Analysing the Component Changes and Microbial Community Structures in Huangjiu undergoing Rancidification

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Abstract

In an effort to develop rapid tests to grade the quality of rancidification characteristics during the traditional pottery jar storage process of Huangjiu, the microbial community structures and associated compositional variation at different stages of the rancidification were analyzed. A comparative analysis of component variations between rancidification and normal Huangjiu samples was carried out by HPLC. Microbial pollution induced rancidification by affecting alcohol strength, acidity, amino acid nitrogen, and conductivity indexes. Moreover, microelements, such as organic acids, amino acids, and bioamine also changed significantly. According to the PacBio RS II analysis of J-10 and J-13, J-10 contained 10 bacteria genera, 3 fungi genera, and 1 unclassified binuclear subkingdom fungus, at a significantly higher relative abundance compared to that of J-13 (p-value<0.01). The relative abundance of only 1 bacterium genus and 1 fungus genus in J-10 was significantly lower compared to those in J-13 (p-value<0.01). This indicated that microbial community structures vary in Huangjiu depending on the rancidification time. Increasing rancidification time caused the relative abundance of microbial community structure to also increase. Collectively, this study can serve as a reference point for researchers and industry partners interested in discussing the influence of rancidification on the quality, health safety, and industrialized development of Huangjiu.

Introduction

As a traditional brewed wine from China, Huangjiu is fermented from rice. The special liquor is rich in nutrients and far less alcoholic than distilled liquor. Moreover, high-quality Huangjiu requires long-term storage in pottery jars to improve the flavors as well as the quality [1]. Therefore, preventing microbial contamination and liquor degradation during the storage of Huangjiu is key in the quality control industry. As early as 800 years ago, the ancient Tang Dynasty adopted wine cooking techniques as a means to sterilize it. This technique is conducive to the long-term storage of Huangjiu [2]. The application of wine sterilization techniques dates back to Louis Pasteur’s study of sterilization and storage techniques of grape wines in the nineteenth century. This sterilization technique could inhibit microorganisms without using preservatives like sulfur dioxide (SO₂) as is done when preserving red wine [3].

Although techniques like pasteurization can assure the storage quality of most Huangjiu, semi-finished products undergo poor preparation and storage as they are operated in semi-open (non-GMP) environments. The sterilized liquor, pottery jar walls, and pottery jar sealing materials all are vulnerable to secondary microbial contamination in air, despite the Huangjiu having been processed by high-temperature sterilization at 85~90°C. Therefore, there are still microbial residues after sterilization [4]. Moreover, pottery jars are likely to suffer sealing damages, wall seepage and other problems during storage, which would only increase the probability of microbial contamination in Huangjiu. The resulting degradation, increased acidity, and development of unpleasant odors lower the quality of the final product [5]. Moreover, the probability of rancidification of Huangjiu increases as the storage time prolongs. Rancidification of Huangjiu during long time storage not only costs time but also creates economic losses to the wine industry [6]. Currently, the quality of Huangjiu is usually examined based on the sensory perception of various characteristics in the industry. Quality monitoring of Huangjiu products is usually performed against physiochemical indexes of online sensors. For example, conductivity is used to study and monitor the quality stability of white wine and grape wine during storage [7,8]. However, why aging raw wine shows rancidification in some pottery jars after long-term storage but not others remain a mystery. This phenomenon has plagued the Huangjiu industry for a long time.

The rancidification process of Huangjiu can generally be divided into two classes: bacterial biofilm rancidification (acetic acid rancidification) and non-bacterial biofilm rancidification (lactic acid rancidification). Of the two methods, lactic acid rancidification causes greater damage to Huangjiu [9]. Recent studies on causes of rancidification in Huangjiu have mainly focused on individual microorganisms, such as members of the Lactobacillus genus, acetic acid bacteria, and Pseudomonas. Due to limitations by technologies, research of microorganisms that cause rancidification of Huangjiu have mainly employed traditional isolation culture, polymerase chain reaction (PCR), and 16S rDNA sequencing [10]. Mao et al.
(2014) separated two new bacterial strains (including members of the Lactobacilli genus) from the aging Huangjiu undergoing rancidification by using a special growth medium [11]. Chen et al. (2014) separated the acid-producing bacteria in raw Huangjiu undergoing rancidification by using Potato Dextrose Agar (PDA) Medium and High Performance Capillary Electrophoresis (HPCE) detection methods. Using these techniques, they separated a total of 8 strains (OM-1~OM-8) [10]. Xie et al. (2008) analyzed how pollutants in rancid Huangjiu affected the wine without including bloating, by using performing PCR and 16S rDNA sequencing of Pseudomonas and Achromobacter strains [12]. Liu et al. (2018) identified 36 key microorganisms that caused rancidification of Huangjiu during storage by using high-throughput sequencing and traditional separation culture technology, including 28 varieties of Lactobacillus acetotolerans and 8 varieties of Lactobacillus fructivorans [13]. High-throughput sequencing technology can determine simultaneous sequences from hundreds of thousands of DNA molecules. The fast development of this technology is currently at fourth-generation iteration [14]. However, third-generation sequencing techniques are relatively advanced and widely applied in microbiology. Some third-generation techniques include Single-Molecule Real-Time (SMRT) and Oxford Nanopore Technologies [15,16]. Despite these advances, very few studies have applied unimolecular sequencing techniques towards the identification and characterization of microorganisms that cause the rancidification Huangjiu. Although Liu et al. (2018) discussed 36 key microbial strains that caused rancidification Huangjiu during storage by combining the third-generation sequencing and traditional separation cultural methods, they determined only two species of Lactobacillus: Lactobacillus acetotolerans and Lactobacillus fructivorans [9]. These studies and techniques provide some basis for the discussion of major microorganisms in the rancidification process. However, the cause of microbial flora-induced rancidification Huangjiu remains unknown. Moreover, the relationship between the rancidification time of Huangjiu and the internal microbial community structure has not been explored.

In this study, changes in key components that might be affected by the rancidification of Huangjiu were analyzed. Using a combination of conductivity assays, a method to rapid detect the quality of Huangjiu was also developed. This novel technique could be used for eliminate spoiled Huangjiu and improve quality control level of Huangjiu. Moreover, unimolecular sequencing techniques (such as PacBio SMRT sequencing technique and PacBio RS II) helped analyze microbial community structure in rancidifying Huangjiu during aging process. Simultaneously, sequencing results were processed by the QIIME platform to determine the microbiological composition (from the phylum level to the species level) in rancidifying Huangjiu. The research outcomes from this study will provide a theoretical basis for the quality control of Huangjiu during storage.

1. Materials And Methods

1.1 Experimental samples

The untreated and rancidifying Huangjiu samples were obtained from Shanghai Jinfeng Wine Co., Ltd. Samples were derived from the same batch of Huangjiu that was stored in pottery jars for 3 years. Of these samples, the sample J-13 had a turbid appearance at sampling as it was undergoing rancidification. However, the sample J-10 was relatively clear in appearance as the rancidification had completed, thus causing abundant bacterial sludge flocculation at the bottom of pottery jars.

1.2 Major reagents and instruments

Organic acids (e.g. ketoglutaric acid, pyruvate, lactic acid, and succinic acid), amino acids (e.g. tyrosine, glutamate, arginine, and histidine), bioamines (β-phenylethylamine, putrescine, cadaverine, histamine, and tyramine) and alkane reagents (e.g. methanol and acetonitrile) used in this study were pre-determined to be chromatographically pure. Other reagents, including phenyl isothiocyanate, triethylamine, n-hexane, acetic acid, phosphoric acid, and potassium dihydrogen phosphate, were also of analytical pure grade. These reagents were purchased from Anpel-chem chemical reagent (Shanghai) Co., Ltd. Waters high-performance liquid chromatography (HPLC) 2695, which was equipped with diode array
detector 2996, was applied to samples. Other instruments that were used include vortex mixers, 10 mL tubes with a plug, a thermostat water bath, an ultrasonic cleaner, a Metler-toledo electronic analytical balance (accuracy to 0.0001 g), a 0.45 μm needle microporous membrane filter (organic phase), a pipette, a Metler-toledo conductivity meter F38, and a Metrohm 702 potentiometric titrator.

1.3 Test of rancidification physiochemical indexes in Huangjiu

Test methods to determine the alcohol content, pH, total acid, and amino acid nitrogen were based on the national standard GB/T 13662-2018 Huangjiu.

Conductivity: Hanna HI 99300 conductivity meter was applied to measure the test conductivity (σt) of samples at a ‘T’ temperature. According to the formula, \( \sigma_{25} = \sigma_t + (25-T) \times 15 \), the test result was transformed to the conductivity of samples at 25°C.

HPLC method of amino acids was also conducted, as previously performed [17]. Derivatives of phenyl isothiocyanate were used for the derivation reactions with free amino acids. An HPLC (Waters HPLC 2695, equipped with diode array detector 2996) was also conducted as a part of this test. The instrumentation included: chromatographic columns: Waters C18 column (5 μm, 250 mm×4.6 mm); column temperature 40 °C; flow velocity: 1.0 mL·min\(^{-1}\); sample size: 10.0 μL; ultraviolet detection wavelength 254 nm; mobile phase A. 16.4 g sodium acetate anhydrous was dissolved in an appropriate amount of water, to which 0.5 mL triethylamine was also dissolved till a final volume of 10 L was reached. Mobile phase B: acetonitrile + water= 8+2 (volume ratio); the elution gradient procedure is listed in Table 1.

**Table 1 Gradient elution procedure for HPLC**

<table>
<thead>
<tr>
<th>Time/min</th>
<th>0</th>
<th>2</th>
<th>10</th>
<th>12</th>
<th>19</th>
<th>21</th>
<th>31</th>
<th>33</th>
<th>36</th>
<th>38</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase A</td>
<td>92</td>
<td>92</td>
<td>90</td>
<td>81</td>
<td>74</td>
<td>65</td>
<td>54</td>
<td>0</td>
<td>0</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Mobile phase B</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>19</td>
<td>26</td>
<td>35</td>
<td>46</td>
<td>100</td>
<td>100</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

HLPC methodology used for assessing bioamines abided by the national standards GB/T 5009.208-2008 Test of Bioamine Content in Foods. HPLC (Waters HPLC 2695, equipped with diode array detector 2996) was used for these tests. The method is introduced as follows: Waters C18 column (5 μm, 250 mm × 4.6 mm); column temperature: 30 °C; flow velocity: 1.0 mL·min\(^{-1}\); sample size: 10.0 μL; UV detection wavelength: 254 nm; Mobile phase A: acetonitrile solution. Mobile phase B: ultrapure aqueous solution. The elution gradient procedure is listed in Table 2.

**Table 2 Gradient elution procedure for HPLC**

<table>
<thead>
<tr>
<th>Time/min</th>
<th>0</th>
<th>9</th>
<th>23</th>
<th>32</th>
<th>37</th>
<th>38</th>
<th>47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase A</td>
<td>50</td>
<td>63</td>
<td>65</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mobile phase B</td>
<td>50</td>
<td>37</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

Organic acids were tested by HPLC (Waters HPLC 2695, equipped with diode array detector 2996) directly. The method is introduced as follows: chromatographic column: Waters C18 column (5 μm, 250 mm×4.6 mm) Column temperature: 30 °C; flow velocity: 1.0 mL·min\(^{-1}\); sample size: 10.0 μL; UV detection wavelength: 210 nm; Mobile phase A: methanol solution. Mobile phase B: 0.01 mol·L\(^{-1}\) monopotassium phosphate solution. Mobile phase C: ultrapure aqueous solution. The elution gradient procedure is listed in Table 3.
1.5 Extraction of microorganism DNA in rancidification samples

1.5.1 Acquisition of thallus

Two rancidification samples (45.0 mL) were collected in 50 mL centrifuge tubes. They were centrifuged (5810, Eppendorf) at 10000 g and 25°C. The supernatant was eliminated and sufficient sediment was collected. The content of each tube was added to a 1.0 mL high salt extract (100 mmol/L Tris-HCl pH8.0, 100 mmol EDTA, 100 mmol/L Na₃PO₄, 1.5 mol/L NaCl) and the mixture was suspended evenly and recollected in 2 mL centrifuge tubes. They were further centrifuged at 12000 g for 10 min at 25°C before obtaining the bacterial sediment.

1.5.2 Microorganism DNA extraction

The bacterial sediments were added to 0.5 mL of above high salt extracts and mixed evenly. The mixture was frozen for 10 minutes at -20°C. Next, the samples were placed in an ice bath and crushed to break open the cell walls, using the JY92-Ⅱ ultrasonic crasher (Scientz Biotechnology Co., LTD) under the following conditions: 10000 g, 15s, repeated 3-5 times. The samples were incubated with lysozyme (10 μL, 50 mg/mL) for 30 minutes at 37°C. 125 μL 10% SDS was added to the mixture, along with 5 μL proteinase K (20 mg/mL). The mixture was incubated at 37°C for 1 h and oscillated gently every 20 min to evenly mix it. 1 mL CTAB buffer solution (1.5 mol/L NaCl, 1% CTAB, 100 mmol/L Tris-HCl pH 8.0, 100 mmol/L EDTA, pH 8.0) was added and the mixture was heated in a 65°C water bath for 1 h, with gentle shaking every 20 min. An equal volume of chloroform-isoamylol (volume ratio=24:1) was added and mixed before the mixture was centrifuged at 12000 g for 10 min at 4°C. The addition of static caused the mixture to separate into three layers. The upper aqueous layer containing DNA was collected in 2 mL centrifuge tubes. The above operation was repeated 2-3 times until the middle layer was devoid of impurities. 0.6 times the volume of isopropanol was added and mixed gently. The mixture was left to sediment at -20°C for at least 1 h before being centrifuged for 10 min at 12000 g and 4°C. The supernatant was removed and the sediment containing nucleic acids was collected. The addition of 1 mL 70% ethanol helped wash the sediment and the procedure was repeated 2-3 times. 50 μL sterile water was used to dissolve the DNA and 1 μL RNA enzyme (10 mg/mL RNaseA) kept the DNA intact. The mixture was incubated in a 37°C water bath for 1-2 h to eliminate RNA. The collected DNA samples were stored at -20°C until they were retrieved for later use.

1.6 PCR amplification of bacterial 16S rDNA gene and fungal ITS regions

Specific primers that complement the bacterial 16S rDNA and fungal ITS regions were used. Primer pairs were designed for each sample. Specific primer information is listed in Table 4. PCR reaction system (50 μL final volume) consisted of 25 μL 2× PrimeSTAR GC Buffer (Mg²⁺ plus), 4 μL dNTP Mixture (2.5 mM each), 0.5 μL Forward Primer, 0.5 μL Reverse Primer, 0.5 μL PrimeSTAR HS DNA polymerase (2.5 U/μL) and 1 μL DNA template. The reaction mixture was supplemented with sterile water to reach a final volume of 50 μL. Names and sequences of bacterial and fungal primers are listed in Table 4.

Table 4 The primer names and corresponding sequences
<table>
<thead>
<tr>
<th>Thallus</th>
<th>Name of primers</th>
<th>Primer sequences (5’~3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>K16S02-65F</td>
<td>TCGTCATACGCTCTAGAGAGTTTGATCMTGGCTCAG</td>
</tr>
<tr>
<td></td>
<td>K16S02-65R</td>
<td>CTAGAGCGTATGACGAACCTTGTTACGACTT</td>
</tr>
<tr>
<td>Fungi</td>
<td>K18SITS202-65F</td>
<td>TCGTCATACGCTCTAGGTAGTCATATGCTTGTCTC</td>
</tr>
<tr>
<td></td>
<td>K18SITS202-65R</td>
<td>CTAGAGCGTATGAGCTCCGCTTATTGATATGC</td>
</tr>
</tbody>
</table>

The following bacterial PCR conditions [18] were used: 3 min of pre-degeneration at 98°C; 30 s of degeneration at 98°C, 30 s of annealing at 58°C, and 2 min of extension at 72°C (Touch down PCR, 10 cycles); 30 s of degeneration at 98°C, 30 s of annealing at 55°C, and 2 min of extension at 72°C (20 cycles); 10 min of final extension at 72°C and permanent insulation at 16°C.

The following fungal PCR conditions were used: 3 min of pre-degeneration at 98°C; 30 s of degeneration at 98°C, 30 s of annealing at 58°C, and 2 min of extension at 72°C (Touch down PCR, 10 cycles); 30 s of degeneration at 98°C, 30 s of annealing at 50°C, and 2 min of extension at 72°C (20 cycles); 10 min of final extension at 72°C and permanent insulation at 16°C.

1.7 PacBio RS II sequencing and analysis

Purified DNA samples were sent to Nextomics Biosciences Co., Ltd, Wuhan for PacBio RS II sequencing. The original post-sequencing data was transformed to sequential data by Base calling, which was then stored in BAM documents. After CCS and Barcode resolution and data quality control, they were transformed to fastq /fasta format for storage. Later, high-accuracy Raw CCS sequences were acquired using CCS of SMRT Link by using the Arrow algorithm and CCS (≥99% accuracy). The target product sequences were matched and intercepted using BLAST software (maintaining primer region in default) and highlighted sequences were corrected. Chimeras were eliminated to obtain Clean CCS data of each sample. Annotations of QIIME, R, Perl, and Python were processed to generate OUT to study Alpha diversity. Analysis of sequencing results was based on QIIME software and implemented under stringent conditions (97% similarity).

1.8 Data analysis

Experimental results were expressed as average with standard deviation (n=3). Statistical and diversity analyses were carried out using elaborate. By function and alpha function of doBy package, vegan package, and picante package in R software. Differential species with significant p-values were validated by t-tests using R software. Differential species analysis was implemented by STAMP software and plotted.

2. Results

2.1 Changes in the physicochemical indexes after rancidification

Table 5

<table>
<thead>
<tr>
<th>Samples</th>
<th>Alcoholic strength /% vol</th>
<th>Total acid content/g L⁻¹</th>
<th>Amino acid nitrogen/g L⁻¹</th>
<th>pH</th>
<th>Conductivity / µS cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18.4 ± 0.2</td>
<td>4.8 ± 0.3</td>
<td>0.69 ± 0.04</td>
<td>4.2 ± 0.2</td>
<td>1766 ± 86</td>
</tr>
<tr>
<td>Rancidification</td>
<td>17.2 ± 0.2</td>
<td>15.0 ± 0.5</td>
<td>0.78 ± 0.05</td>
<td>3.7 ± 0.2</td>
<td>2684 ± 124</td>
</tr>
</tbody>
</table>
The total acid content in the normal sample was 4.8 g/L. The markedly higher acid content of 15.0 g/L in rancid samples indicated that the organic acid composition in *Huangjiu* had changed. Moreover, except for the abnormal growth of acidity, the amino acid nitrogen of wine increased, while the alcoholic content and pH declined (Table 5). Additionally, the conductivity index also increased dramatically. The conductivity of samples undergoing rancidification was unusually higher than that of normal samples. The metabolic process of pollutant microorganisms in wine might have led to the formation of metal ions complexes which caused proteins to change their binding state and thus increase the conductivity of rancidifying *Huangjiu*.

The lactic acid content in normal samples and samples undergoing rancidification was the highest, followed by acetic acids (Fig. 1). After rancidification, most organic acids increased to some extent. In particular, the lactic acid increased the most (12.05 g/L – 25.38 g/L), followed by acetic acid (2.15 g/L – 6.05 g/L). Citric acid increased to a modest extent. However, other organic acids did not increase significantly. This observation indicated that contamination pollutants mainly produce lactic acid, acetic acid, and citric acid through metabolism, and it was consistent with previous research [21].

The rapid propagation of microorganisms in *Huangjiu* produces excessive organic acids that increase the acidity of the wine and trigger rancidification. The accumulation of metabolites in the wine may also change the free amino acid content and alter the harmful bioamine composition in the wine. In this study, the amino acid composition differed between samples that had experienced rancidification compared to those that hadn't (Fig. 2). In normal and rancidification samples, the total amino acid content was 5275.9 mg/L and 5815.4 mg/L, respectively. The amino acid content for these samples was 2784.6 mg/L and 2816.3 mg/L respectively, which accounted for more than 50% of total amino acid content. The amino acids that give wine sweet, umami, and astringent qualities accounted for the second-highest proportion in terms of total amino acid content. Amino acids associated with bitter taste were relatively unchanged after rancidification, but their total content was quite high. Other amino acids, such as the aspartic acid and glutamic acid that give wine its umami taste, increased by 47.27% from 671.6 mg/L to 989.1 mg/L in rancidification samples compared with that in normal samples.

Some bioamines, such as putrescine, cadaverine, histamine and tyramine, increased significantly in samples undergoing rancidification (Table 6). For example, histamine content increased from 2.4 mg/L in the normal sample to 24.9 mg/L in the rancidifying sample. This suggests that the metabolic reaction of contaminating microorganisms greatly influences the quality of wine. Since contamination microbial community is heavily under the influence of environmental factors, large differences in metabolic reactions may occur. These microorganisms may decompose and use proteins in *Huangjiu* to generate free amino acids and metabolite amino acids to generate various bioamines.

<table>
<thead>
<tr>
<th>Samples</th>
<th>β-phenylethylamine</th>
<th>Putrescine</th>
<th>Cadaverine</th>
<th>Histamine</th>
<th>Tyramine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.2</td>
<td>14.2</td>
<td>2.5</td>
<td>2.4</td>
<td>4.4</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Rancidification</td>
<td>0.2</td>
<td>25.7</td>
<td>6.0</td>
<td>24.9</td>
<td>16.5</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Notes: Tryptamine is not detected.

### 2.3 Microbial diversity in *Huangjiu* with different rancidification progresses

#### 2.3.1 α-diversity indexes of bacteria and fungi
The α-diversity indexes of bacteria and fungi are shown in Table 7. It was quite apparent that the number of the observed bacterial and fungal species in the J-13 sample was much smaller than those in J-10. In particular, the Shannon index and Simpson index of bacteria in the J-13 sample was dozens of times lower than those in the J-10 sample. However, the advantages of this phenomenon remain unknown and require further research. According to the diversity index of fungi, the abundance declined significantly upon rancidification. In the J-13 and J-10 samples, 10 and 14 fungi species were observed, respectively. Possible experimental errors might have caused the number of fungi species in the two samples to decline dramatically. However, it is also possible that the rancidification process inhibited fungal growth for unknown reasons. With increased rancidification, these inhibited fungi might gain resistance and grow slowly. On the other hand, these microorganisms might have material exchange with the external world during storage, thus introducing new microorganisms.

### Table 7

<table>
<thead>
<tr>
<th>Flora</th>
<th>Samples</th>
<th>Observed.otus</th>
<th>Shannon</th>
<th>Simpson</th>
<th>Chao1</th>
<th>Ace</th>
<th>Goods.coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>J-13</td>
<td>35</td>
<td>0.254</td>
<td>0.044</td>
<td>85</td>
<td>86.003</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>J-10</td>
<td>87</td>
<td>3.000</td>
<td>0.668</td>
<td>165.4</td>
<td>186.965</td>
<td>0.951</td>
</tr>
<tr>
<td>Fungi</td>
<td>J-13</td>
<td>10</td>
<td>2.259</td>
<td>0.666</td>
<td>11.000</td>
<td>12.491</td>
<td>0.953</td>
</tr>
<tr>
<td></td>
<td>J10</td>
<td>14</td>
<td>2.978</td>
<td>0.829</td>
<td>28.000</td>
<td>35.030</td>
<td>0.862</td>
</tr>
</tbody>
</table>

#### 2.3.2 Abundance distribution curve of bacteria

Each broken lines of the abundance distribution curves (Fig. 3) represent OTU abundance distribution in samples. In other words, the degree of abundance of species is reflected by the curve length on the x-axis. The greater curve span represents the greater composition of species. Species richness was higher in J-10 samples, which exhibited more than 75 species (Fig. 3). However, the J-13 samples harbored relatively fewer species, at only 30.

#### 2.3.3 Abundance distribution curve of fungi

After performing quality control of fungal sequences, 28 operation taxonomic units (OTUs) were collected from both the J-10 and J-13 samples. Each broken lines of abundance distribution curves (Fig. 4) represent OTUs abundance distribution of the sample, while the degree of species abundance is reflected by curve length on the x-axis. The higher curve span indicated a higher abundance of species. Although the fungal composition of both samples was generally similar, there were also some differences (Fig. 4). Despite the higher degree of rancidification in J-13, the sample had fewer OUTs than J-10. Therefore, other indexes need to be included to further analyze differences and similarities between normal and rancidification samples.

#### 2.4 Microbial community structure in Huangjiu with different rancidification progresses

##### 2.4.1 Community structure of bacteria

The phyla, genera, and species of microorganisms with a relative abundance of ≥ 1% were defined as the dominant bacteria of the group. The top 10 groups were plotted to show relative abundance of dominant bacterial composition in all samples. In the J-13 sample, Firmicutes dominated the group (98.06% abundance), followed by Epsilonbacteraeota (Fig. 5). Surprisingly, in the J-10 sample, Firmicutes only accounted for 18.29% of the bacteria, while Bacteroidetes accounted for 5.52% and Proteobacteria accounted for 10.55%. Moreover, Epsilonbacteraeota accounted for more than 65.32% of the bacterial genus. In addition, some WPS-2 fungi were detected in J-13 samples. This microbe, which cannot be cultured, might instead be a bacteriophyta that had been mislabeled by the detection technology.
At the genus level (Fig. 6), the dominant bacteria genera *Lactobacillus* was very abundant (≥ 98.05%). In J-10, *Lactobacillus* only accounted for 16.08% of the bacteria, while *Arcobacter* accounted for 65.33% and *Comamonas* accounted for 5.53%. Other bacteria genera that displayed much lower relative abundance (less than 1%) included *Prevotella 7, Acetobacter, Acinetobacter*, and *Proteiniphilum*.

At the species level (Fig. 7), most *Lactobacillus* species belonged to the *Lactobacillus acetotolerans* DSM 20749 variety, which accounted for 98.79% of the bacteria in J-13. As other species were less abundant (less than 0.1%), statistical analyses on those samples were challenging. In J-10, *Lactobacillus acetotolerans*, uncultured *Arcobacter*, *Comamonas terrigena*, uncultured *Prevotella* and uncultured *Proteiniphilum* accounted for 11.56%, 64.42%, 4.82%, 2.01% and 0.51%, respectively. In addition, the relative abundance of other bacterial species was less than 1%. However, the species richness in the samples caused the total relative abundance to be greater than 12.86%. A combination of other relevant studies [9] revealed that L.s acetotolerans has relatively high similarity to *Lactobacillus fructivorans*, homofermentative *Lactobacillium*, and other *Lactobacillus*. Therefore, the common relative abundance of these microorganisms is often gained in identification.

The t-tests revealed significant differential species based on genera level are shown in Fig. 8. According to t-tests and comparison of the relative abundance of different microorganisms, 10 bacterial genera including *Arcobacter, Comamonas, Prevotella 7, Acetobacter*, and *Acinetobacter* were significantly higher in J-10 than that in J-13 (p<0.01). However, *Lactobacillus* was significantly lower in J-10 than that in J-13 (p<0.01). This trend is also apparent from the relative abundance diagram of the genera level. Collectively, these indicated that the degree of rancidification might affect the abundance of these significantly different bacterial genera in *huangjiu* contaminated by microorganisms during storage.

### 2.4.2 Fungal community structures

It can be seen from the relative abundance diagram of the *Eumycophyta* level that *Ascomycota, Basidiomycota* and unidented *Dikarya* made up 63.8%, 5.2%, and 31.0% of the J-10 sample, respectively (Fig. 9). The relative abundance of *Ascomycota* in J-13 was quite high (87.5%) while the relative abundance of *Basidiomycota* and unidented *Dikarya* were only 3.13% and 9.4%, respectively.

The relative abundance of fungal genera was similar to that observed at the *Eumycophyta* level, which in turn inherited the relative abundance of *Eumycophyta* level to some extent (Fig. 10). Moreover, there are similar bacterial genera between the samples. Except for the consistently abundant unidentified *Dikarya*, *Aureobasidium* had a relatively high abundance (24.1%) in the J-10 sample. Additionally, the abundance of *Aspergillus* and *Debaryomyces* were 15.5% and 12.1%, respectively. The relative abundance of *Penicillium*, *Rhodotorula*, and *Cladosporium* were 5.2%, 5.1% and 1.7%, respectively. In J-13, *Aspergillus* was the most abundant at 54.7%. *Saccharomyces* accounted for 15.6% of the microbial content. The abundance of *Aspergillus* and *Debaryomyces* was close to that observed in J-10 and *Malassezia* (approximately 3.1%).

The relative abundance of fungal species was also summarized (Fig. 11), *Aspergillus niger*, the major *Aspergillus* fungi, accounted for 15.5% and 54.7% of the J-10 and J-13 samples, respectively. J-10 consisted of 24.1% *Aureobasidium pullulans*, 12.1% *Debaryomyces hansenii*, 5.2% *Rhodotorula mucilaginosa*, and 3.4% *Penicillium commune*. Other fungi with a relative abundance higher than 1% included *Pichia manshurica* (1.7%) and *Penicillium decumbens* (1.7%). In J-13, the abundance of *Debaryomyces hansenii* decreased by 2.8% compared to that observed in J-10. On the other hand, the abundance of *Saccharomyces cerevisiae* increased by 15.6%. Moreover, *Penicillium decumbens* accounted for 4.7%. Other fungi included *Malassezia sp LCP_2008a* which accounted for 3.1% and *Penicillium chrysogenum* which accounted for 1.6%.

The t-tests determining significantly different fungal species based on genera are shown in Fig. 12. Based on the comparison of differential microorganisms, it is apparent that the relative abundance of *Penicillium* in J-13 was
significantly higher than that of J-10 (p<0.01). However, the relative abundances of *Pichia, Rhodotorula, Debaryomyces*, and unidentified *Dikarya* in J-10 were significantly higher than those observed in J-13 (p<0.01). This indicated that the degree of rancidification might influence the propagation of these fungal genera in *huangjiu* contaminated by microorganisms during storage.

### 3. Discussions

As a fermented wine, *Huangjiu* is rich in nutrients and low in alcoholic concentration. The amino acid content in *Huangjiu* is significantly higher than those observed in other wines [19]. Therefore, any microbial contamination that might occur during the storage of *Huangjiu* is likely to propagate rapidly such that the metabolites generated as byproducts would cause the rancidification of the wine [9].

During the storage of *Huangjiu*, the initiation process of rancidification is relatively complicated. For the same batch of 3-year *Huangjiu*, most samples are normal and devoid of rancidification or only display weak rancidification from microbial pollution. Among the rancidifying samples, the time of occurrence of rancidification differs significantly. Some samples underwent rancidification from microbial pollution. The rest of the samples completed rancidification after at least half a year. These samples appeared turbid and clear, respectively. According to a comparison of physicochemical indexes and microcomponent detection results between normal and rancidification samples, the changes in major microorganisms and compositions observed in this study were consistent with previous studies (Liu et al. year; Feng, 2010; Liu, Chen and Xu, 2018). Different pollution microorganisms and environmental conditions influence the process of rancidification process. Proteins, amino acids, and other components in *Huangjiu* further complicate metabolic reactions. After rancidification occurs, most organic acids increased to a certain extent. In particular, lactic acid increased the most, followed by acetic acid. Citric acid also increased to some extent (Fig. 1). Free amino acids such as aspartic acid and glutamic acid also increased greatly. An increase in these components only affected the taste of *Huangjiu*. However, the increase of some boamines, such as putrescine, cadaverine, histamine, and tyramine (Table 6) affected the quality of *Huangjiu* and could pose a potential food safety risk.

*Huangjiu* utilizes open fermentation through the usage of cereals and wheat koji as raw materials [20]. During this storage process, various factors may cause the rancidification of *Huangjiu* and give rise to industrial problems. In this study, the identification of microorganism categories and relative abundances in samples undergoing rancidification was performed using PacBio SMRT sequencing technique. Analyses of uncultured samples serve as an exploration of the microorganism species that may influence rancidification progress.

When evaluating the diversity indexes, it was observed that the microorganism content in *Huangjiu* increased significantly after rancidification, which might have been related to the alcohol content of the wine (Table 5). In normal *Huangjiu* samples, alcohol content naturally inhibits the growth of microorganisms. However, the low alcohol content of samples undergoing rancidification caused the previously inhibited microorganisms to slowly grow and produce damaging acids. In this way, the growth environment of microorganisms is changed (in other words, acid production induces rancidification of *Huangjiu*) to be favor the growth of microorganisms. In this process, the inhibited microorganisms can grow normally. Whether external microorganisms were introduced in this study may be explained through the combination of further experiments and data. The multiplicity of bacteria-fungi interactions may further enhance the rancidification of samples [9]. As there were only a few samples, this speculation could not be validated. However, it was determined that *Lactobacillus acetotolerans* is the major bacteria responsible for causing the rancidification of *Huangjiu*. Fungi responsible for the rancidification process included uncommon mildew and yeasts in *Huangjiu*, including black yeast, *Aspergillus*, and *Hansenula polymorpha*. Interestingly, a study [9] demonstrated that the sequence similarity between *Lactobacillus acidophilus* and *Lactobacillus fructivorans* is higher than 99%. Therefore, differences between these bacteria cannot be easily ascertained. According to experimental results, both these bacteria cause the rancidification of *Huangjiu*, albeit to varying degrees. According to the microbial community structural analysis of *Huangjiu* at different stages of
rancidification, J-10 which had undergone the longest duration of rancidification, showed significantly more microorganisms (both bacteria and fungi) compared with J-13, which had a shorter rancidification time. These studies reveal that increased exposure to rancidification enriches the microbial community structures in Huangjiu. Moreover, many Arcobacter species and other pathogenic microorganisms were detected in J-10. Therefore, it is worth investigating the safety value of Huangjiu during aging process.

The research presented in this paper demonstrated that most microorganisms causing the rancidification of Huangjiu in traditional pottery jars and mechanical pots experienced difficulty growing in their existing media. Although it was known that rancidification of Huangjiu is mainly caused by contamination, improper wine cooking temperature or damaged packaging during production could also cause contamination and should be studied to understand their mechanism of action. Based on two Huangjiu samples with different rancidification degrees, microorganism differences were analyzed by the third generation sequencing and PCR technique. This study provides clear guidance for future identification of microorganisms that causes rancidification of Huangjiu. Moreover, studying conductivity provides an effective means of monitoring microbial pollution during Huangjiu storage.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
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<tr>
<td>HPCE</td>
<td>High performance capillary electrophoresis</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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Declarations

CRediT authorship contribution statement


Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References


**Figures**

![Comparison of organic acid composition before and after rancidification](image)

**Figure 1**

Comparison of organic acid composition before and after rancidification
Figure 2

Comparison of amino acid compositions before and after rancidification.

Figure 3

Relative Abundance(%)

OTUs Rank

J-10

J-13
Bacterial abundance distribution curve

Figure 4

Fungal abundance distribution curves

Figure 5

Relative abundance of bacteriophyta

Figure 6
Relative abundance of bacteria genera

Figure 7

Relative abundance of bacterial species levels

Figure 8
Figure 9
Relative abundance of *Eumycophyta* level

Figure 10
Relative abundance of *Eubacterium* level
Figure 11

Relative abundance of fungal species level

Figure 12

T-test diagram of fungal significant differential species