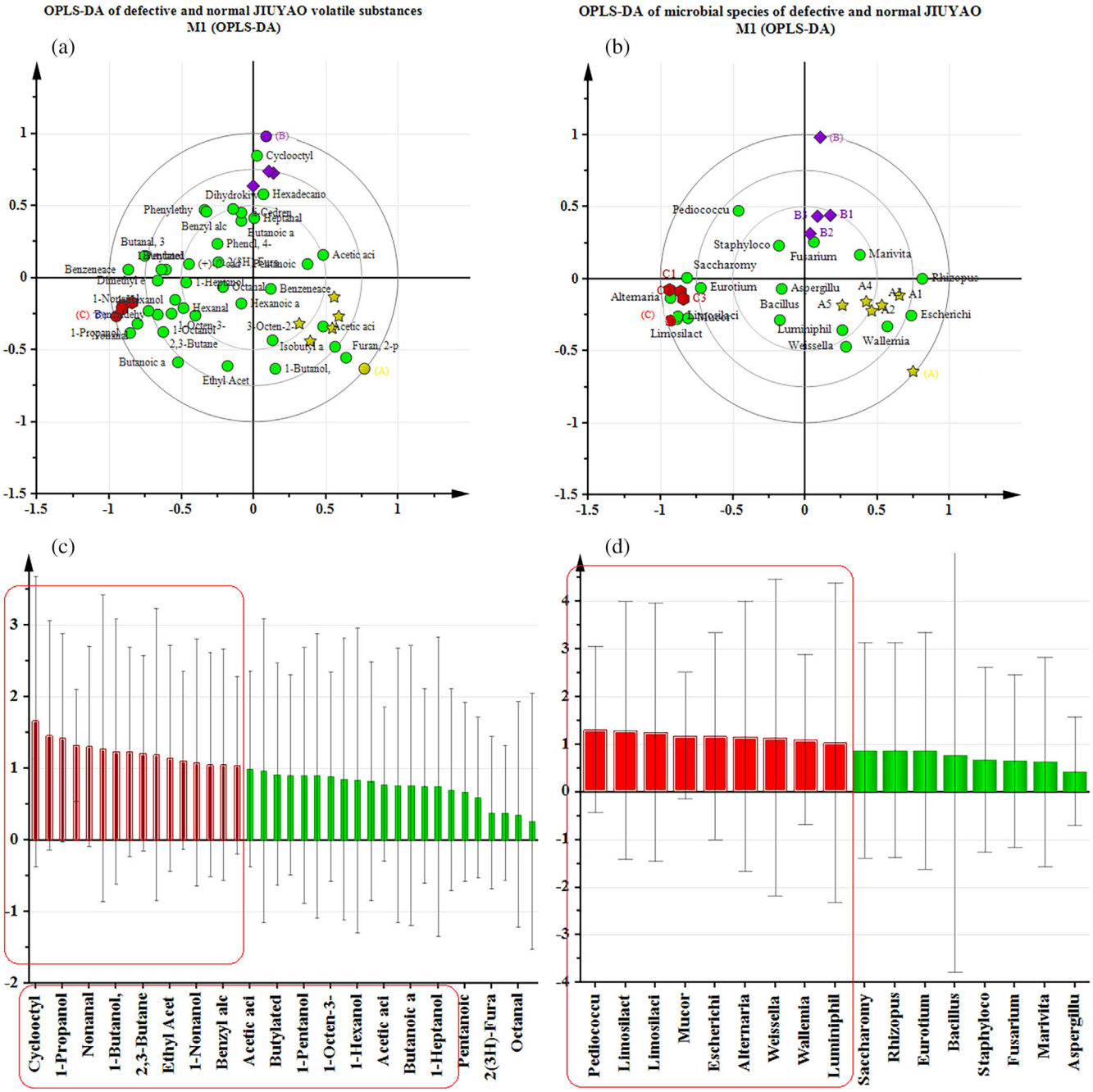

Figure 4. Orthogonal partial least-squares discrimination analysis of substandard and normal jiuyao. (a), (b) score chart, substance; microorganism (green circle), A (normal jiuyao, yellow, pentacle); B (type I substandard jiuyao, purple, diamond); C (type II substandard jiuyao, red, hexagonal), (c) Variable importance in project value of volatile substance, (d) Variable importance in project value of microbial genus.

Saccharomycopsis were highly abundant in type II substandard jiuyao (Fig. 3(b)).

Orthogonal partial least-squares discrimination analysis of substandard and normal jiuyao

Orthogonal partial least-squares discrimination analysis is a supervised analysis model that uses partial least squares regression to establish volatile components.[26] By establishing the relationship model between indicators and sample categories, combined with data dimension reduction, the relationship between samples can be better established.[27] In addition, OPLS-DA can predict the sample categories. By constructing classification prediction models, OPLS-DA can be further used to identify more samples. This cannot be achieved by principal component analysis (PCA).[28]

The OPLS-DA of the jiuyao is shown in Fig. 4. The cyclooctyl alcohol, 2-pentyl-furan, 1-nonanol, and so forth, in the volatile substances of jiuyao are in the 75–100% confidence interval and can be explained by this model. *Limosilactobacillus*, *Rhizopus*, and *Alternaria* in microorganisms are in the 75–100% confidence interval, which can be explained well by the model. Variable importance in project (VIP) was used to screen markers that could characterize the difference between substandard and normal

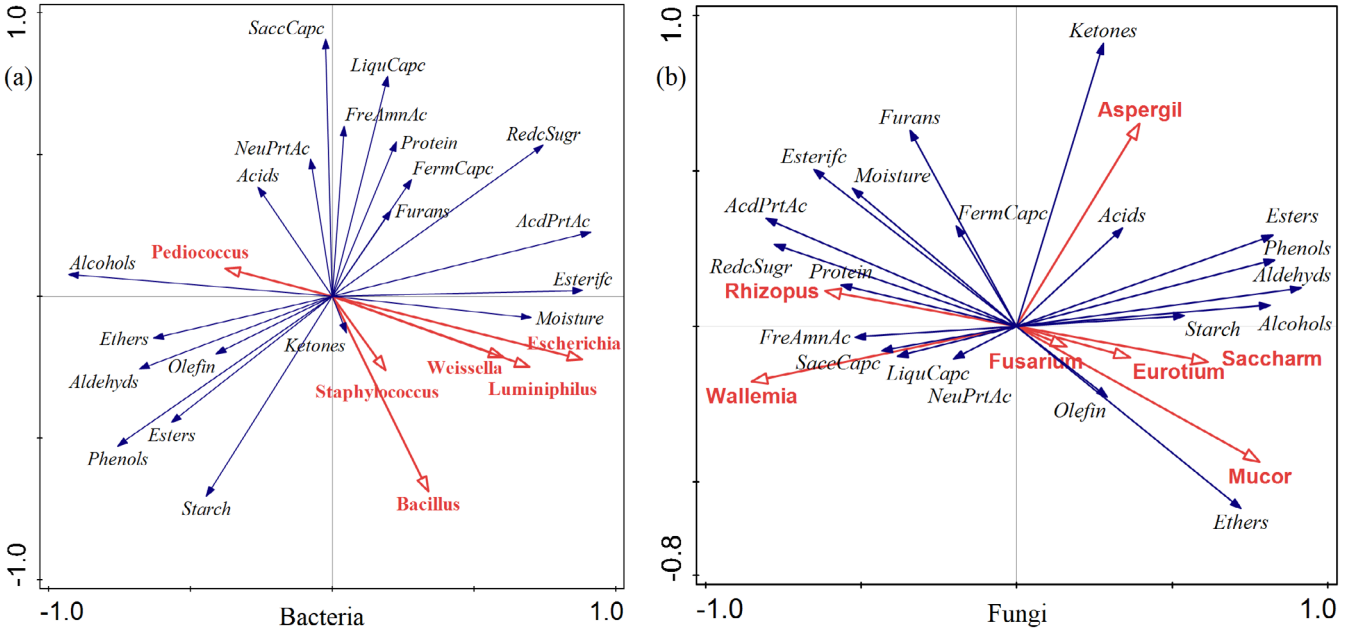

Figure 5. Correlation of jiuyao indicators with representative microorganisms. The length of arrows in the figure represents the correlation size, and the angle between the center point and the corresponding microbial genus and the factors is acute to indicate a positive correlation, whereas the obtuse angle indicates a negative correlation. The red arrows represent jiuyao microbial genera, and the blue arrows represent jiuyao correlation indices.

jiuyao.[29] The components with a VIP value greater than 1 were the main marker components reflecting the differences between samples, when the VIP values less than or equal to 1, it means the samples was hard to discrimination.[30] The VIP results are shown in Fig. 4(c), (d).

Sixteen volatile substances with VIP values greater than 1 were screened, such as cyclooctyl alcohol (VIP = 1.65); 2-pentylfuran (VIP = 1.46); 1-pentanol (VIP = 1.46); butanoic acid, ethyl ester (VIP = 1.32) and so forth, 9 microbial genus with VIP value greater than 1, such as *Pediococcus* (VIP = 1.31); *Mucor* (VIP = 1.18); *Weissella* (VIP = 1.10) and so forth.

According to Fig. 4 and Table 1, the aroma components – cyclooctyl alcohol, hexadecanoic acid, ethyl ester in type I substandard jiuyao was significant positive correlation to *Alternaria*, and 1-nonanol, 1-pentanol, nonanal were mainly relate to *Mucor*. Normal jiuyao were significantly positively correlated with 2-pentylfuran, acetic acid, *Weissella* and *Wallemia*.

Redundancy analysis of the microbial and physicochemical indicators of different jiuyao

Redundancy analysis is a type of constrained ranking analysis and a variant of PCA. It has strong advantages in the analysis of datasets composed of species and environmental factors.[31] Environmental changes can affect the distribution of microbial community, and the analysis of environmental factors can provide an important theoretical basis for standardizing production and improving jiuyao fermentation control.

The RDA of the jiuyao is shown in Fig. 5. It involved six bacterial species, seven fungal species, and 20 physicochemical factors. Regarding bacterial communities in the jiuyao, the physicochemical factors were as follows: 35.6% for bacterial community species distribution, 55.3% for water content ($P = 0.04$), and 5.9% for esterification capacity ($P = 0.056$). *Lamellococcus* and *Pediococcus* were significantly correlated with alcohols. *Bacillus* and *Staphylococcus* showed a significant correlation with ketones. *Weissella*, *Escherichia*, and *Luminiphilus* were significantly correlated with moisture content and esterification. For fungi, fungal species distribution explained 46.2%, with acid protease activity explaining 46.7% ($P = 0.038$), liquefaction capacity explaining 17.1% ($P = 0.166$), and esterification power explaining 15.4% ($P = 0.003$) of the fungi. *Rhizopus* was strongly correlated with protein content, reducing sugar content, and acid protease activity. *Wallemia* was strongly correlated with liquefaction capacity, saccharification capacity, and free amino acid content. *Aspergillus* was significantly associated with acids and ketones. *Saccharomycopsis*, *Fusarium*, *Mucor*, and *Eurotium* were associated with ethers, olefins, and alcohols.

DISCUSSION

Visual variation was most evident in the physicochemical parameters of different quality jiuyao. Water content was a common controllable parameter in the fermentation process, and we found strong correlations between water content and microbial composition as well as the abundance of various jiuyao by RDA. The highest water content was approximately 10% in the normal group of jiuyao (Fig. 1), significantly higher than that in the substandard group ($P < 0.05$). Specifically, water content was highest in the normal jiuyao, followed by type I substandard jiuyao and type II substandard jiuyao.

Enzyme activity can indicate the fermentation ability of jiuyao. Jiuyao plays a bilateral fermentation role in the brewing process of huangjiu, and the difference in saccharification capacity of jiuyao affects the quality of huangjiu. Significant differences were observed among the three groups of jiuyao regarding saccharification capacity, and the highest saccharification capacity of type I substandard jiuyao was 231.85 U g^{-1}. Type I substandard jiuyao showed the best saccharification capacity, followed by the normal jiuyao and type II substandard jiuyao.

Eleven volatile substances differed significantly between the substandard and normal jiuyao, among which the contents of

hexadecanoic acid, ethyl ester, and phenethyl alcohol were significantly higher in the substandard jiuyao group than in the normal group.

Taken together, we suggested that the water content, saccharification capacity, hexadecanoic acid, ethyl ester, and phenethyl alcohol content could be regarded as crucial markers to evaluate the quality of jiuyao. The jiuyao type of defects can be determined by detecting the contents of cyclooctyl alcohol and 1-nonanol. Determining the differences between jiuyao by monitoring these indicators is intuitive and efficient.

Rhizopus possesses strong amylase activity[32] and can produce organic acids such as fumaric, malic, and succinic acids.[33] The abundance of *Rhizopus* in the substandard jiuyao was significantly lower than that in the normal group, which may result in insufficient amylase activity to hydrolyze starch into reducing sugars.[34] As *Rhizopus* can produce esterase,[35] this might be responsible for the significant difference between esterification capacities of the normal and substandard jiuyao group. *Rhizopus* can produce aromatic esters[36] the abundance of *Rhizopus* in the two substandard groups was significantly lower than that in the normal group, which may lead to the low content of isobutyl acetate, 1-Butanol, 3-methyl-, acetate and other esters in the substandard jiuyao.[36]

Bacillus is a gram-positive genus that produces strong proteases, causing differences in the efficiency of protein hydrolysis to amino acids in jiuyao,[37] and changes in the abundance of *Bacillus* may affect protein and amino acid content of the substandard jiuyao. *Bacillus* and ketones exhibited a significant positive correlation, and the abundance of *Bacillus* in the substandard jiuyao was significantly higher than that in the normal group, which probably led to the increase in 3-octen-2-one content in type II substandard jiuyao.

The abundance of *Weiss* spp. was significantly higher in the normal group than that in the substandard group, which may have contributed to the higher acidic protease activity and fermentation capacity,[38] observed in the normal jiuyao group.

Aerobic *Pediococcus* showed a higher abundance in type I substandard jiuyao than in type II jiuyao, whereas strictly aerobic *Marivita* showed a higher abundance in type I substandard jiuyao than in type II substandard jiuyao.[39]

Type II substandard jiuyao contained a large abundance of anaerobic or partially anaerobic *Algiphilus* and *Saccharomycopsis*; *Mucor* can grow well under poorly ventilated conditions,[40] and *Rhizopus* can grow without oxygen, so the poor aeration facilitated the growth and reproduction of *Rhizopus*. According to the growth of different microorganisms in the substandard jiuyao, we speculated that oxygen concentration in the production process of type I substandard jiuyao was higher than that in the normal group, whereas the amount of oxygen in type II substandard jiuyao was lower than that in the normal group. The difference in oxygen content may have led to the change of microbial species and abundance in the jiuyao, affecting the mycelial growth of the jiuyao and thus causing differences in jiuyao quality. *Eurotium* can grow under low water activity conditions.[41] As type II substandard jiuyao had the lowest water content, *Eurotium* was highly abundant in type II substandard jiuyao, suggesting that water content may be another important factor in the production of substandard jiuyao.

The quality of jiuyao can be controlled by managing oxygen concentration and moisture content in the environment to reduce the production of substandard jiuyao during subsequent fermentation.

CONCLUSION

In this study, the quality differences between normal and substandard jiuyao were compared. Various key indicators and core microbial communities of the normal jiuyao were determined, and water content was the principal contributor to the production of the substandard jiuyao. Water content, glycolytic enzyme activity, esterification force and cyclooctyl alcohol, nonanal, and 2-pentylfuran content can be used as key indicators for rapid discrimination of jiuyao quality in the future. *Marivita*, *Mucor*, and *Alternaria* can be used as representative microorganisms in substandard jiuyao. The analysis of microbial abundance and growth characteristics in the substandard jiuyao revealed a potential relationship between the microbial abundance and oxygen requirements of different substandard jiuyao, and oxygen concentration during the maturation of surface jiuyao may also be an important reason for the difference in the quality of jiuyao. However, the most suitable oxygen concentration for microbial growth in jiuyao remains poorly defined, and further research is required to understand the effect of moisture content and oxygen concentration on the growth and metabolism of jiuyao microbial communities.

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DECLARATION OF COMPETING INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Guoliang Zhao: Methodology, software, investigation, writing the original draft. Zhilei Zhou: Supervision, writing – review. Zhijiang Li: Formal analysis, validation. Shuangping Liu: Writing – editing. Zhichu Shan: Sampling. Fei Cheng: Sampling. Weibiao Zhou: Writing – editing. Jian Mao: Project administration, funding acquisition, conceptualization.

CONFLICT OF INTEREST

No conflict of interest exits in the submission of this manuscript and all authors are aware of and accept responsibility for the manuscript. The work has not been published previously and it is not under consideration for publication elsewhere, either in whole or in part. All authors have approved the manuscript.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Xu EB, Long J, Wu ZZ, Li H, Wang F, Xu X *et al.*, Characterization of volatile flavor compounds in Chinese rice wine fermented from enzymatic extruded rice. *J Food Sci* **80**:C1476–C1489 (2015).
2 Liu ZB, Wang ZY, Lv XC, Zhu XP, Chen LL and Ni L, Comparison study of the volatile profiles and microbial communities of Wuyi Qu and

Gutian Qu, two major types of traditional fermentation starters of Hong Qu glutinous rice wine. *Food Microbiol* **69**:105–115 (2018).
3 Liu SP, Mao J, Liu YY, Meng XY, Ji ZW, Zhou ZL *et al*., Bacterial succession and the dynamics of volatile compounds during the fermentation of Chinese rice wine from Shaoxing region. *World J Microbiol Biotechnol* **31**:1907–1921 (2015).
4 Liu SP, Chen QL, Zou HJ, Yu Y, Zhou Z, Mao J *et al*., A metagenomic analysis of the relationship between microorganisms and flavor development in Shaoxing mechanized huangjiu fermentation mashes. *Int J Food Microbiol* **303**:9–18 (2019).
5 Huang ZR, Guo WL, Zhou WB, Li L, Xu JX, Hong JL *et al*., Microbial communities and volatile metabolites in different traditional fermentation starters used for Hong Qu glutinous rice wine. *Food Res Int* **121**:593–603 (2019).
6 Xie GF, Li WJ, Lu J, Cao Y, Fang H, Zou HJ *et al*., Isolation and identification of representative fungi from Shaoxing rice wine wheat Qu using a polyphasic approach of culture-based and molecular-based methods. *J Inst Brewing* **113**:272–279 (2007).
7 Zhao G and Zhang G, Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *J Appl Microbiol* **99**:333–338 (2005).
8 Jia G, Yueming T, Fangyao L, Yuxiao T and Jinghong L, Quality changes of fresh-cut yams under different shelf-life temperatures. *Food Ferment Ind* **6**:98–102 (2022).
9 Ouzouni PK, Petridis D, Koller WD and Riganakos KA, Nutritional value and metal content of wild edible mushrooms collected from West Macedonia and Epirus, Greece. *Food Chem* **115**:1575–1580 (2009).
10 Zheng J, Ma L, Wang F and Shi Y, Determination of amino acids in healthcare wine by HPLC method. *J Instrum Anal* **29**:970–973 (2010).
11 Song S, Zhang J and Wang H, Determination total nitrogen in the Kjeldahl digests of soil samples by continuous flow analyzer in comparison with automated distillation-titration instrument. *Soil Fert Sci China* **5**:207–212 (2019).
12 Luo K, Kim NG, You SM and Kim YR, Colorimetric determination of the activity of starch-debranching enzyme via modified Tollens' reaction. *Nanomaterials* **9**(9):1291 (2019).
13 Li P, Lin WF, Liu X, Wang XW and Luo LX, Environmental factors affecting microbiota dynamics during traditional solid-state fermentation of Chinese Daqu starter. *Front Microbiol* **7**:253 (2016).
14 Yong Y, Yanrong L, Lei J, Yawei J and Qibiao W, Study on the difference and change regulation of biochemical indices between parts of medium and high temperature Daqu. *Food Ferment Ind* **19**:73–78 (2019).
15 He GQ, Dong Y, Huang J, Wang X, Zhang S, Wu C *et al*., Alteration of microbial community for improving flavor character of Daqu by inoculation with Bacillus velezensis and Bacillus subtilis. *LWT--Food Sci Technol* **111**:1–8 (2019).
16 Kasana RC, Salwan R and Yadav SK, Microbial proteases: detection, production, and genetic improvement. *Crit Rev Microbiol* **37**:262–276 (2011).
17 Guo M, Chai C, Lu X, Wang T and Fan H, Development and applications of HS-SPME-GC-MS technology on detection of volatile flavor components in aquatic product. *Sci Technol Food Ind* **35**:368 (2014).
18 Chunlin Z, Zonghua AO, Weiqiang C, Caihong S, Wenyi TAO and Suyi Z, Rapid analysis of volatile compounds in Daqu by headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry. *Food Sci* **32**:137–140 (2011).
19 Babushok VI and Zenkevich IG, Retention indices for Most frequently reported essential oil compounds in GC. *Chromatographia* **69**:257–269 (2009).
20 Zhang X, Bai B, Cheng S, Luo F and Ji S, Effects of endogenous enzyme on the volatile component of broccoli by GC-MS-AMDIS combined with retention index. *J Chin Inst Food Sci Technol* **20**:278–286 (2020).
21 Chen C, Liu Y, Tian H, Ai L and Yu H, Metagenomic analysis reveals the impact of JIUYAO microbial diversity on fermentation and the volatile profile of Shaoxing-jiu. *Food Microbiol* **86**:103326 (2020).
22 Wu D, Wei ZM, Zhao Y, Zhao X, Mohamed TA, Zhu L *et al*., Improved lignocellulose degradation efficiency based on Fenton pretreatment during rice straw composting. *Bioresour Technol* **294**:122132 (2019).
23 Langille MGI, Zaneveld J, Caporaso JG *et al*., Predictive functional profiling of microbial communities using 16 S rRNA marker gene sequences. *Nat Biotechnol* **31**:814 (2013).
24 Xu W, Jiang Y, Wu D, Zhao R and Zhu Q, RNA sequencing analysis of fungi community diversity in mildew raw dark tea. *J Tea Sci* **37**:483–492 (2017).
25 Estaki M, Jiang L, Bokulich NA, McDonald D, González A, Kosciolek T *et al*., QIIME 2 enables comprehensive end-to-end analysis of diverse microbiome data and comparative studies with publicly available data. *Curr Protoc Bioinformatics* **70**:e100 (2020).
26 Bylesjo M, Rantalainen M, Cloarec O, Nicholson JK, Holmes E and Trygg J, OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. *J Chemom* **20**:341–351 (2006).
27 Chung IM, Kim JK, Han JG, Kong WS, Kim SY, Yang YJ *et al*., Potential geo-discriminative tools to trace the origins of the dried slices of shiitake (Lentinula edodes) using stable isotope ratios and OPLS-DA. *Food Chem* **295**:505–513 (2019).
28 Li SY, Zhu BQ, Reeves MJ and Duan CQ, Phenolic analysis and theoretic design for Chinese commercial wines' authentication. *J Food Sci* **83**:30–38 (2018).
29 Yu S, Liu G, Mei C and Cheng J, VIP optimal neural network inverse system soft sensingmethod in bio-fermentation process. *J Nanjing Univ Sci Technol* **39**:447–451 (2015).
30 Zhou H-X, Liu H, Han X, Nie SJ, Zhang RP, Yu JY *et al*., Application of UPLC-QTOF-MS in analysis of non-targeted urine metabolomics in rats with Yunaconitine poisoning. *Fa Yi Xue Za Zhi* **37**:653–660 (2021).
31 Wu XG, Wang Y, Zhu Y, Tian H, Qin X, Cui C *et al*., Variability in the response of bacterial community assembly to environmental selection and biotic factors depends on the immigrated bacteria, as revealed by a soil microcosm experiment. *Msystems* **4**(6):e00496–00419 (2019).
32 Zong-wen P, Jing-juan L, Hai-xia SU and Yu-xiu CEN, Effect of amylase system from Rhizopus and Aspergillus oryzae on ethanol fermentation with raw cassava starch. *Sci Technol Food Ind* **30**:189 (2009).
33 Kloetzer L, Tucaliuc A, Galaction AI and Cascaval D, Fractionation of dicarboxylic acids produced by Rhizopus oryzae using reactive extraction. *Sci Rep* **12**:2020 (2022).
34 Lei C, Zhen-Ming C, Zhe C and Mei L, Amylase production by Saccharomycopsis fibuligera A11 in solid-state fermentation for hydrolysis of cassava starch. *Appl Biochem Biotechnol* **162**:252–263 (2010).
35 Zhuang SW, Smart K and Powell CD, The relationship between wort sugar concentration and yeast carbon partitioning during brewing fermentations. *J Am Soc Brew Chem* **77**:225–234 (2019).
36 Ning Z, Gao G and Li S, Effects of three starters on volatile flavor compounds in Chongming rice wine. *Ind Microbiol* **46**:8–15 (2016).
37 Shizhang Y, Shuai SHI, Haixiang XU and Xingpu QI, Protein degradation in fermented duck. *Jiangsu J Agric Sci* **27**:401–404 (2011).
38 Shu-kun W, Jun X, Tie-yuan C, Chun-hui W, Zhi-guo H and Jie D, Correlation between quality index and bacterial community of Luzhou flavor daqu. *Food Res Dev* **40**:158–164 (2019).
39 Budinoff CR, Dunlap JR, Hadden M and Buchan A, Marivita roseacus sp nov., of the family Rhodobacteraceae, isolated from a temperate estuary and an emended description of the genus Marivita. *J Gen Appl Microbiol* **57**:259–267 (2011).
40 Kurakov AV, Khidirov KS, Sadykova VS and Zviagintsev DG, Anaerobic growth ability and alcohol fermentation activity of microscopic fungi. *Prikl Biokhim Mikrobiol* **47**:187–193 (2011).
41 Gock MA, Hocking AD, Pitt JI and Poulos PG, Influence of temperature, water activity and pH on growth of some xerophilic fungi. *Int J Food Microbiol* **81**:11–19 (2003).