The changes of microbial diversity and flavor compounds during the fermentation of millet Huangjiu, a traditional Chinese beverage

Yi Yan¹,², Haiyan Chen¹,², Leping Sun¹,², Wei Zhang³, Xin Lu⁴, Zhenpeng Li⁴, Jialiang Xu¹,²*, Qing Ren¹,²*

¹ School of Light Industry, Beijing Technology and Business University, Beijing, China, ² Key Laboratory of Brewing Molecular Engineering of China Light Industry, Beijing, China, ³ College of Food Science and Technology, Hebei Agricultural University, Baoding, China, ⁴ State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China

* xujialiang@btbu.edu.cn (JX); renqing@th.btbu.edu.cn (QR)

Abstract

Huangjiu is a national alcoholic beverage in China. Millet has congenital advantages in development and utilization of nutrient. Brewing Huangjiu with millet can increase the value of millet. Microbial community plays crucial roles in millet Huangjiu fermentation. Flavor compounds reflect the quality and health function of Huangjiu. The flavor compounds of Huangjiu are complex and their formation is closely associated with microorganisms, but the relationship between them during fermentation has been unknown. In this research, this relationship during millet Huangjiu fermentation were deeply investigated. Totally 86 volatile compounds were detected. Bacillus, Weissella, Paenibacillus, Klebsiella, Prevotella was investigated as the dominant microbes through high-throughput sequencing. 537 correlations between major flavor compounds and microbes were established to reflect the dynamic change during millet Huangjiu fermentation. The top five dominant genus of flavor producing microbes were Chryseobacterium, Sporolactobacillus, Psychrobacter, Sphingobium and Anoxybacillus. The content of malic acid and citric acid was gradually improved all through the millet Huangjiu fermentation. Malic acid and citric acid generated from millet Huangjiu fermentation shows healthy properties as liver protection and eliminating fatigue. Our research provides essential information on microbial community succession and the flavor formation during millet Huangjiu fermentation, and beneficial for development of Huangjiu products.

Introduction

Huangjiu is one of the oldest wine types and most famous liquors in China. Over the past several years, the joint efforts of the entire industry promoted the rapid development of rice wine, which also attract notable attention [1]. Glutinous rice was usually used as the brewing raw materials, such as Guyue Longshan rice wine in southern China. Non-glutinous rice, indica
rice and glutinous rice mentioned earlier are three typical raw materials for traditional Huangjiu brewing, barleys and millet with prominent regional characteristics also beginning to emerge [2, 3]. The key raw material in Northern Huangjiu is millet, the content of crude protein and starch in which is much higher than that of rice and wheat [4, 5]. As excellent natural functional food with high nutritious value, millet shows unique advantages in food development and application, which has been validated in yogurt, bread and baby supplementary foods [6, 7]. All these superior qualities make it very suitable for Northern Huangjiu fermentation. Furthermore, dietary fiber, flavonoids, polyphenols, inositol, sterols and other nutrients are also found rich in millet meanwhile [8, 9]. On the one hand, current studies predominantly focus on the improvement of millet brewing technology, the flavor formation in which was comparatively less well understood [10–12]. On the other hand, the exploitation and utilization of millet resources has been limited due to its indigestion [13]. Huangjiu brewed by millet could increase the value of millet, which also have some implications for the development of Huangjiu products.

The aroma is the best characterized feature of Huangjiu, determined by diverse volatile flavor compounds, and over 900 different kinds of various volatile flavor compounds have been confirmed according Chen et al’s study, comprising primarily esters, alcohols, phenols, aldehydes, ketones and acids [14, 15]. Though the classification of Huangjiu varies according to region or aroma types, the production process could be generally divided into six major stages [3, 15–17]: the pretreatment of raw materials (especially soaking in millet Northern Huangjiu, while glutinous rice steaming in Southern Huangjiu), saccharification (primary fermentation), alcoholization (secondary fermentation), filtering, sterilizing and aging. In other words, almost all the aroma compounds have been achieved through the above complicated fermentation process with the help of raw materials and Qu (fermentation starter) [18], during which the microbial diversity plays the most critical and indispensable role [19, 20]. Microbiota and flavor dynamics during Huangjiu brewing mainly refers to simultaneous saccharification fermentation [3, 21]. Several studies have demonstrated that Bacillus, Leuconostoc, Lactococcus, Weissella, Thermoactinomyces, Pseudomonas, Saccharopolyspora, Staphylococcus, Enterobacter and Lactobacillus were the dominant genera during the fermentation of Shaoxing rice wine [21]. Analogously, five Bacillus species and three lactic acid bacteria were identified as the dominant bacteria in Hong Qu rice wine, which is another eminent Chinese Huangjiu as well [22]. These microorganisms have been proved essential during fermentation and the generation of special flavors. Chen found that the main fungi species during wheat Qu storage were Aspergillus oryzae, Absidia corymbifera, Rhizomucor pusillus, Clavispora lusitaniae and Saccharomycopsis fibuligera [23]. Another study combined traditional microorganism isolation methods and PCR-Denaturing Gradient Gel Electrophoresis (DGGE) technology obtained Thermomyces lanuginosus and Fusarium sp., which had not been reported in Shaoxing Huangjiu wheat Qu [24]. According to Huang et al, Lactobacillus, Leuconostoc, and Bacillus from bacteria, and Weissella, Saccharomyces, Rhizopus, Aspergillus and Candida of fungi are the core functional microorganisms during Wuyi Hongqu Huangjiu fermentation [16]. It has been reported that six microbial genera (Saccharomyces, Aspergillus, Saccharopolyspora, Staphylococcus, Lactobacillus, and Lactococcus) were most intimately linked to the major flavor components—amino acids, alcohols, acids, phenols and esters [25].

This study aims at monitoring the bacterial succession via high throughput sequencing (HTS) and the detection of the volatile compound dynamics with the help of headspace solid phase micro-extraction combined with gas chromatography-mass spectrometry (HS-SPME/GC-MS) during brewing. We also considered to find the relationship between volatile compounds and the bacterial flora, expecting to provide promising perspectives on flavor and functional microbes in millet Huangjiu fermentation for the first time. Usage of millet brewed...
Huangjiu can realize the development and utilization of millet resources, and is beneficial for the development of novel Huangjiu products.

**Materials and methods**

**Sample collection**

The fermentation assay was displayed at a constant temperate of 28˚C and the fermentation stage lasted for twelve days. The fermented mash of each sample was collected at 0, 2, 4, 6, 8, 10, 12 fermentation stages. Each sample has six replications. Each sample was conducted for flavor test and high-throughput sequencing.

**Determination of reducing sugar, alcohol, acidity, volatile compounds and organic acids**

The dinitrosalicylic acid method (DNS) was used to detect the content of reducing-sugar with glucose as standard substance [26]. The alcohol degree and the acidity assay could evaluate the quality of Huangjiu, which was measured based on the standard of GB/T13662-2008.

The HS-SPME-GC-MS was used to analyze the characters of volatile compounds. The volatile compounds were collected by a 50/30µm DVB/CAR/PDMS (Superco, Bellefonte, PA, USA). Each Huangjiu sample (8 mL) was set in a 15 mL SPME glass vial in addition with 2.5 g NaCl and 5 mL internal standards (65.76 mg/L 2-octanol). Then the water bath and ultrasonic wave were applied for the treatment of the mixture for 45 min at 50˚C. The volatile compounds were identified via a Shimadzu-QP2010 Plus-GCMS. The carrier gas helium was circulated at the speed of 1 mL/min with the split-flow mode, the split ratio of which was set as 50/1. The settings of the oven temperature program were as follows: 35˚C 4 min; five centigrade per minute ramp to 150˚C and time-keeping for 2 min; 3˚C/min ramp to 210˚C. The temperatures of detector and injector were both 230˚C and that of ion source was 200˚C. The ion energy for electron impact (EI) was adjusted to 70 eV. The detection and monitor of the total ion currents were performed to record the chromatograms in 30–350 mass range. 2-octanol was used as the internal standard to determine semi-quantitatively the content of the volatile compounds [27].

The HPLC analysis was used to analyze organic acids. Each wine sample of 5 mL was put in tube and centrifuged at 10,000 r/min for 20 min, then filtrated through a microporous membrane, which was 0.45 mm. Chromatographic conditions were referred to the method proposed by Ye et al with some modifications [28]. The separations were conducted on Agilent 1260 Infinity II equipped with a 250 mm x 4.6 mm and 5 µm Welch ultimate XB-C18 column. The temperature of the column was set at 30˚C. A mixture of phosphate buffer (0.01 mol/L (NH₄)₂HPO₄), adjusted with the solution of phosphoric acid to pH 3.0 was employed as the mobile phase with a flow rate of 0.7 mL/min. The detection wavelength was 215 nm.

**DNA extraction, and high-throughput sequencing**

Total genomic DNA of fermented mash samples was extracted via CTAB method, which was further adjusted to a uniform final concentration of 1 ng/µL by sterile water.

Hypervariable regions of V3-V4 on 16S rRNA gene of bacteria were amplified through PCR with specific primers of 338 F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806 R (5’-GGACTACHVGGGTWTCTAAAT-3’). Primers of ITS1 (5’-AxxxCTTGTCATTGATATGC-3’) and ITS2 (5’-BGCTGCGTTCTTCATGG-3’) were used to amplified ITS1 and ITS2 region of fungus. All PCR reactions were performed in Phusion High-Fidelity PCR Master Mix (NEB). The mixture of PCR products was then purified using Qiagen Gel Extraction Kit.
Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer’s recommendations. The library quality was assessed on the Agilent Bioanalyzer 2100 system. Finally, the library mentioned above was sequenced with the help of an Illumina HiSeq2500 platform.

Paired-end reads were assigned to samples on account of their unique barcode and truncated through cutting off the primer sequences and barcode. Paired-end reads were merged via FLASH (V1.2.7) [29]. According to the QIIME (V1.7.0), the high-quality clean reads were obtained after the raw reads filtered under specific filtering conditions [30, 31]. Chimera sequences were detected through the reference database (Gold database) using UCHIME algorithm, and then removed out [32, 33].

**OTU cluster, species annotation and phylogenetic analysis**

Sequences were performed with Uparse software (Uparse v7.0.1001) [34]. Sequences with ≥97% similarities were assigned to be the same OTUs. GreenGene Database based on RDP 3 classifier (Version 2.2) was applied to annotate the taxonomic information for each representative OTU [35, 36]. Alpha and beta diversity analysis was performed on account of the normalized data. The phylogenetic relationship of different OTUs was investigated by multiple sequence alignment by the MUSCLE software (Version 3.8.31) [37]. Alpha diversity was analyzed via the usage of six indices, including Observed-species, Chao1, Shannon, Simpson, ACE and coverage. All the indices were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Beta diversity was calculated by both weighted and unweighted UniFrac via QIIME software (Version 1.7.0).

**Correlations between microorganisms and flavor compounds**

Correlations between the microorganisms and flavor compounds during the fermentation of millet Huangjiu were established by Pearson correlation coefficient (r). R programming language was used to construct the correlation network. The P value was adjusted by FDR using the Benjamini-Hochberg method. P value and the adjusted P value lower than 0.05 was regarded as significant difference.

**Results and discussion**

**The acid, reducing sugar and alcohol were altered during millet Huangjiu fermentation**

The contents of acids, reducing sugar and alcohol were detected based on National Standard of the People’s Republic of China [38]. The results showed acid concentration continued to climb with a rapid growth since 8th day then began to flatten at day 10. The content of reducing sugar reached peak value on the 2nd day, and decreased progressively with the process of fermentation. The alcohol also tended to increase gradually and exhibited a fast increase from day 0 to day 2, with a steady growth rate from 4th day to 12th day (Fig 1).

Studies on Huangjiu using traditional rice as raw material have shown that the liquor output rate was negatively correlated to the fat content in rice. In the meantime, the reduction of protein in raw materials could effectively control the peculiar smell raised by excessive higher alcohols, and the contents of harmful substances such as ethyl urethane and biogenic amine in wine. Furthermore, the starch granules expanded after water absorption, facilitating the accumulation of reducing sugar, helpful for further saccharification and fermentation in millet Huangjiu. Therefore, the application of millet with low concentration of protein and fat, as well as high content of starch as raw material for Huangjiu brewing would be a better choice [39–42].
Organic acid in millet Huangjiu fermentation

Acid is an important flavoring substance of wine. Its main component organic acid, an essential precursor of flavors, could interact with other aroma & flavor-producing substances then collectively form the unique flavor and fragrance in Huangjiu [43, 44]. An appropriate amount of organic acid helps harmonize and stabilize the taste and aroma of wine, making it refreshing and palatable. Moreover, organic acids also hold considerable implications for improving intestinal function, resisting fatigue and other health benefits [45]. The alcoholic and malolactic fermentation and oxidation of the ethanol contributed a lot to the formation of organic acids.

The main organic acids identified in millet Huangjiu fermentation were oxalic acid, tartaric acid, pyruvic acid, malic acid, α-ketoglutaric acid, lactic acid, citric acid and succinic (S1 Table). The concentration of oxalic acid, tartaric acid, malic acid, lactic acid, citric acid and succinic acid were showed to be upward in general as the fermentation time extended compared to day 0. Most of them have increased sharply from 8th day to 10th day. As a whole, the total content of organic acids reached the peak at the end of the fermentation day 12, while the valley was detected at the initial day 0. The concentration of succinic acid and lactic acid were significantly higher than others, especially the former. Pyruvic acid reached the highest concentration at 6th day, subsequently underwent a decline then increased a bit on day 10 and 12 but still lower than the value of day 6. α-ketoglutaric acid reached its peak on 2nd day, and then kept decreasing over the remnant days, becoming the least abundant substance al last.
Lactic acid, malic acid, citric acid, tartaric acid and succinic acid are typical non-volatile acid [46]. Lactic acid bacteria presented in Huangjiu fermentation could produce massive lactic acid, which contribute a lot to the excellent flavor of Huangjiu. Malic acid is widely known for anti-fatigue, liver protection and cardiovascular fitness enhancement, while citric acid mainly serves as delaying senility, lowering blood pressure and eliminating fatigue. They have shown important effects on the quality of Huangjiu.

**Volatile compounds in in millet Huangjiu fermentation**

In this study, 95 volatile flavor compounds were finally detected during the whole fermentation process, including 31 esters, 23 alcohols, 13 alkanes, 7 ketones, 6 acids, 3 phenols, 2 aldehydes and 10 other kinds of volatile compounds (S2 Table). Hexadecanoic acid ethyl ester, 9,12-octadecadienoic acid ethyl ester, decanoic acid ethyl ester and hexanoic acid ethyl ester were the dominant ester with high concentration in the last day of fermentation. Among all detectable alcohols, 3-methyl-1-butanol, 2,3-butanediol, phenylethyl alcohol and glycerin were major alcohols during the fermentation process. In addition, 2-methoxy-4-vinylphenol, pentacosane and 2-octanone were the dominant phenol, alkane and ketones, respectively. At the last day of fermentation, 48 compounds were detected, including twenty-four esters, thirteen alcohols, three ketones, three acids, one alkane, one aldehyde, one phenol, and three other volatile compounds. The contents and varieties of those flavor compounds reached their peak at 10th day.

The types and quantities of flavor compounds were fluctuated during the different brewing stages. The content of alcohol was relatively steady in different brewing periods; the changes of esters and alcohol were stable except for a mild decrease at the end of brewing. While the content of acids and alkanes were gradually declined with the development of brewing.

The characteristic flavor compounds in fermented millet Huangjiu are 2-methyl-1-propanol, 3-methyl-1-butanol, phenethyl alcohol, hexanoic acid ethyl ester, octanoic acid ethyl ester, decanoic acid ethyl ester, 2-hydroxy-propanoic acid ethyl ester, hexadecanoic acid ethyl ester, (E)-9-octadecenoic acid ethyl ester, 9,12-octadecadienoic acid ethyl ester.

**Bacterial community dynamics during millet Huangjiu fermentation**

The initial fermentation had significant difference in bacterial richness and community diversity compared with later fermentation. Bacterial richness was not altered during the middle and final stages of fermentation, but the community diversity in the two stages were dramatically different (Table 1 and S1 Fig).

An average of 2,455,812 high-quality reads were obtained through high-throughput sequencing. Totally, 26 phyla, 45 classes, 90 orders, 142 families and 284 genera of bacteria participated in millet Huangjiu fermentation. The dominant phyla in millet Huangjiu fermentation is Firmicutes, which predominated over 90% in the whole samples (Fig 2A). Followed by Proteobacteria and Bacteroidetes. Firmicutes was gradually declined in the first six days. Proteobacteria appeared its maximum quantity on the second day, and then slightly decreased. The peak of Bacteroidetes was appeared at the fourth day.

From the genus level (Fig 2B), Bacillus was predominant, represented by 57.60–98.69% among the bacteria. The second abundant genus was Paenibacillus, with a decline on the sixth day. Weissella was the third abundant genus, which reached the highest quantity on the second day, and then slightly decreased. The peak of Bacillales was appeared at the fourth day.

From the genus level (Fig 2B), Bacillus was predominant, represented by 57.60–98.69% among the bacteria. The second abundant genus was Paenibacillus, with a decline on the sixth day. Weissella was the third abundant genus, which reached the highest quantity on the 2nd day, then decreased and gradually leveled off on the 8th day.

**Fungal community dynamics during millet Huangjiu fermentation**

Four phyla, 9 classes, 13 orders and 15 genera of fungus were identified and characterized. The results showed that approximately 99% fungus belong to the phylum of
Ascomycota (Fig 3A). *Saccharomyces*, *Issatchenka* and *Lichtheimia* occupied the top three at genus level (Fig 3B). *Saccharomyces* and *Lichtheimia* reached the highest level on the second day. The quantity of *Saccharomyces* was declined on the fourth day, and then gradually increased at remaining fermentation stage. The quantity of *Issatchenka* was varied slowly in the fermentation process.

### Relationship between microorganisms and flavor compounds during millet Huangjiu fermentation

Totally 537 correlations were established between flavor compounds and microorganisms during millet Huangjiu fermentation. 153 microorganisms were relevant to the formation of main flavor compounds (*P* < 0.05) (Fig 4A). *Rheinheimera, Psychrobacter, Sporolactobacillus*, and *Chryseomicrobium* participated in the formation of more than 10 flavor compounds. *Acetobacter*, *Asticcacaulis, Bradyrhizobium, Brevibacillus, Enterococcus, Aquabacterium, Methyllobacterium, Myxococcus, Novosphingobium, and Sphingomonas* were only associated with the formation of 4-ethyl-2-methoxy-phenol. *Clostridium_sensu_stricto_7, Exiguobacterium, Finegoldia, Lysobacter*, was related to the generation of octadecanoic acid ethyl ester, hexanoic acid ethyl ester, octadecanoic acid ethyl ester and 3-methyl-1-butanol, respectively. There are more than 15 genera were associated with the formation of acetic acid phenylethyl ester, decanoic

Table 1. Comparison of α diversity and β diversity during millet Huangjiu fermentation.

| Between-group          | P-values (α Diversity Index) | P-values (β Diversity Index) |
|------------------------|------------------------------|------------------------------|
|                        | Observed species | Chao1 | ACE | Shannon | Simpson | Weighted UniFrac distance |
| Day0 vs Day2           | **               | *     | *   | ***     | NS      | NS                            |
| Day0 vs Day4           | *                | NS    | NS  | **      | ***     | **                          |
| Day0 vs Day6           | *                | NS    | NS  | **      | NS      | NS                          |
| Day0 vs Day8           | NS               | NS    | NS  | NS      | NS      | NS                          |
| Day0 vs Day10          | **               | **    | *   | *       | *       | **                          |
| Day0 vs Day12          | *                | *     | *   | ***     | ***     | *                            |
| Day2 vs Day4           | NS               | NS    | NS  | *       | **      | NS                          |
| Day2 vs Day6           | NS               | NS    | NS  | NS      | NS      | *                           |
| Day2 vs Day8           | NS               | NS    | NS  | NS      | **      | NS                          |
| Day2 vs Day10          | NS               | NS    | NS  | NS      | *       | NS                          |
| Day2 vs Day12          | NS               | NS    | NS  | NS      | NS      | NS                          |
| Day4 vs Day6           | NS               | NS    | NS  | NS      | NS      | NS                          |
| Day4 vs Day8           | NS               | NS    | NS  | NS      | NS      | NS                          |
| Day4 vs Day10          | NS               | NS    | NS  | NS      | **      | NS                          |
| Day4 vs Day12          | NS               | NS    | NS  | NS      | NS      | NS                          |
| Day6 vs Day8           | NS               | NS    | NS  | *       | ***     | NS                          |
| Day6 vs Day10          | NS               | NS    | NS  | NS      | **      | NS                          |
| Day6 vs Day12          | NS               | NS    | NS  | NS      | NS      | NS                          |
| Day8 vs Day10          | *                | *     | NS  | NS      | NS      | NS                          |
| Day8 vs Day12          | NS               | NS    | NS  | NS      | **      | NS                          |
| Day10 vs Day12         | NS               | NS    | NS  | NS      | *       | NS                          |

Significance: NS > 0.05,
* <0.05,
** ≤0.01,
*** ≤0.001

https://doi.org/10.1371/journal.pone.0262353.t001
acid ethyl ester, 4-ethyl-2-methoxy-phenol, hexanoic acid ethyl ester, octanoic acid ethyl ester, oxalic acid, tartaric acid, glycerin and 3-hydroxy-2-butanone.

P value related to microorganisms and the major flavor compounds was adjusted to obtain closer correlation. 56 correlations were established by filtering P adjust less than 0.05 (Fig 4B). More than 20 microorganisms exhibited correlations to the formation of 4-ethyl-2-methoxy-phenol and hexanoic acid ethyl ester. *Sphingobium* showed a closer correlation with the generation of dodecanoic acid ethyl ester, tetradecanoic acid ethyl ester and decanoic acid ethyl ester. 2-methyl-1-propanol was showed a closer correlation with *Virgibacillus*. Dodecanic acid ethyl ester is colorless oil liquid with a waxy, rum aroma and cream flavors, commonly found in cognac, rum, Irish whiskey and Chinese traditional beverages [47–49], also detected in our millet Huangjiu. It could be used in daily fragrance and flavoring essence for the manufacture of soft drinks, ice creams, candies and baking food, also applied to produce lubricants, plasticizers and softeners. The traditional catalyst for synthesizing dodecanic acid ethyl ester is concentrated sulfuric acid, although cheap and easy to obtain, there have still been many side reactions [50]. The accumulation of carboid is inevitable and the color of the esterified products is too much darker. Moreover, the product quality is seriously affected and the post-reaction treatment is also complicated. Therefore, the use of biotechnology methods, such as direct microbial fermentation and enzymatic catalysis exhibit lower energy consumption and higher product quality [51]. Above all, it is more environmentally friendly than traditional chemical synthesis methods. In this study, after P value adjust (Fig 4B), we found that *Sphingobium* still has a strong correlation with the formation of dodecanic acid ethyl ester, which suggests that
we could subsequently isolate and cultivate this strain and optimize the fermentation conditions to achieve the large-scale synthesis of dodecanic acid ethyl ester \textit{in vivo}, overcoming the defects of the original production process.

Open fermentation of Huangjiu could result in the diverse microorganisms in the brewing process [52]. Microorganisms in the wheat Qu and external environment together provide essential enzymes and metabolites for the fermentation process, which has also formed the unique flavor of Huangjiu. In the present study, the main bacteria in the fermentation of millet Huangjiu were \textit{Bacillus}, \textit{Brevibacillus} and \textit{Weissella}. \textit{Bacillus} could secret carbohydrate degradation enzymes, such as glucanase, pectinase and cellulase, which could destroy the cell walls of plant cells and release the nutrients. \textit{Bacillus} could also synthesize many different kinds of organic acids, physiological active substances and nutrients, most of which are flavor precursors and flavor compounds, which explains the dominant reason for \textit{Bacillus} [53]. \textit{Brevibacillus} could degrade starch, xylan, cellulose, non-starch polysaccharides and improve the utilization rate of raw materials. The function of \textit{Weissella} in fermentation was decompose glucose to carbon dioxide and ethanol [54].

Higher alcohols and aromatic esters are the dominant and important volatile flavor components in Huangjiu. They reflect the quality and the flavor of Huangjiu. In this study, the main alcohols were 2-methyl-1-propanol, 3-methyl-1-butanol, 2-3-butanediol and \(\beta\)-phenethyl alcohol, most of which were the degradation products of amino acids [55]. 2-methyl-1-propanol could be obtained from natural fermentation of carbohydrates, or biosynthesized by genetic engineering techniques [56–58]. Shota Atsumi reported intermediates \textit{Escherichia coli} amino acid synthesis pathway could generate 2-methyl-1-propanol through expressed \textit{kind} and
Fig 4. The correlation analysis between microorganisms and flavor compounds during the fermentation of millet Huangjiu. The blue pie represents microbial composition; the red pie indicates flavor compounds composition; the green line means correlation between microorganisms and flavor compounds. a: \( P < 0.05 \); b: \( P \) adjust < 0.05.
https://doi.org/10.1371/journal.pone.0262353.g004
ADH2 (14). In this study, we found the formation of 2-methyl-1-propanol was closely related to Candidatus Nitrosopumilus, Modestobacter, Oceanobacillus and Virgibacillus. 3-methyl-1-butanol could affect the quality of the Huangjiu, and is also harmful to human toxicity [59]. Our study suggested that Gulosibacter, Lysobacter, Virgibacillus were related to the production of 3-methyl-1-butanol. It is quite necessary to decrease the content of 3-methyl-1-butanol by regulating the three strains during Huangjiu fermentation. 2,3-butanediol has sweet taste and has been widely used to improve liquor flavor. K. oxytoca and B. polymyxa displayed a relative higher production ability of 2-3-butanediol [60, 61]. In the process of millet Huangjiu brewing, Anoxybacillus, Peptoniphilus, Sphingobium, Sporolactobacillus and Stenotrophomonas were closely related to the synthesis of 2,3-butanediol. Phenethyl alcohol is an aromatic higher alcohol, which is extensively used in various alcoholic beverages [27]. Previous studies showed that phenethyl alcohol was mainly produced by yeast metabolism and growth [62, 63]. In addition, Helicobacter, Peptoniphilus, Sphingobium, Sporolactobacillus and Stenotrophomonas were also involved in the synthesis of beta phenethyl alcohol [64].

Higher alcohols are very important precursors for ester formation. Esters can impact the quality and flowery flavors of alcohol beverages. The main esters during fermentation are identified as hexanoic acid ethyl ester (strong aroma for liquor blending), octanoic acid ethyl ester (colorless transparent liquid with an odor similar to brandy), decanoic acid ethyl ester (colorless transparent liquid with fruity and bouquet aroma) [65], hexadecanoic acid ethyl ester (light yellow oily liquid with the aroma of oil, sweet and mellow, increase the mellow feeling of the wine) [66], 9,12-octadecadienoic acid ethyl ester (responsible for reducing blood fat, preventing and curing atherosclerosis). The formation of these esters is closely related to alcohols, fatty acids, coenzyme A, etc. [67]. Their synthesis pathways are concerned with yeast species. In the present research, we found Acidaminococcus, Anoxybacillus, Bordetella, Deinococcus, Fusobacterium, Gluconobacter, Peptoniphilus, Serratia and Sphingobium were involved in the synthesis of various esters during millet Hunagjiu fermentation, which also indicated that the biosynthesis of esters was associated with microorganisms.

As one of the extremely important flavoring substances in Huangjiu [68] and Baijiu [69, 70], hexanoic acid ethyl ester (ethyl hexanoate) is also the representative of the characteristic flavor component in Baijiu, the content of which directly determines the quality of strong-flavor Baijiu. Esters produced during the fermentation process are mainly synthesized by the following two pathways based on present research: one is that microorganisms synthesize esters under the action of their own intracellular enzymes [71–73]; while the more frequent method is the catalysis by the extracellular enzymes (microbial lipases) secreted by microorganisms when organic acids react with alcohols in outside surrounding environment for synthesis [74, 75]. In addition, the acidic substances and alcohols in the wine body can also undergo esterification under natural conditions, but the rate and yield are far lower than the above two methods [76]. Bacillus spp., Alcaligenes spp., and Pseudomonos spp., of bacteria and Penicillium spp., Fusarium spp., Aspergillus spp., of fungi are screened as candidates for large scale of the production of lipases mentioned above [77]. However, the selection and modification of high-yield ethyl caproate strains mainly focus on Saccharomyces cerevisiae [78–80] and Monascus purpureus [81–83]. In our study, we established the closer relationship between 21 novel genus of bacterium (such as Aericardovia, Atojobium and Paracoccis) and the formation of ethyl hexanoate after P value adjust (Fig 4B). The isolation and cultivation of them would provide a solid theoretical basis for the identification of bacteria producing ethyl hexanoate esterase and the optimization of fermentation conditions for further higher yield.

With herbal aroma and warm spicy taste, 4-ethyl-2-methoxyphenol has been widely used in food additive and fragrance, especially in wine and soy sauce [84]. As Fig 4B showed, the formation of 4-ethyl-2-methoxyphenol could be related to 26 different genera of bacterium with
less abundance during millet Huangjiu fermentation, suggesting that the generation of flavor compounds is sophisticated and fantastic, and it is worth noting that the interaction between different, especially those less abundant microbes.

Acid is an important flavor compounds of Huangjiu, and organic acid of which is mainly responsible for the sour taste in Huangjiu. Moreover, organic acids are critical to improve and enhance intestinal function, anti-fatigue and other health functions. Malic acid plays important roles in anti-fatigue, liver protection and cardiovascular fitness enhancement. The main function of citric acid is delaying senility, lowering blood pressure and eliminating fatigue. Most Lactobacillus species have been proved indispensable for producing lactic acid, ethanol and acetic acid. The genus Saccharomyces, Pichia, and Zygosaccharomyces could convert lactic acid to pyruvate. Moreover, there have been evidences that they could convert pyruvate to acetyl-CoA, acetaldehyde in liquor production [85]. Microorganisms include several yeast, Aspergillus, Penicillium and Candida could accumulate citric acid [86]. The microorganisms that could produce malic acid were Saccharomyces and Aspergillus [87, 88]. The genera Bacteroides, Porphyromonas and Sedimentibacter have been reported to produce succinic acid, propionic acid and alcohols [89–91]. Besides the microorganisms reported above, many other microorganisms are also involved in the formation of organic acids according to our study. Aeromonas, Brachybacterium, Haemophilus, Weissella can utilize glucose to produce acid during fermentation. Fusobacterium can hydrolyze sugars and proteins, and usually produce mixed organic acids and alcohols with sugar or peptone participation. Furthermore, there are numbers of microbial species such as Buchnera, Cetobacterium, Chryseobacterium, Macrococcus, Gaiella, otgalbacillus, Marinimicrobium, Oxalophagus, Wolinella, Pelotomaculum, Psychrobacter all related to organic acid production, but have not yet been fully investigated in detail. The relationship between these microorganisms and their corresponding acids needs to be verified in future. The existence of carbonyl and carboxyl groups in pyruvate acid makes its participation in various biochemical reactions, especially in Tricarboxylic acid cycle and microbial metabolism, further promoting more microbial involvement during fermentation, also the direct or indirect precursor of many high value-added products in millet Huangjiu aroma. The principally well-known pyruvate bioproduction microorganisms are fungus like Torulopsis glabrata [92, 93] and Saccharomyces cerevisiae [94], with less research on bacterium. For example, Corynebacterium pyruvicproduens was isolated as candidate strains to produce pyruvate [95, 96] and Escherichia coli acetF mutant strains were genetically engineered to ferment pyruvate [97, 98]. In our study, Corynebacterium was the only genus predicted association with the formation of pyruvic acid. This strong relevance indicated it could be a high-productive pyruvate strain during millet Huangjiu fermentation, also inspired us for its further isolation and extensive application.

Millet, a kind of high-quality healthy grain, has not been paid enough attention to its utilization. Although as the main raw material for Huangjiu fermentation, it has seldom been reported. Northern Huangjiu using millet can realize the development and utilization of millet resources, and also beneficial for the development of novel Huangjiu products. On the other hand, the way to promote the quality and flavor of Huangjiu is a consistent problem. Our work could provide more suggestive information on flavor and functional microbes during millet Huangjiu fermentation. Future investigation would focus on the improvement millet Huangjiu quality by synthetic microbial communities closely relevant to aroma compounds.

Conclusions

Millet Huangjiu is a national alcoholic beverage in China. In this study, basic physicochemical parameters, 95 flavor compounds (31 esters, 23 alcohols, 13 alkanes, 7 ketones, 6 acids, 3
phenols, 2 aldehydes and 10 other kinds of volatile compounds) and microorganism profiles (284 and 15 genera of bacteria and fungus were detected, respectively) have been investigated during millet Huangjiu fermentation, their correlations were also established and analyzed. *Bacillus* was principal followed by *Paenibacillus* and *Weissella* form the genus level. Overall, 537 correlations were established between flavor compounds and microbes during millet Huangjiu fermentation. 153 microorganisms were relevant to the formation of main flavor compounds (*P*<0.05). The top five dominant genus of flavor producing microbes were *Chryseobacterium*, *Sporolactobacillus*, *Psychrobacter*, *Sphingobium* and *Anoxybacillus*. Our research provides essential information on the relationship between microbial community and the flavor formation during millet Huangjiu fermentation, and is beneficial for the development of Huangjiu products.

**Supporting information**

S1 Table. Dynamic change of organic acids found in Huangjiu brewed from millet during fermentation.

(XLSX)

S2 Table. Dynamic change of volatile compounds in Huangjiu brewed from millet during fermentation.

(XLSX)

S1 Fig. Distributions of α diversity indices (A: observed species index, B: Shannon diversity index) during fermentation stages

(TIF)

**Acknowledgments**

We are grateful to all wine producers that collaborate with this study.

**Author Contributions**

Funding acquisition: Yi Yan, Wei Zhang.

Methodology: Yi Yan, Qing Ren.

Supervision: Xin Lu.

Visualization: Zhenpeng Li.

Writing – original draft: Haiyan Chen, Leping Sun, Jialiang Xu.

Writing – review & editing: Yi Yan, Haiyan Chen, Leping Sun, Wei Zhang.

**References**

1. Wang J, Shen Y, Lu W, Qian Y. Current situation and development trend of Chinese medicine information research. China Brewing 2013; 33(4):559–64.

2. Yang Y, Hu W, Xia Y, Mu Z, Tao L, Song X, et al. Flavor Formation in Chinese Rice Wine (Huangjiu): Impacts of the Flavor-Active Microorganisms, Raw Materials, and Fermentation Technology. Front Microbiol. 2020; 11:580247. https://doi.org/10.3389/fmicb.2020.580247 PMID: 33281774; PubMed Central PMCID: PMC7691429.

3. Chen G-M, Huang Z-R, Wu L, Wu Q, Guo W-L, Zhao W-H, et al. Microbial diversity and flavor of Chinese rice wine (Huangjiu): an overview of current research and future prospects. Current Opinion in Food Science. 2021.

4. Hilu K, De Wet J, Seigler D. Flavonoid patterns and systematics in Eleusine. Biochemical Systematics and Ecology. 1978; 6(3):247–9.
5. Gao X, Li B, Mei J. Research progress in active components and functional properties of yellow rice wine. Brewing Technol. 2018; 1:91–6.
6. Shi LL, Li A, Mu FT, Zhang WG. Effect of different kinds of millet on the flavor of Huangjiu. China Brewing. 2021; 40(3):54–63.
7. Ojediran J, Adamu M, Jim-George DJA JA GA. Some physical properties of Pearl millet (Pennisetum glaucum) seeds as a function of moisture content. 2021; 6(1).
8. Shi H, Shi G, Yang C, Hou G, Chen Y. Study on the Nourishment Health Care of Millet and Food Treat Value. Rain Fed Crops. 2007.
9. Chethan S, Malleshi N. Finger millet polyphenols: Optimization of extraction and the effect of pH on their stability. Food Chem. 2007; 105(2):862–70.
10. Liu JK, Zhao W, Fu HQ, Zhang YZ. A Study on Brewing Technology for Millet Yellow Wine. Cereal & Feed Industry. 2009;(12):22–3+7.
11. Amadou I. Millet Based Fermented Beverages Processing. Fermented Beverages: Elsevier; 2019. p. 11.
12. Kalse S, Swami S, Sawant A, Thakor NJ. Development and quality evaluation of jamun seed powder fortified biscuit using finger millet. 2016; 7(633):2.
13. Ren Q, Sun L, Wu H, Wang Y, Wang Z, Zheng F, et al. The changes of microbial community and flavor compounds during millet Huangjiu fermentation. Food Res Int. 2020; 134:109238. Epub 2020/06/11. https://doi.org/10.1016/j.foodres.2020.109238 PMID: 32517941.
14. Chen S, Xu Y, Qian MC. Journal F. Comparison of the aromatic profile of traditional and modern types of Huang Ji (Chinese rice wine) by aroma extract dilution analysis and chemical analysis. 2018; 33(3):263–71.
15. Wang J, Yuan C, Gao X, Kang Y, Huang M, Wu J, et al. Characterization of key aroma compounds in Huangjiu from northern China by sensory-directed flavor analysis. Food Res Int. 2020; 134:109238. Epub 2020/06/11. https://doi.org/10.1016/j.foodres.2020.109238 PMID: 32517941.
16. Hu Y, Lei X, Zhang X, Guan T, Wang L, Zhang Z, et al. Characteristics of the Microbial Community in the Production of Chinese Rice-Flavor Baijiu and Comparisons With the Microflora of Other Flavors of Baijiu. Front Microbiol. 2021; 12:673670. Epub 2021/05/18. https://doi.org/10.3389/fmicb.2021.673670 PMID: 33995338; PubMed Central PMCID: PMC8116502.
17. Zhu J, Lin JL, Palomec L, Wheeldon I. Microbial host selection affects intracellular localization and activity of alcohol-2-acetyltransferase. Microb Cell Fact. 2015; 14:35. Epub 2015/04/17. https://doi.org/10.1186/s12934-015-0221-9 PMID: 25880435; PubMed Central PMCID: PMC4367896.
18. Chen T, Wu F, Guo J, Ye M, Hu H, Guo J, et al. Effects of glutinous rice protein components on the volatile substances and sensory properties of Chinese rice wine. J Sci Food Agric. 2020; 100(8):3297–307. Epub 2020/02/23. https://doi.org/10.1002/jsfa.10343 PMID: 32086813.
19. 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. 2015; 31(12):1907–21. https://doi.org/10.1007/s11274-015-1931-1 PMID: 26492888.
20. Yu LJ, Ding F, Ye H. Analysis of characteristic flavour compounds in Chinese rice wines and representative fungi in wheat Qu samples from different regions. J I Brewing. 2012; 118(1):114–9. https://doi.org/10.1002/jib.13 PubMed PMID: WOS:000306959000016.
21. Ji Z, Jin J, Yu G, Mou R, Mao J, Liu S, et al. Characteristic of filamentous fungal diversity and dynamics associated with wheat Qu and the traditional fermentation of Chinese rice wine. 2018; 53(7):1611–21.
22. Liu S, Chen Q, Zhou H, 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. 2019; 308:3–18. Epub 2019/05/19. https://doi.org/10.1016/j.ijfoodmicro.2019.05.001 PMID: 31162963.
23. Chen J. Study on the factors of Shaoxing Yellow Wine maiqu quality and its influence. Zhejiang Province: Jiangnan University; 2008.
24. Ji Z, Jin J, Yu G, Mou R, Mao J, Liu S, et al. Characteristic of filamentous fungal diversity and dynamics associated with wheat Qu and the traditional fermentation of Chinese rice wine. 2018; 53(7):1611–21.
25. Liu S, Chen Q, Zhou H, 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. 2019; 308:3–18. Epub 2019/05/19. https://doi.org/10.1016/j.ijfoodmicro.2019.05.001 PMID: 31162963.
26. Hong XT, Chen J, Liu L, Wu H, Tan HQ, Xie GF, et al. Metagenomic sequencing reveals the relationship between microflora composition and quality of Chinese Rice Wine. Sci Rep-Uk. 2016;6: doi: ARTN 26621 https://doi.org/10.1038/srep26621 PubMed PMID: WOS:000376874300001. PMID: 27241862.
27. Luo T, Fan WL, Xu Y. Characterization of volatile and semi-volatile compounds in Chinese rice wines by headspace solid phase microextraction followed by gas chromatography-mass spectrometry. J I Brewing. 2008; 114(2):172–9. [10.1002/j.2050-0416.2008.tb00323.x] PubMed PMID: WOS:000258479800013.

28. Fu-Rong YE, Xi-Dan C, Xia-Hong N. Study of Analytical Method for Organic acids in Yellow Rice Wines by High Performance Liquid Chromatography. Liquor Making. 2013; 40:80–5.

29. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011; 27(21):2957–63. [10.1093/bioinformatics/btr507] PubMed PMID: WOS:000296099300005.

30. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JL, Knight R, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods. 2013; 10(1):57–U11. [10.1038/nmeth.f.303] PubMed PMID: WOS:000312810100005. PMID: 23202435

31. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010; 7(5):335–6. [10.1038/nmeth.f.303] PubMed PMID: WOS:000277175100003. PMID: 20383131

32. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011; 27(16):2194–200. [10.1093/bioinformatics/btr381] PubMed PMID: WOS:000293620800004. PMID: 21700674

33. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Ganiotou G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 2011; 21(3):494–504. [10.1011/gr.112730.110] PubMed PMID: WOS:000287841100015. PMID: 21212162

34. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013; 10(10):996+. [10.1038/nmeth.f.2604] PubMed PMID: WOS:000325073800023. PMID: 23955772

35. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimerach- checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microb. 2006; 72(7):5069–72. [10.1128/AEM.03006-05] PubMed PMID: WOS:000238961000071. PMID: 16820507

36. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microb. 2007; 73(16):5261–7. [10.1128/AEM.03006-05] PubMed PMID: WOS:000238961000071. PMID: 17586664

37. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32(5):1792–7. [10.1103/nar/2004.00062-05] PubMed PMID: WOS:0002200487200025. PMID: 15034147

38. Council CNLI. Chinese rice wine. Beijing: China Standard Press; 2008.

39. Ran X. Preliminary study on the selection criteria of raw materials for the Chinese Rice Wine [Dissertation for the Degree of Master]: Zhejiang A&F University; 2015.

40. Rong ZX, Zhou JD, Qian B, Ma C, Jiang YJ. Effect of the polishing degree of rice on higher alcohols content during the main fermentation of Chinese rice wine. China Brewing. 2013; 32(01):28–32.

41. Fang RS. The metabolism mechanism and inhibition method of ethyl carbamate formation during traditional Chinese rice wine fermentation [PhD dissertation]: Zhejiang University; 2017.

42. Song Y, Dong Q. Research progress in formation and control of the biogenic amine in Chinese rice wine. Science and Technology of Food Industry. 37(08):387–91.

43. Lin X, Wei W, He Z, Lin X. Determination of organic acids in rice wine by ion-exclusion chromatography. Chinese Journal of Chromatography. 2014; 32(3):304. [10.3724/sp.j.1123.2013.11023] PubMed PMID: 24984473

44. Robles A, Fabjanowicz M, Chmiel T, Plotka-Wasylka J. Determination and identification of organic acids in wine samples. Problems and challenges. Trac-Trend Anal Chem. 2019; 120. doi: ARTN 115630 [10.1016/j.trac.2019.115630] PubMed PMID: WOS:000501178370009.

45. Sun HL, Liu SP, Mao JQ, Yu ZW, Lin ZJ, Mao J. New insights into the impacts of huangjiu components on intoxication. Food Chem. 2020;317. doi: ARTN 126420 [10.1016/j.foodchem.2020.126420] PubMed PMID: WOS:000517839800039. PMID: 32101783

46. Yu HY, Zhao J, Li FH, TianHX, Ma X. Characterization of Chinese rice wine taste attributes using liquid chromatographic analysis, sensory evaluation, and an electronic tongue. J Chromatogr B. 2015; 997:129–35. [10.1016/j.jchromb.2015.05.037] PubMed PMID: WOS:000358816400019. PMID: 26113454

47. Kumar MH, Prabhu K, Rao M, Shanthi B, Kavimani N, Dinakar S, et al. Gas chromatography mass spectrometry analysis of one Ayurvedic skin oil, Eladi Kera Thailam. 2019; 11:2657–60.
48. Li L, Nandi I, Kim KH. Development of an ethyl laurate-based microemulsion for rapid-onset intranasal delivery of diazepam. Int J Pharm. 2002; 237(1–2):77–85. Epub 2002/04/17. https://doi.org/10.1016/s0378-5173(02)00029-7 PMID: 11955806.

49. Li H, Qin D, Wu Z, Sun B, Sun X, Huang M, et al. Characterization of key aroma compounds in Chinese Guojing sesame-flavor Baijiu by means of molecular sensory science. Food Chem. 2019; 284:100–7. Epub 2019/02/13. https://doi.org/10.1016/j.foodchem.2019.01.102 PMID: 30744833.

50. Daviot L, Len T, Lin CSK, Len CJC. Microwave-assisted homogeneous acid catalysis and chemoenzymatic synthesis of dialkyl succinate in a flow reactor. 2019; 9(3):272.

51. Gawas SD, Jadhav SV, Rathod VK. Characterization of key aroma compounds in Chinese Guojing sesame-flavor Baijiu by means of molecular sensory science. Food Chem. 2019; 284:100–7. Epub 2019/02/13. https://doi.org/10.1016/j.foodchem.2019.01.102 PMID: 30744833.

52. Daviot L, Len T, Lin CSK, Len CJC. Microwave-assisted homogeneous acid catalysis and chemoenzymatic synthesis of dialkyl succinate in a flow reactor. 2019; 9(3):272.

53. Gawas SD, Jadhav SV, Rathod VK. Characterization of key aroma compounds in Chinese Guojing sesame-flavor Baijiu by means of molecular sensory science. Food Chem. 2019; 284:100–7. Epub 2019/02/13. https://doi.org/10.1016/j.foodchem.2019.01.102 PMID: 30744833.
gas chromatography-ion mobility spectrometry. Food Res Int. 2021; 145:110421. Epub 2021/06/12. https://doi.org/10.1016/j.foodres.2021.110421 PMID: 34112423.

69. Lin H, Kang WC, Jin HJ, Man ZX, Chen QS. Discrimination of Chinese Baijiu grades based on colorimetric sensor arrays. Food Sci Biotechnol. 2020; 29(8):1037–43. Epub 2020/07/17. https://doi.org/10.1007/s10068-020-00757-z PMID: 32670657; PubMed Central PMCID: PMC7447373.

70. Zhang Y, Gu J, Ma C, Wu Y, Li L, Zhu C, et al. Flavor classification and year prediction of Chinese Baijiu by time-resolved fluorescence. Appl Opt. 2021; 60(19):5480–7. Epub 2021/07/16. https://doi.org/10.1063/5.0042015 PMID: 34263834.

71. Chen Y, Luo W, Gong R, Xue X, Guan X, Song L, et al. Improved ethyl caproate production of Chinese liquor yeast by overexpressing fatty acid synthesis genes with OPI1 deletion. J Ind Microbiol Biotechnol. 2016; 43(9):1261–70. Epub 2016/06/28. https://doi.org/10.1007/s10295-016-1795-x PMID: 27344573.

72. Dong J, Hong KQ, Zhang CY, Dong SS, Li X, Chen YF, et al. Increased Acetate Ester Production of Polyploid Industrial Brewer's Yeast Strains via Precise and Seamless "Self-cloning" Integration Strategy. Iran J Biotechnol. 2019; 17(2):e1990. Epub 2019/08/29. https://doi.org/10.21859/ijb.1990 PMID: 31457054; PubMed Central PMCID: PMC6697848.

73. Saerens SM, Verstrepen KJ, Van Laere SD, Voet AR, Van Dijck P, Delvaux FR, et al. The Saccharomyces cerevisiae EHT1 and EE81 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J Biol Chem. 2006; 281(7):4446–56. Epub 2005/12/20. https://doi.org/10.1074/jbc.M51208200 PMID: 16361250.

74. Martinez-Ruiz A, Tovar-Castro L, Garcia HS, Saucedo-Castaneda G, Favela-Torres E. Continuous ethyl oleate synthesis by lipases produced by solid-state fermentation by Rhizopus microsporus. Biosensour Technol. 2018; 265:52–8. Epub 2018/06/08. https://doi.org/10.1016/j.biotех.2018.05.080 PMID: 29879651.

75. Luo W. Research of Improving Ethyl Caproate Production in Saccharomyces Cerevisiae by Strengthening Fatty Acid Synthesis: Tianjin University of Science and Technology; 2016.

76. Ren DQ, Tang YM, Yao W, Liu Y, Zhuo Y, Xu K, et al. Research on the Kinetics of Esterifying Enzyme. Liquor-making Science & Technology. 2006; 144(06):39–40.

77. Chandra P, Singh R, Arora PKJMF. Microbial lipases and their industrial applications: a comprehensive review. 2020; 19(1):1–42.

78. Fan G, Liu P, Chang X, Yin H, Cheng L, Teng C, et al. Isolation and Identification of a High-Yield Ethyl Caproate-Producing Yeast From Daqu and Optimization of Its Fermentation. Front Microbiol. 2021; 12:663744. Epub 2021/06/18. https://doi.org/10.3389/fmicb.2021.663744 PMID: 34135875; PubMed Central PMCID: PMC8200637.

79. Takahashi T, Ohara Y, Sueno K. Breeding of a sake yeast mutant with enhanced ethyl caproate productivity in sake brewing using rice milled at a high polishing ratio. J Biosci Bioeng. 2017; 123(6):707–13. Epub 2017/03/14. https://doi.org/10.1016/jbiosc.2017.01.014 PMID: 28286120.

80. Yoda T, Ogura A, Saito T. Influence of Ethyl Caproate on the Size of Lipid Vesicles and Yeast Cells. Biomimetics (Basel). 2020; 5(2). Epub 2020/05/01. https://doi.org/10.3390/biomimetics5020016 PMID: 32349293; PubMed Central PMCID: PMC7344887.

81. Jiao J, Zheng Z, Liu Z, You C. Study of the Compositional, Microbiological, Biochemical, and Volatile Profile of Red-Veined Cheese, an Internal Monascus-Ripened Variety. Front Nutr. 2021; 8:649611. Epub 2021/06/04. https://doi.org/10.3389/fnut.2021.649611 PMID: 33937306; PubMed Central PMCID: PMC8085271.

82. Teng W, Li G, Liu X, Wang Q, Gu Q, Yu X. Isolation and identification of ethyl caproate esterifying enzyme in Daqu and its fermentative technology optimization. J Food Sci. 2016;35(9).

83. Xu Y, Wang X, Liu X, Li X, Zhang G, Li W, et al. Discovery and development of a novel short-chain fatty acid ester synthetic biocatalyst under aqueous phase from Monascus purpureus isolated from Baijiu. Food Chem. 2021; 338:128025. Epub 2020/09/15. https://doi.org/10.1016/j.foodchem.2020.128025 PMID: 32927200.

84. Rayne S, Eggers NJ. Quantitative determination of 4-ethylphenol and 4-ethyl-2-methoxyphenol in wines by a stable isotope dilution assay. J Chromatogr A. 2007; 1167(2):195–201. Epub 2007/09/14. https://doi.org/10.1016/j.chroma.2007.08.049 PMID: 17850808.

85. Song Z, Du H, Zhang Y, Xu Y. Unraveling Core Functional Microbiota in Traditional Solid-State Fermentation by High-Throughput Amplicons and Metatranscriptomics Sequencing. Front Microbiol. 2017; 8:1294. Epub 2017/08/05. https://doi.org/10.3389/fmicb.2017.01294 PMID: 28769888; PubMed Central PMCID: PMC5509801.

86. Papagianni M. Advances in citric acid fermentation by Aspergillus niger: biochemical aspects, membrane transport and modeling. Biotechnol Adv. 2007; 25(3):244–63. Epub 2007/03/06. https://doi.org/10.1016/j.biotechadv.2007.01.002 PMID: 17937335.
87. Peleg Y, Barak A, Scrutton M, Goldberg I. Malic acid accumulation by Aspergillus flavus. Applied Microbiology and Biotechnology. 1989; 30:176–83.

88. Pines O, Even-Ram S, El Nathan N, Battat E, Aharonov O, Gibson D, et al. The cytosolic pathway of L-malic acid synthesis in Saccharomyces cerevisiae: the role of fumarase. Appl Microbiol Biotechnol. 1996; 46(4):393–9. Epub 1996/11/01. https://doi.org/10.1007/BF00166235 PMID: 8987728.

89. Hu X, Hai D, Cong R, Yan X. Illuminating Anaerobic Microbial Community and Co-occurrence Patterns across a Quality Gradient in Chinese Liquor Fermentation Pit Muds. Appl Environ Microb. 2016;82. https://doi.org/10.1128/AEM.03409-15 PMID: 26896127

90. Isar J, Agarwal L, Saran S, Kaushik R, Saxena RKJA. A statistical approach to study the interactive effects of process parameters on succinic acid production from Bacteroides fragilis. 2007; 13(2):50–6.

91. Kawamura Y, Kuwabara S, Kania SA, Kato H, Hamagishi M, Fujiwara N, et al. Porphyromonas pogo-nae sp. nov., an anaerobic but low concentration oxygen adapted coccobacillus isolated from lizards (Pogona vitticeps) or human clinical specimens, and emended description of the genus Porphyromonas Shah and Collins 1988. Syst Appl Microbiol. 2015; 38(2):104–9. Epub 2014/12/08. https://doi.org/10.1016/j.syapm.2014.11.004 PMID: 25481042.

92. Li Y, Hugenholtz J, Chen J, Lun SY. Enhancement of pyruvate production by Torulopsis glabrata using a two-stage oxygen supply control strategy. Appl Microbiol Biotechnol. 2002; 60(1–2):101–6. Epub 2002/10/17. https://doi.org/10.1007/s00253-002-1064-y PMID: 12382048.

93. Miyata R, Yonehara T. Breeding of high-pyruvate-producing Torulopsis glabrata with acquired reduced pyruvate decarboxylase. J Biosci Bioeng. 1999; 88(2):173–7. Epub 2005/10/20. https://doi.org/10.1016/s1389-1723(99)80197-2 PMID: 16232593.

94. van Maris AJ, Geertman JM, Vermeulen A, Groothuisen MK, Winkler AA, Piper MD, et al. Directed evolution of pyruvate decarboxylase-negative Saccharomyces cerevisiae, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl Environ Microbiol. 2004; 70(1):159–66. Epub 2004/01/09. https://doi.org/10.1128/AEM.70.1.159-166.2004 PMID: 14711638; PubMed Central PMCID: PMC321313.

95. Goldenberger D, Hinic V, Turan S, Schultheiss E, Pacheco AL, Frei R, et al. Extended characterization of Corynebacterium pyruviciproducens based on clinical strains from Canada and Switzerland. J Clin Microbiol. 2014; 52(9):3180–3. Epub 2014/06/22. https://doi.org/10.1128/JCM.00792-14 PMID: 24951802; PubMed Central PMCID: PMC4313134.

96. Tong J, Liu C, Summanen PH, Xu H, Finegold SM. Corynebacterium pyruvicproduens sp. nov., a pyruvic acid producer. Int J Syst Evol Microbiol. 2010; 60(Pt 5):1135–40. Epub 2009/08/12. https://doi.org/10.1099/ijss.0.011793-0 PMID: 19666796.

97. Causey TB, Shannugam KT, Yomano LP, Ingram LO. Engineering Escherichia coli for efficient conversion of glucose to pyruvate. Proc Natl Acad Sci U S A. 2004; 101(8):2235–40. Epub 2004/02/26. https://doi.org/10.1073/pnas.0308171100 PMID: 14982993; PubMed Central PMCID: PMC356934.

98. Tomar A, Eiteman MA, Altman E. The effect of acetate pathway mutations on the production of pyruvate in Escherichia coli. Appl Microbiol Biotechnol. 2003; 62(1):76–82. Epub 2003/07/02. https://doi.org/10.1007/s00253-003-1294-6 PMID: 12839524.