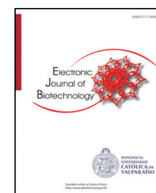
Electronic Journal of Biotechnology 43 (2020) 23–31

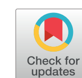
Contents lists available at ScienceDirect

Electronic Journal of Biotechnology



Research article

Dynamic changes of total acid and bacterial communities during the traditional fermentation of Hong Qu glutinous rice wine



Zhangcheng Liang [a,b], Xiaozi Lin [a,b], Zhigang He [a,b,*], Weixin Li [a,b], Xiangyun Ren [a,b], Xiaojie Lin [a,b]

[a] *Institute of Agricultural Engineering Technology, Fujian Academy of Agricultural Sciences, Fuzhou, Fujian, China*
[b] *Fujian Key Laboratory of Agricultural Products (Food) Processing, Fuzhou, Fujian, China*

ARTICLE INFO

Article history:
Received 14 August 2019
Accepted 2 December 2019
Available online 10 December 2019

Keywords:
Acetobacter
Bacterial communities
Glutinous rice wine
Hierarchical clustering analysis
Lactobacillus
Pediococcus
Principal component analysis
Total acid production
Traditional fermentation

ABSTRACT

Background: Hong Qu glutinous rice wine (HQGRW) is brewed under non-aseptic fermentation conditions, so it usually has a relatively high total acid content. The aim of this study was to investigate the dynamics of the bacterial communities and total acid during the fermentation of HQGRW and elucidate the correlation between total acid and bacterial communities.
Results: The results showed that the period of rapid acid increase during fermentation occurred at the early stage of fermentation. There was a negative response between total acid increase and the rate of increase in alcohol during the early fermentation stage. Bacterial community analysis using high-throughput sequencing technology was found that the dominant bacterial communities changed during the traditional fermentation of HQGRW. Both principal component analysis (PCA) and hierarchical clustering analysis revealed that there was a great difference between the bacterial communities of Hong Qu starter and those identified during the fermentation process. Furthermore, the key bacteria likely to be associated with total acid were identified by Spearman's correlation analysis. *Lactobacillus*, unclassified *Lactobacillaceae*, and *Pediococcus* were found, which can make significant contributions to the total acid development ($|r| > 0.6$ with FDR adjusted $P < 0.05$), establishing that these bacteria can associate closely with the total acid of rice wine.
Conclusions: This was the first study to investigate the correlation between bacterial communities and total acid during the fermentation of HQGRW. These findings may be helpful in the development of a set of fermentation techniques for controlling total acid.
How to cite: Liang Z, Lin X, He Z, et al. Dynamic changes of total acid and bacterial communities during the traditional fermentation of Hong Qu glutinous rice wine. Electron J Biotechnol 2020;43. https://doi.org/10.1016/j.ejbt.2019.12.002

© 2019 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Chinese rice wine, one of the most ancient wines in the world, is deeply loved by the Chinese people [1]. Hong Qu glutinous rice wine (HQGRW), produced from glutinous rice and 'Hong Qu' (a traditional Chinese fermentation starter), had unique flavor and rich functional nutrients. However, when Hong Qu was inoculated with pure *Monascus purpureus* (a mold genus), and since the process was carried out in an open, non-sterile environment in which different microbes adhered to the surface of the rice, a significant difference in the yeast and lactic acid bacteria present was observed for each different location, fermentation method, and batch [2]. At present, HQGRW is produced under non-aseptic fermentation conditions, which can lead to a lack of stability and controllability in the quality of the wine during fermentation [2]; for example, the total acid content of rice wine is usually too high. Suitable organic acid content could promote the formation of flavor compounds of rice wine and enhance the taste [3]; however, if the total acid content is too high, the acidity in the rice wine can be overpowering, and rice wine becomes unpalatable. Nowadays, more than 20% of HQGRW is discarded every year because of excessive acidity. When the total acid content is too high, there is a lack of consistency in the quality of rice wine, which gives consumers a negative impression.

Different Hong Qu glutinous rice starters contain different kinds of microbes, such as filamentous fungi, yeasts, and bacteria [4,5], so the rice wine made from these different starters has different levels of acidity. In rice wine, filamentous fungi can produce acid via saccharification of starch and yeasts can produce acid via fermentation of sugars during fermentation, but they produce a very low amount of acid [6]. Some researchers have looked at which bacteria were the

* Corresponding author:
E-mail address: njgzx@163.com (Z. He).

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2019.12.002

0717-3458/© 2019 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

most important for producing acid during fermentation in rice wine [3,7,8]. The structure of the bacterial communities experienced dynamic changes during the fermentation of rice wine. For example, *Bacillales*, *Actinomycetales*, and *Lactobacillales* species have been reported to be the dominant bacterial communities in Fen Liquor wine starter [9]. Bacterial community dynamics revealed *Lactobacillus brevis*, *Lactobacillus plantarum*, *P. pentosaceus*, and *P. acidilactici* to be present during the traditional fermentation process in HQGRW, and their abundances varied across different stages of the fermentation process [2]. In general, bacteria can play an important function in organic acid and flavor development during fermentation [10,11]. However, to the best of our knowledge, most of the research has focused on correlation analysis between fermentation quality and the microbial communities of the wine starters such as DaQu and HongQu; thus, correlation analysis between total acid and the bacterial communities during fermentation has not yet been studied.

High-throughput sequencing technology based on Illumina MiSeq sequencing platform, which can accurate taxonomic classification of the microbial diversity, used to investigate the microbial relative abundance and community structure of some traditional fermented food, such as pure tea, Fu brick tea, soyfood Douchi, Chinese vinegar, and Chinese rice wine [6]. In this study, three different varieties of Hong Qu wine fermentation starters were used, which in our previous studies have each produced wine with different levels of acidity. Also, this study is focused on dynamic changes of total acid and bacterial communities during the fermentation of HQGRW by High-throughput sequencing technology. Furthermore, the potential correlations between bacteria and total acid were investigated by Spearman's correlation analysis for the first time. The results may be useful to understand the bacteria and their contribution to acid production during traditional fermentation, which may be helpful in the development of a set of fermentation techniques for controlling total acid.

2. Materials and methods

2.1. Traditional HQGRW fermentation method

The three kinds of Hong Qu starters that were used from three wine factories in the Fujian Province of China in this study, were (A) LJ2, (B) CK1, and (C) SH3. Fig. 1 shows a flow chart describing the HQGRW fermentation process. At the beginning of the fermentation process, 2.0 kg of glutinous rice was washed and soaked in water for 4–8 h at room temperature, and then cooked and steamed for 30 min at 100°C. After the steamed rice was cooled to room temperature, it was mixed with 100 g of fermentation Hong Qu starter and transferred to a traditional Chinese wine jar. Finally, sterilized water was added to make a total volume of 2.0 L, and the wine underwent fermentation at 20 ± 1°C for 30 d. In this study, the three groups of HQGRW were labeled as LJ2 Hong Qu (group A), CK1 Hong Qu (group B), and SH3 Hong Qu (group C). Fermentation was conducted using the same starters (LJ2, CK1, and SH3 Hong Qu) in three independent experiments.

2.2. Sample collection

Wine samples weighing 200 g were collected from each jar at different stages of fermentation (0, 2, 5, 12, 16, 20, 25, and 30 d) under aseptic conditions. Solid–liquid separation was performed, and the samples were centrifuged to obtain the supernatant for future lactate and alcohol determination. For the analysis of bacterial communities, 50 g of wine samples was collected from each jar at 0, 2, 5, and 16 d under aseptic conditions and stored at -80°C.

2.3. Measurement of pH value and total acid content (titratable acidity) of samples

The pH of the samples was determined using a Hach pH meter (Hach, Loveland, CO, US). The method of measuring the total acid content (titratable acidity) was titration to the endpoint of pH 8.2 with 0.05 M NaOH [12].

2.4. Measurement of alcohol content by full evaporation headspace gas chromatography

Full evaporation headspace gas chromatography (FE HS-GC) measurements were used to assess the level of alcohol in rice wine [13].

2.5. DNA extraction

Total bacterial genomic DNA samples were extracted using the Fast DNA SPIN Extraction Kits (MP Biomedicals, Santa Ana, CA, US), then stored at -20°C for further analysis. The quantity of extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, US). The quality of extracted DNA was measured using agarose gel electrophoresis.

2.6. 16S rRNA amplicon sequencing

PCR amplification of the bacterial 16S rRNA genes V4–V5 region was performed using the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) [14]. Sample-specific 7-bp barcodes

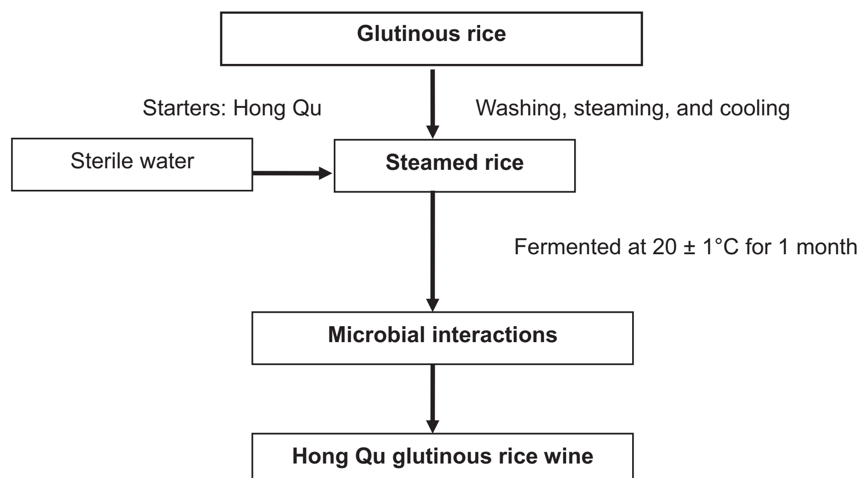


Fig. 1. Flowchart for the traditional brewing of Hong Qu glutinous rice wine.

were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μL of Q5® reaction buffer (5×), 0.25 μL of Q5® High-Fidelity DNA Polymerase (5 U/μL), 5 μL of Q5® High-Fidelity GC buffer (5×), 2 μL of DNA template, 1 μL (10 μM) each of forward and reverse primer, 2 μL (2.5 mM) of dNTPs, and 8.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 98°C for 2 min, followed by 25 cycles of denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension of 5 min at 72°C. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Brea, CA, US). Purified liquids were quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, US). Finally amplicons were pooled in equimolar ratios and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform with a MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

2.7. Sequence analysis

The sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline [15]. Briefly, raw sequencing reads with exact matches to the barcodes were assigned to their respective samples and identified as valid sequences. The low-quality sequences were filtered under the following conditions [16]: sequences that had average Phred scores of <20, sequences that had a length of <150 bp, sequences containing mononucleotide repeats of >8 bp, and sequences containing ambiguous bases. Paired-end reads were assembled using FLASH [17]. Chimeras were detected, and the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST [18]. Using default parameters, a representative sequence was selected from each OTU. Using the best hit, OTU taxonomic classification was conducted by BLAST-searching the representative sequences set against the Greengenes Database. An OTU table was generated, and the abundance of each OTU in each sample and the taxonomy of these OTUs were recorded. An OTU table was generated to record the abundance of each OTU in each sample and the taxonomy of these OTUs. OTUs containing <0.001% of the total sequences across all samples were discarded. Under 90% of the minimum sequencing depth, a rarefied, rounded, averaged OTU table was generated by averaging 100 evenly resampled OTU subsets to minimize the difference of sequencing depth across samples.

2.8. Bioinformatics analysis

Sequence data analyses were mainly performed via QIIME (version 1.17) under the following conditions: (a) the 300 bp reads were truncated at any site receiving an average quality score less than 20 over a 50 bp sliding window, and the truncated reads that were shorter than 50 bp were discarded; (b) using exact barcode matching, that is, if there were two nucleotide mismatches in primer matching, and removed the reads containing ambiguous characters; and (c) according to their overlap sequence; we only considered assembled sequences that overlapped more than 10 bp. We discarded the reads that could not be assembled. OTU-level ranked abundance curves were generated to allow comparison to the evenness and richness of OTUs among samples.

2.9. Statistical analysis

All the experiments were performed in triplicate. Data were represented as mean ± standard deviation (±s). Statistical analyses were performed by SPSS 15.0 software using one-way analysis of variance, and P indicated the statistical significance difference ($P < 0.05$). A rarefaction curve was constructed for the number of reads sampled randomly extracted with their representative OTU number using the MOTHUR program (v.1.34.0). The Shannon–Wiener curve analysis was constructed using the number of read sequences and the Shannon index using the method of random sampling of the sequences. We then conducted alpha and beta diversity statistics. Alpha diversity was analyzed with total OTUs and the relative abundance of each OTU to estimate the with-in sample species richness using MOTHUR program (v.1.34.0), and revealed by Chao index for estimating the species richness, Goods Coverage (determines the depth of coverage of the clone libraries), observed species (estimates the number of unique OTUs found in each sample), and Shannon index (a means of comparing the diversity between two or more samples). Principal component analysis (PCA) was performed based on the genus-level [19]. Using hierarchical clustering analysis, we determined the similarity among the bacterial communities during fermentation [20] in which the unweighted pair group method with arithmetic mean (UPGMA) was used. The relative abundances of the representative taxa were further visualized as a heatmap, using SPSS 15.0 software. Spearman's correlation coefficient (r) was calculated by using R software (ver. 3.5.3) with the “pheatmap” package, and $|r| > 0.6$ with FDR-value (FDR < 0.05) was considered a robust correlation [21].

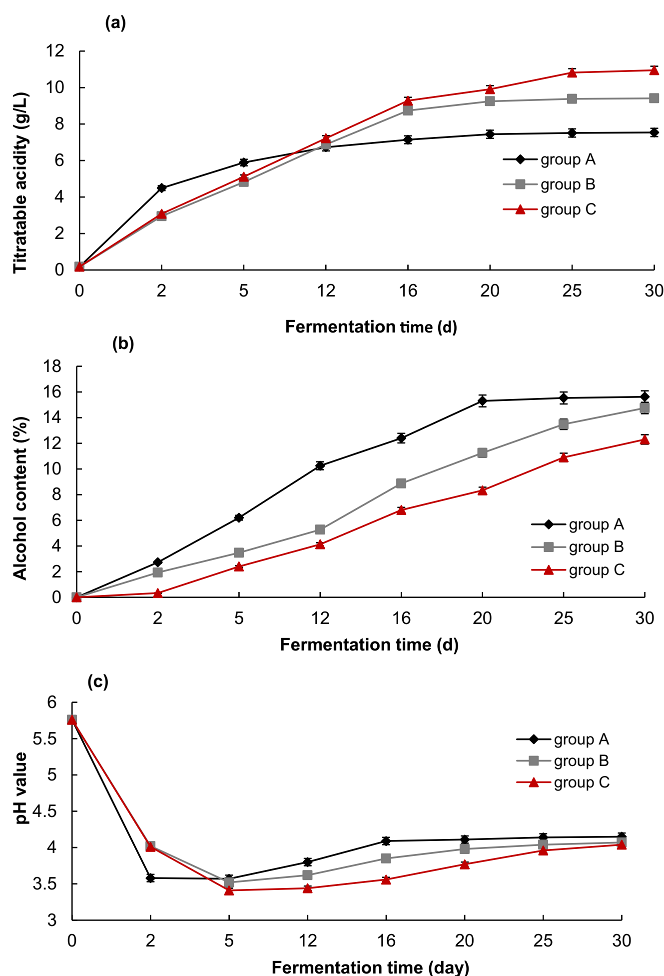


Fig. 2. Change of (a) total acid content, (b) alcohol content, and (c) pH value in different starter samples during fermentation. Data points (A, B, and C) are indicated: (A) LJ2 Hong Qu group, (B) CK1 Hong Qu group, and (C) SH3 Hong Qu group. Data points (1, 2, and 3) represent the means of three biological repeats, and error bars represent standard deviations.

3. Results

3.1. Change of total acid content, pH value, and alcohol content during fermentation

The change of total acid content and pH value in rice wine during the sequential fermentation stages is shown in Fig. 2(a) and (b). The total acid content of the samples all first rose rapidly and then tended to stabilize in all three of the groups. The pH value of the samples all first declined rapidly during the first 2 d of fermentation in all three of the groups. It is interesting that the pH value of the samples all showed a slowly increasing trend. There are significant differences ($P < 0.05$) in the total acid content and pH value between each group. Group A had the lowest total acid content and the highest pH followed by group B, whereas group C had the highest total acid content and lowest pH. The change in the amount of alcohol in rice wine during the sequential fermentation stages is shown in Fig. 2(c). In all three groups of samples, the amount of alcohol tended to increase. There were significant differences ($P < 0.05$) in the alcohol content between each group. During fermentation, group A had the fastest increase in alcohol content followed by group B; group C had the slowest increase. There was a negative response between total acid increase and the rate of increase in alcohol during the early fermentation stage. The results showed that the early fermentation stage was the period of rapid total acid growth during fermentation, and a certain concentration of alcohol can inhibit the increase of total acid.

3.2. Changes in the relative abundance of bacterial communities during fermentation

The sequencing quality data such as rarefaction curve (Fig. S1), abundance grade curve (Fig. S2) and alpha diversity indexes of microbial communities based on Illumina MiSeq sequencing (Table S1). At the genus level, the changes in the relative abundance of bacterial communities during fermentation are shown in Fig. 3. In the beginning of fermentation, the dominant bacterial communities of Hong Qu glutinous rice starters were *Bacillales* (groups B and C), *Gluconobacter* (group A), and *Burkholderiales* (group C). Lactic acid bacteria (LAB) accounted for only a small portion of the initial fermentative flora (all less than 3%). Population dynamics in many other traditional fermented rice wines have been demonstrated [17]. In addition, a transition in the bacterial communities occurred during fermentation under different environmental conditions. Environmental conditions can promote the dynamics of bacterial communities during

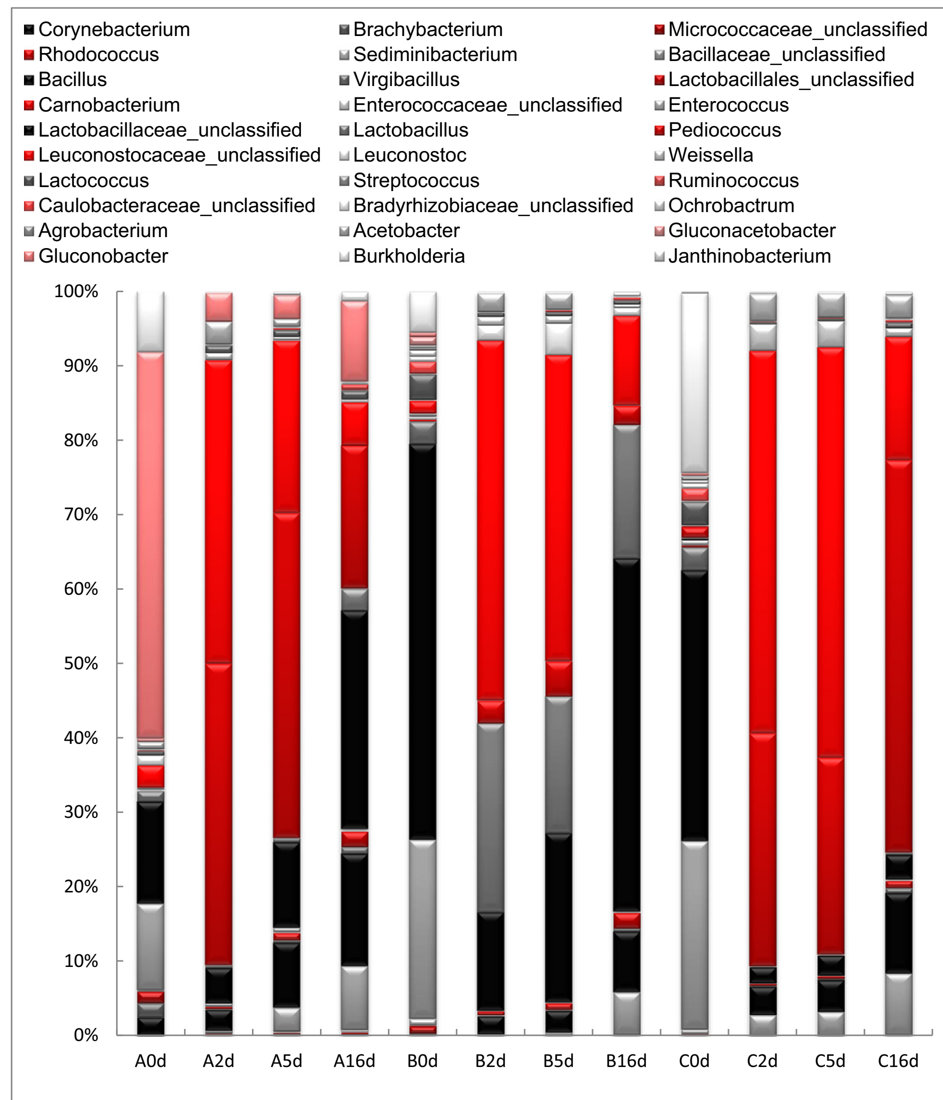


Fig. 3. Change of bacterial community among different starter samples during fermentation. Data points (A, B, and C) are indicated: (A) LJ2 Hong Qu group, (B) CK1 Hong Qu group, and (C) SH3 Hong Qu group. Data points (1, 2, and 3) represent the means of three biological repeats, and error bars represent standard deviations.

HQGRW fermentation process [2]. In this study, during the first 2 d of fermentation, LAB grew quickly and were determined to be unclassified *Leuconostocaceae*, *Pediococcus*, and *Lactobacillus*. The dominant bacteria at the early fermentation stage were from those three genera, and their relative abundance all increased more than 10%. The growth of other bacteria was inhibited for all groups. To the best of our knowledge, various physico-chemical factors (alcohol and pH) are known to affect the growth of bacteria in wine. Alcohol is generally regarded as the principal inhibitor of bacterial growth in wine [22], and concentrations of 13% or greater would limit bacterial growth [23]. As the fermentation progressed, the growth of LAB declined rapidly under the unfavorable conditions created by low pH [24]. In this study, as total acid and alcohol increased during fermentation, the growth of some LAB was inhibited ($P < 0.05$) such as unclassified *Leuconostocaceae* and *Lactobacillus*; thus its abundance was reduced. It is interesting that *Pediococcus* and unclassified *Lactobacillaceae* became the dominant bacteria genera at the later fermentation stage. They together became either the dominant bacteria genus (group A) or individually became the dominant bacteria genus (group B or C). Thus, we inferred that alcohol and acid tolerance of *Pediococcus* and unclassified *Lactobacillaceae* were stronger than *Lactobacillus* and unclassified *Leuconostocaceae*, but further research is needed to prove it.

The results show that the dominant bacteria were unclassified *Leuconostocaceae*, *Pediococcus*, unclassified *Lactobacillaceae*, and *Lactobacillus*; the dominant bacterial communities changed during the traditional fermentation of HQGRW. It is worth noting that *Acetobacter* grew quickly during the first 2 d of fermentation (their relative abundance ranges from 2% to 4%), but their growth was inhibited later. This is due to that most of LAB can produce antibacterial materials such as bacteriocin, which can inhibit the growth of spoilage and pathogenic microbes during fermentation such as *Acetobacter*, which is helpful in preventing the acetification of wine.

3.3. Cluster analysis

PCA demonstrated that the four types of structure that communities can have could be separated using the genus abundance dataset (Fig. 4), and the similarity among the bacterial communities was determined (Fig. 5). The transformation of the relative abundance of bacterial communities in the fermentation process of HQGRW is divided into four stages. The four stages are (i) 0 d for all the groups; (ii–iv) 2, 5, and 16 d for each of the three groups A, B, and C, respectively. It is interesting that the bacterial communities of the samples that were fermented for 16 d had a high difference compared with those fermented for 2 and 5 d in groups A, B, and C (Fig. 5). The results show that the bacterial communities involved in the fermentation process of different Hong Qu rice wines were each different, and there

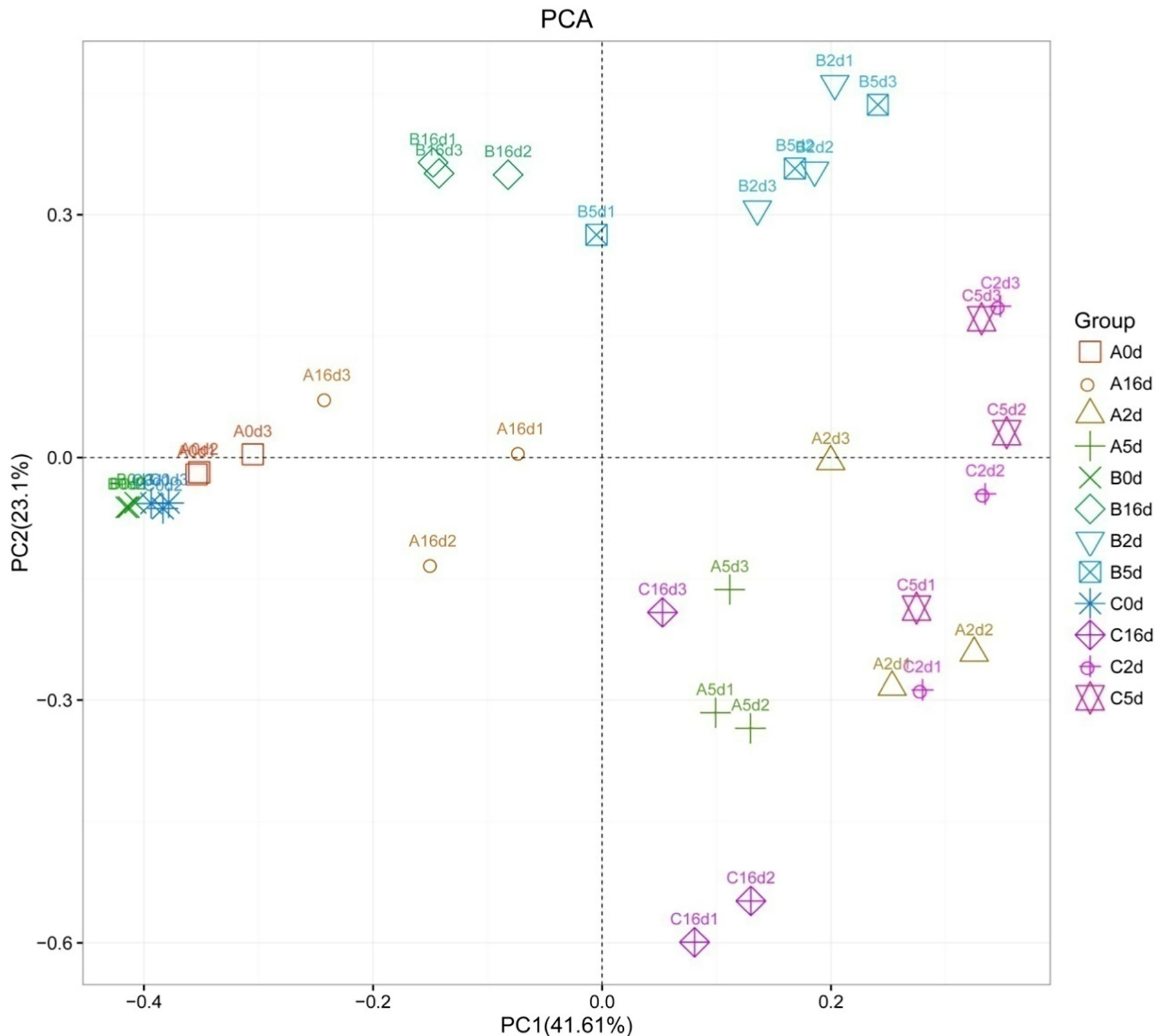


Fig. 4. PCA analysis of different starter samples during fermentation. Data points (A, B, and C) are indicated: (A) LJ2 Hong Qu group, (B) CK1 Hong Qu group, and (C) SH3 Hong Qu group. Data points (1, 2, and 3) are the means of three biological repeats.

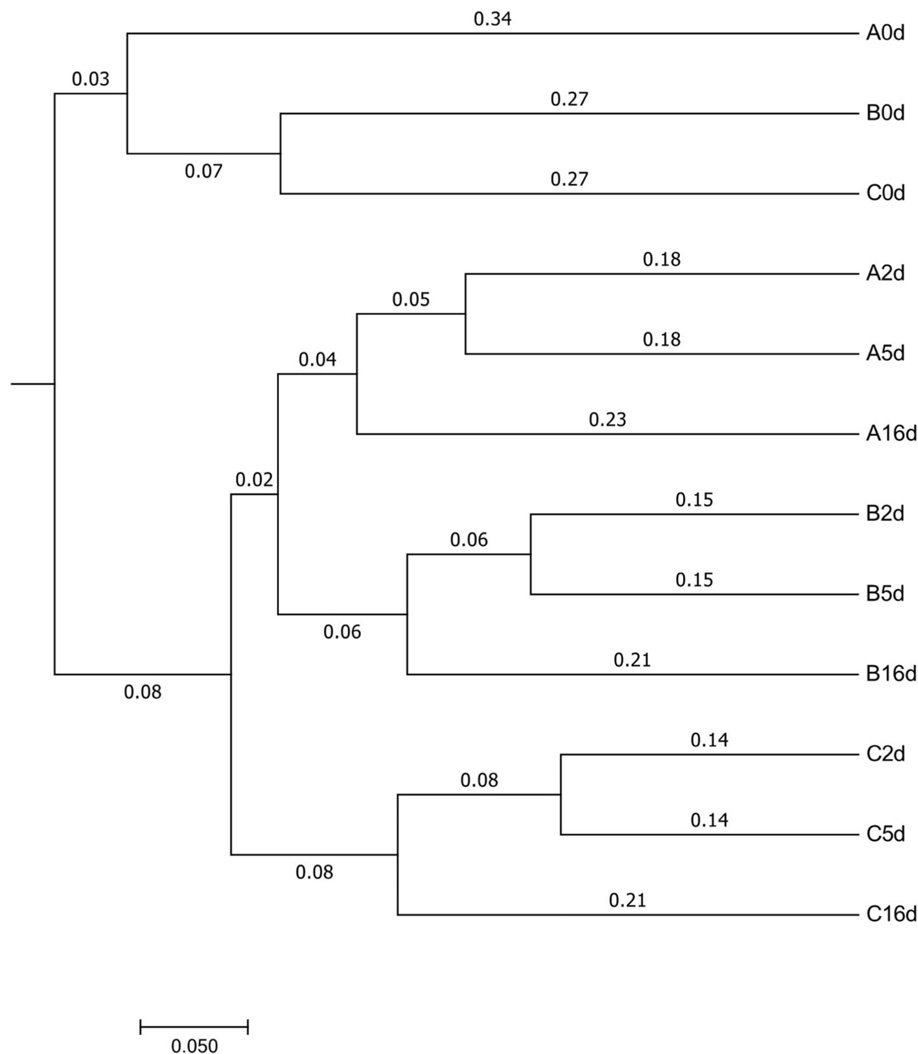


Fig. 5. Hierarchical clustering with unweighted UniFrac UPGMA clustering analysis diagram. It based on an UniFrac distance matrix. According to the similarity of each other, the shorter the branch length, the more the similarity between the samples.

was a great difference between the bacterial communities of Hong Qu rice starter and those identified during the fermentation process.

3.4. Correlation analysis between the abundance of different types of bacteria, and the abundances of bacteria and total acid

The correlations between the abundances of bacteria and bacteria, and the abundances of bacteria and total acid (TA) were elucidated based on Spearman's correlation analysis. A Spearman's rank correlation matrix of bacterial genus with >0.01% abundance is shown in Fig. 6. LAB including unclassified *Leuconostocaceae*, *Pediococcus*, unclassified *Lactobacillaceae*, *Lactobacillus*, and *Weissella*, appeared almost antagonist to *Gluconobacter*, *Bacillus*, *Burkholderia*, unclassified *Bacillaceae*, *Acetobacter*, and other miscellaneous bacteria such as *Carnobacterium* and *Enterococcus*. The results show that the growth of LAB can inhibit the growth of miscellaneous bacteria and acetic acid bacteria, which is helpful in preventing the deterioration and acetification of wine.

Spearman's correlation analysis between bacteria and total acid indicated that total acid was correlated positively with *Lactobacillus*, unclassified *Lactobacillaceae*, *Pediococcus*, *Janthinobacterium*, *Weissella*, unclassified *Lactobacillales*, unclassified *Leuconostocaceae*, *Acetobacter*, *Ruminococcus,* unclassified *Caulobacteraceae*, *Agrobacterium*, and *Ochrobactrum*. Total acid exhibited a negative correlation with *Gluconacetobacter*, *Corynebacterium*, *Brachybacterium*, *Gluconobacter*, *Burkholderia*, unclassified *Enterococcaceae*, and so on. Most of them were pathogenic and harmful bacteria. Moreover, *Lactobacillus*, unclassified *Lactobacillaceae*, and *Pediococcus* were highly associated with total acid ($|r| > 0.6$ with FDR-value ($FDR < 0.05$) was considered as a robust correlation), and they were the key bacteria responsible for acid production during fermentation, explaining that these bacteria may be closely associated with the total acid of rice wine.

4. Discussion

Traditionally, Hong Qu starter is produced through fermentation inoculating Monascus to the rice under non-sterile conditions, so Hong Qu often showed complexity and diversity, which might contain various kinds of microbes, such as filamentous fungi, yeasts, and bacteria [25]. The acid-producing ability of different kinds of bacteria is different [24]; therefore, different types of Hong Qu starters might lead to the different total acid contents of rice wine. This study indicated that total acid was correlated positively with *Lactobacillus*, unclassified *Lactobacillaceae*, *Pediococcus*, *Janthinobacterium*, *Weissella*, unclassified *Lactobacillales*, unclassified *Leuconostocaceae*, *Acetobacter*, *Ruminococcus*, unclassified *Caulobacteraceae*, *Agrobacterium*, and *Ochrobactrum*. Moreover, *Lactobacillus*, unclassified *Lactobacillaceae*, *Pediococcus*, unclassified *Leuconostocaceae*, *Weissella*, and unclassified

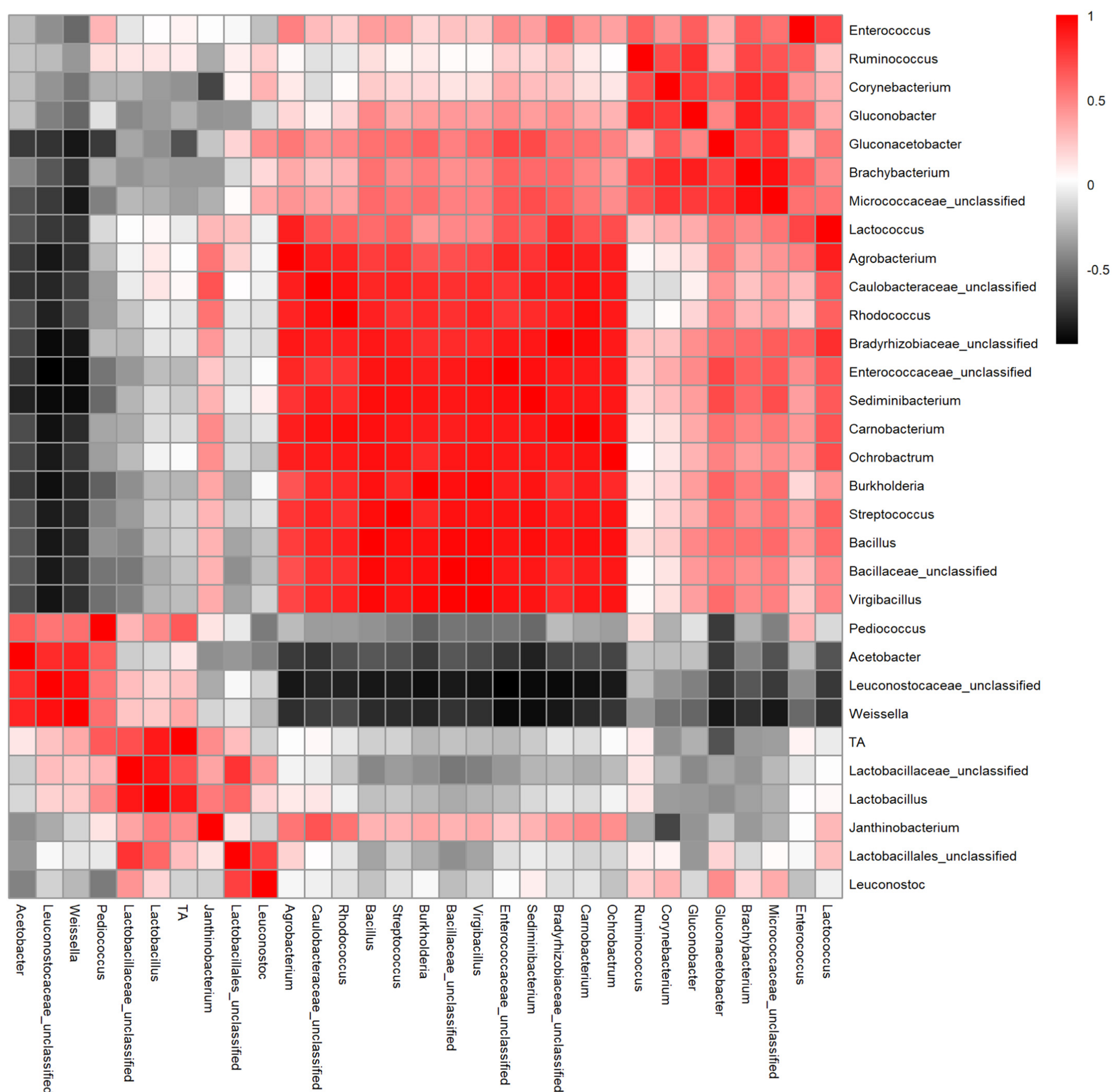


Fig. 6. Correlations between the abundance of different types of bacteria, and the abundances of bacteria and total acid. The figure presents a Spearman's rank correlation matrix of bacterial/fungal species with >0.01% abundance. The color of the scale bar denotes the nature of the correlation with 1 indicating a perfect positive correlation (light red) and -1 indicating a perfect negative correlation (light black).

Lactobacillales were LAB. And LAB may have important functions in the production of some fermented foods such that they can produce lactate and promote flavor [26]. *Acetobacter* is well-known for oxidizing alcohols to ace acids [27]. *Janthinobacterium* is one of the best folate producers [28,29]. *Ruminococcus* is an enriched microbial consortia for the fermentation of sugarcane vinasse toward short-chain organic acids [30]. Apart from these bacteria, some other low-abundant taxa, for example, unclassified *Caulobacteraceae*, *Agrobacterium*, and *Ochrobactrum*, during fermentation were first discovered to be related to the acid production. Future studies are recommended with the aim of screening the mechanism of acid production by isolating the key bacteria species responsible for acid production during fermentation.

To the best of our knowledge, some researchers have looked at the structure of the bacterial communities that experienced dynamic changes during the fermentation of rice wine. For example, Lv et al. obtained bacterial dynamic information via polymerase chain reaction-denaturing gradient gel electrophoresis PCR-DGGE) revealed the relative proportions of *Bacillus sp.*, *P. acidilactici*, *L. brevis*, and *P. pentosaceus* detected at early fermentation stage decreased as the fermentation progressed, while *L. plantarum* was consistently detected with high light band intensity throughout the fermentation process [2]. They also investigated the microbial compositions of fermented samples using a culture-dependent approach (conventional plate count technique combined with molecular identification), and showed that the relative proportions of *Lactococcus lactis* subsp. *lactis*, *Staphylococcus pasteuri*, *Bacillus* sp., and *P. pentosaceus* detected at the early brewing stage decreased as the fermentation progressed, while *L. brevis, L. plantarum* and *Lactobacillus paracasei* became the

predominant species during the late brewing period [4]. Huang et al. demonstrated bacteria change during the traditional fermentation process by microbiological analysis based on high-throughput sequencing (HTS) technology. *Bacillus* spp., *Staphylococcus* spp., *Weissella* spp., and *P. acidilactici* were detected at the initial brewing stage, and their populations decreased as the fermentation progressed, while those of *Lactobacillus, Gluconacetobacter*, and *Leuconostoc* increased to become the predominant genera at the final stage [31]. These findings all verify that LAB was dynamically changed during fermentation in rice wine; some of the LAB were alive in high concentration of lactic acid and alcohol such as Lactobacillus, by the PCR-DGGE, culture-dependent approach, and high-throughput sequencing (HTS) technology. In this study, we found that *Pediococcus* and unclassified *Lactobacillaceae* became the dominant bacteria genera at day 16; at the same time, from Fig. 2 it was found that from days 16 to 30 of fermentation, the total acid in groups A, B, and C still grows but very slowly. On one hand, the higher level of alcohol and acid content can inhibit some of the lactate-producing enzyme activity of LAB, including glucokinase (GLK), pyruvate kinase (PYK), 6-phosphofructokinase (PFK), phosphoglucose isomerase (PGI), and lactate dehydrogenase (LDH) [32], so some LAB are alive in high concentration of lactic acid, but they only produced a little acid. On the other hand, the relative abundance of bacterial flora at a later stage of fermentation may be increased by the DNAs from the dead bacteria in the process of fermentation. Because high-throughput sequencing technology does not allow complete characterization of fermenting microbial communities and activities unless it is complemented by RNA-based evaluations of community compositions. All of the microbial taxa in cereal fermentations are culturable in HQGRW and DNA is a very stable molecule, wherein short sequences can be detected long after cell death. Thus, in fermentations where succession of different microorganisms occur, microbial taxa can remain detectable via DNA sequencing long after cell death. Therefore, during fermentation where succession of different microorganisms occurs, microbial taxa can remain detectable via DNA sequencing long after cell death. Recently, the utilization of an RNA-based 16S rRNA gene sequencing analysis for PCR reactions can allow the evaluation of presumably active microbial components of communities [33,34]. This method allows for a significant reduction in the detection of contaminant sequences, thereby rendering RNA-based 16S rRNA gene sequencing an ideal strategy for studying microbial ecosystems. Further study will use the RNA-based method to study the dynamics of the closed microbial ecosystem present during HQGRW fermentation.

From Fig. 2, it was found that at 2 and 5 d of fermentation, the total acid and pH value in each group changed rapidly, meaning that the key bacteria for acid production can grow and produce acid under a suitable acidity, alcohol, and pH value condition; from d 16 to 30 of fermentation, the total acid in group A (7.14 g/L up to 7.54 g/L), group B (8.74 g/L up to 9.41 g/L), and group C (9.28 g/L up to 10.95 g/L) was still growing slowly. This means that the key bacteria may exist, which can grow and product acid under a high acidity and alcohol and low pH condition at 16 d. From d 25 to 30 of fermentation, the total acid and pH value in each group would be difficult to change, meaning that the dominate bacteria probably were not necessarily related to acid production, because at this time, the key bacteria for acid production may be no longer alive or functioning. Therefore, in this study, days 0, 2, 5, and 16 were chosen as the time points at which to study the relationship between total acids and bacterial genus.

It was found from Fig. 5 that group B is more closely related to group C than group A at day 0 by hierarchical clustering analysis. It may be due to the fact that at day 0, the dominant bacteria of groups B and C all were *Bacillus* (49.09% and 33.60%) and *Bacillaceae*_unclassified (22.19% and 23.40%), the dominant bacteria of group A were Gluconobacter (50.14%) with *Bacillus* and *Bacillaceae*_unclassified only (13.28% and 11.21%) from Fig. 3 and Table S2. Then, at the early stage of fermentation (d 2 and 5), the alcohol in groups A and B increased more rapidly than those in group C (Fig. 2). It caused that the low alcohol-tolerant bacteria were inhibited more quickly, and LAB became the dominant bacteria more quickly in groups A and B than those in group C. Furthermore, as the fermentation progressed, the alcohol increases to a level that can inhibit the growth of some LAB more quickly in groups A and B than those in group C. Also, the dominant bacteria in groups A and B change into alcohol-tolerant bacteria more quickly than those in group C (Fig. 2). Thus, group B is more closely related to group A than group C in the cluster of days 2, 5, and 16 (Fig. 5). The results showed that the influence of environment on dominant flora in the fermentation process is much greater than that of bacterial flora carried out by Hong Qu starters. Therefore, future research will focus on the optimization of fermentation process conditions to achieve the regulation of the total acid in rice wine.

Most of LAB can produce antibacterial materials such as bacteriocin, which can inhibit the growth of spoilage and pathogens microbes during fermentation [35]. Therefore, in this study, the growth of LAB can inhibit the growth of acetic acid bacteria and miscellaneous bacteria (Fig. 6), which is helpful to prevent the deterioration and acetification of wine. Some studies have revealed that LAB discovered rapid growth during the fermentation of HQGRW [2], inferring that it may promote the formation of organic acid. However, the spoilage of rice wine has been reported to be associated with the rapid growth of LAB at the beginning of fermentation [36]. Because most of LAB produce lactic acid and acetic acid, the pH of the environment may decline, which could finally inhibit the growth of other microorganisms, such as yeasts and Monascus [37]. Thus, the growth rate of LAB should be controlled at a certain level to improve the quality of rice wine. In this study, there was a negative response between total acid increase and the rate of increase in alcohol during the early fermentation stages. Therefore, we can try to control the growth of LAB by adding a certain amount of alcohol at the beginning of fermentation, so as to achieve the purpose of controlling total acid and improving the quality of HQGRW in future work. In conclusion, this is the first study to investigate the total acid dynamics, as well as the dynamics of the bacterial communities, during the fermentation of HQGRW and provide the correlation between bacterial communities and total acid during traditional fermentation. Results could facilitate better understanding of the acid-production mechanism during the fermentation process to improve the quality and safety of HQGRW. Future studies are recommended to screen the mechanism of acid production by isolating the key bacterial species responsible for acid production during fermentation and it should be performed using a multi-omics approach, including metatranscriptomics, metagenomics, and metaproteomics.

https://doi.org/10.1016/j.ejbt.2019.12.002

Financial support

This study was financially supported by the Natural Science Foundation of Fujian Province, China [No. 2016J01124] and the Regional Development Project of Fujian Province, China [No. 2019N3017]. We would like to thank Shanghai Personal Biotechnology Co., Ltd. for Illumina MiSeq sequencing and analysis.

Declaration of competing interest

The authors have no conflicts of interest to declare.

References

[1] Liu ZB, Wang ZY, Lv XC, et al. 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 2018;69:105–15. https://doi.org/10.1016/j.fm.2017.07.019 PMid: 28941890.

[2] Lv XC, Huang RL, Chen F, et al. Bacterial community dynamics during the traditional brewing of *Wuyi* Hong Qu glutinous rice wine as determined by culture-independent methods. Food Control 2013;34(2):300–6. https://doi.org/10.1016/j.foodcont.2013.05.003.
[3] Xie GF, Li WJ, Lu J, 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 2007;113(3):272–9. https://doi.org/10.1002/j.2050-0416.2007.tb00287.x.
[4] Lv XC, Chen ZC, Jia RB, et al. Microbial community structure and dynamics during the traditional brewing of *Fuzhou* Hong Qu glutinous rice wine as determined by culture-dependent and culture-independent techniques. Food Control 2015;57: 216–24. https://doi.org/10.1016/j.foodcont.2015.03.054.
[5] Cai HY, Zhang T, Zhang Q, et al. Microbial diversity and chemical analysis of the starters used in traditional Chinese sweet rice wine. Food Microbiol 2018;73:319–26. https://doi.org/10.1016/j.fm.2018.02.002 PMid: 29526219.
[6] Lv XC, Huang XL, Zhang W, et al. Yeast diversity of traditional alcohol fermentation starters for Hong Qu glutinous rice wine brewing, revealed by culture-dependent and culture-independent methods. Food Control 2013;34(1):183–90. https://doi.org/10.1016/j.foodcont.2013.04.020.
[7] Wang PX, Mao J, Meng XY, et al. Changes in flavour characteristics and bacterial diversity during the traditional fermentation of Chinese rice wines from Shaoxing region. Food Control 2014;44:58–63. https://doi.org/10.1016/j.foodcont.2014.03.018.
[8] Lv XC, Li Y, Qiu WW, et al. Development of propidium monoazide combined with real-time quantitative PCR (PMA-qPCR) assays to quantify viable dominant microorganisms responsible for the traditional brewing of Hong Qu glutinous rice wine. Food Control 2016;66:69–78. https://doi.org/10.1016/j.foodcont.2016.01.040.
[9] Shi JH, Xiao YP, Li XR, et al. Analyses of microbial consortia in the starter of fen liquor. Lett Appl Microbiol 2009;48(4):478–85. https://doi.org/10.1111/j.1472-765X.2009.02554.x PMid: 19187491.
[10] Li XR, Ma EB, Yan LZ, et al. Bacterial and fungal diversity in the traditional Chinese liquor fermentation process. Int J Food Microbiol 2011;(1)146:31–7. https://doi.org/10.1016/j.ijfoodmicro.2011.01.030 PMid: 21377228.
[11] Zheng XW, Yan Z, Han BZ, et al. Complex microbiota of a Chinese "*Fen*" liquor fermentation starter (*Fen-Daqu*), revealed by culture-dependent and culture-independent methods. Food Microbiol 2012;31(2):293–300. https://doi.org/10.1016/j.fm.2012.03.008 PMid: 22608236.
[12] Wu JJ, Ma YK, Zhang FF, et al. Biodiversity of yeasts, lactic acid bacteria and acetic acid bacteria in the fermentation of "Shanxi aged vinegar", a traditional Chinese vinegar. Food Microbiol 2012;30(1):289–97. https://doi.org/10.1016/j.fm.2011.08.010. 22265314.
[13] Zhang CY, Lin NB, Chai XS, et al. A rapid method for simultaneously determining ethanol and methanol content in wines by full evaporation headspace gas chromatography. Food Chem 2015;183:169–72. https://doi.org/10.1016/j.foodchem.2015.03.048. 25863625.
[14] Costa OYA, Souto BM, Tupinambá DD, et al. Microbial diversity in sugarcane ethanol production in a Brazilian distillery using a culture-independent method. J Ind Microbiol Biotechnol 2015;42(1):73–84. https://doi.org/10.1007/s10295-014-1533-1. 25404204.
[15] Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6. https://doi.org/10.1038/nmeth.f.303. 20383131.
[16] Chen H, Jiang W. Application of high-throughput sequencing in understanding human oral microbiome related with health and disease. Front Microbiol 2014;5: 508. https://doi.org/10.3389/fmicb.2014.00508.
[17] Magoc T, Salzberg S FLASH. Fast length adjustment of short reads to improve genome assemblies. Bioinformatics 2011;27(21):2957–63. https://doi.org/10.1093/bioinformatics/btr507. 21903629.
[18] Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26(19):2460–1. https://doi.org/10.1093/bioinformatics/btq461. 20709691.
[19] Ramette A. Multivariate analyses in microbial ecology. FEMS Microbiol Ecol 2007;62(2):142–60. https://doi.org/10.1111/j.1574-6941.2007.00375.x. 17892477.
[20] Jiang XT, Peng X, Deng GH, et al. Illumina sequencing of 16S rRNA tag revealed spatial variations of bacterial communities in a mangrove wetland. Mic Ec 2013;66(1): 96–104. https://doi.org/10.1007/s00248-013-0238-8 PMid: 23649297.
[21] Huang ZR, Guo WL, Zhou WB, et al. Microbial communities and volatile metabolites in different traditional fermentation starters used for Hong Qu glutinous rice wine. Food Res Int 2018;121:593–603. https://doi.org/10.1016/j.foodres.2018.12.024. 31108786.
[22] Britz TJ, Tracey RP. The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos*. J Appl Microbiol 1990;68(1):23–31. https://doi.org/10.1111/j.1365-2672.1990.tb02544.x.
[23] Lafon-Lafourcade S, Carre E, Ribéreau-Gayon P. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl Environ Microbiol 1983;46(4):874–80. 16346401.
[24] Xiong T, Guan QQ, Song SH, et al. Dynamic changes of lactic acid bacteria flora during Chinese sauerkraut fermentation. Food Control 2012;26(1):178–81. https://doi.org/10.1016/j.foodcont.2012.01.027.
[25] Hwa Park K, Liu ZB, Park CS, et al. Microbiota associated with the starter cultures and brewing process of traditional *Hong Qu* glutinous rice wine. Food Sci Biotechnol 2016;25(3):649–58. https://doi.org/10.1007/s10068-016-0115-6. 30263319.
[26] Simonen M, Palva I. Protein secretion in Bacillus species. Microbiol Rev 1993;57(1): 109–37. 8464403.
[27] Jonas R, Farah LF. Production and application of microbial cellulose. Polym Degradation Stab 1998;59(1–3):101–6. https://doi.org/10.1016/S0141-3910(97)00197-3.
[28] Kariluoto S, Edelmann M, Herranen M, et al. Production of folate by bacteria isolated from oat bran. Int J Food Microbiol 2010;143(1–2):41–7. https://doi.org/10.1016/j.ijfoodmicro.2010.07.026. 20708290.
[29] Herranen M, Kariluoto S, Edelmann M, et al. Isolation and characterization of folate-producing bacteria from oat bran and rye flakes. Int J Food Microbiol 2010;142(3): 277–85. https://doi.org/10.1016/j.ijfoodmicro.2010.07.002. 20678822.
[30] Bruna SM, Graciete MS, Tiago PD, et al. Enriched microbial consortia for dark fermentation of sugarcane vinasse towards value-added short-chain organic acids and alcohol production. J Biosci Bioeng 2019;127(5):594–601. https://doi.org/10.1016/j.jbiosc.2018.10.008 PMid: 30420331.
[31] Huang Z-R, Hong J-L, Xu J-X, et al. Exploring core functional microbiota responsible for the production of volatile flavour during the traditional brewing of *Wuyi* Hong Qu glutinous rice wine. Food Microbiol 2018;76:487–96. https://doi.org/10.1016/j.fm.2018.07.014. 30166178.
[32] Zhang QL, Brashears M, Yu ZM, et al. Effect of ultrafiltered fractions from casein on lactic acid biosynthesis and enzyme activity in yoghurt starter cultures. Int J Food Sci Tech 2013;48(7):1474–82. https://doi.org/10.1111/ijfs.12115.
[33] Chen K-H, Liao H-L, Arnold AE, et al. RNA-based analyses reveal fungal communities structured by a senescence gradient in the moss *Dicranum scoparium* and the presence of putative multi-trophic fungi. New Phytol 2018;218(4):1597–611. https://doi.org/10.1111/nph.15092. 29604236.
[34] Wuyts S, Van Beeck W, Oerlemans EFM, et al. Carrot juice fermentations as man-made microbial ecosystems dominated by lactic acid Bacteria. Appl Environ Microbiol 2018;84:e00134–00118. https://doi.org/10.1128/AEM.00134-18 PMid: 29654180.
[35] Cappello MS, Zapparoli G, Logrieco A, et al. Linking wine lactic acid bacteria diversity with wine aroma and flavour. Int J Food Microbiol 2017;243:16–27. https://doi.org/10.1016/j.ijfoodmicro.2016.11.025. 27940412.
[36] Hong XT, Chen J, Liu LD, et al. Metagenomic sequencing reveals the relationship between microbiota composition and quality of Chinese Rice wine. Sci Rep 2016;6: 26621. https://doi.org/10.1038/srep26621. 27241862.
[37] Edwards C, Reynolds A, Rodriguez AV, et al. Implication of acetic acid in the induction of slow/stuck grape juice fermentations and inhibition of yeast by *Lacobacillus* sp. Am J Enol Viticult 1999;50:204–10.