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# A metagenomic analysis of the relationship between microorganisms and flavor development in Shaoxing mechanized huangjiu fermentation mashes

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## ABSTRACT

Complex microbial metabolism is responsible for the unique flavor of Shaoxing mechanized huangjiu. However, the relationship between the microorganisms present during fermentation and the formation of specific flavor components is difficult to understand. In this study, gas chromatography–mass spectrometry and high-performance liquid chromatography were used to identify flavor components, and a metagenomic sequencing approach was used to characterize the taxonomic and functional attributes of the Shaoxing mechanized huangjiu fermentation microbiota. The metagenomic sequencing data were used to predict the relationship between microorganisms and flavor formation. The chromatographic analysis identified amino acids, alcohols, acids, phenols and esters as major flavor components, and six microbial genera (*Saccharomyces*, *Aspergillus*, *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, and *Lactococcus*) were most closely related to the production of these flavor components. This study helps clarify the different metabolic roles of microorganisms in flavor formation during Shaoxing huangjiu fermentation.

## 1. Introduction

Huangjiu, also called Chinese rice wine, is a very popular traditional fermented alcoholic beverage in China (Chen and Xu, 2013). Shaoxing huangjiu is a typical huangjiu with regional characteristics. Although the hand producing process of Shaoxing huangjiu is still widely used, the mechanized producing process with controllable, industrial and efficient advantage gradually becomes the mainstream in Shaoxing. Like the traditional producing process, the fermentation process of Shaoxing mechanized huangjiu also uses glutinous rice, water, and wheat qu as raw materials, and is also a multispecies fermentation with a simultaneous saccharification and fermentation. However, rapid-fermenting yeast was inoculated into the shaoxing mechanized huangjiu fermentation mash (MHJFM) at the beginning of fermentation. The fermentation equipment for MHJFM is fermentation tank not ceramic jar. Cold water was kept in cooling jacket of the fermentation tank to control fermentation temperature automatically (He et al., 2015; Xu et al., 2010). The producing process includes a pre-fermentation period that lasts four to five days, a post-fermentation period that lasts 15 to 20 days, and then a series of refining processes that includes pressing, filtration, boiling, ageing, and blending. During the pre-fermentation period, the temperature is maintained between 27 and 33 °C. During the post-fermentation period, the temperature is kept below 18 °C.

A variety of volatile and non-volatile compounds affect the sensory characteristics and quality of huangjiu, and complex microbial metabolism is an important source of the complicated flavor (Chen et al., 2013). Non-volatile compounds can be converted to volatile compounds. For instance, phenylalanine, 4-coumaric acid and ferulic acid can be converted to phenylethanol, 4-vinylphenol and 4-vinylguaiacol. The relationship between flavor formation and microbial metabolism has been studied to reveal the primary functional microorganisms and metabolic pathways associated with specific flavor substances. In general, huangjiu flavor compounds include esters, alcohols, amino acids and organic acids (Wang et al., 2014). *Saccharomyces* is the main

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alcohol-producing microorganism in huangjiu. Wine fermented with different strains of *Saccharomyces cerevisiae* have differences in the kinds and amounts of alcohols and esters, which influence the sensory evaluation (Yang et al., 2017). The genus *Lactobacillus,* which are acid-producing bacteria, may also take an active part in huangjiu flavor development, but rapid growth of *Lactobacillus brevis* during the early stage of fermentation results in spoilage (Hong et al., 2016; Wang et al., 2014). In one effort to evaluate the effect of bacteria on volatile compounds formation, partial least squares regression was used to analyze the volatile compounds and bacterial community present during the fermentation (Liu et al., 2015). This analysis indicated that members of the genus *Pseudomonas, Thermoactinomyces*, *Bacillus* and *Lactococcus* were involved in synthesis of volatile compounds. Although some microorganisms that inhabit mechanized huangjiu fermentation mashes are known to be associated with the production of flavors, it is not fully understood which metabolic pathways are relevant to flavor formation, and what roles various microorganisms play. In particular, the genus *Saccharopolyspora* were recently found to be the dominant bacteria present during huangjiu fermentation. Huangjiu produced in good fermentations tended to have more *Saccharopolyspora* than that produced in poor fermentations (Hong et al., 2016). The high abundance and benefit of the genus *Saccharopolyspora* in huangjiu is unique among the world's fermented alcoholic beverage. *Staphylococcus* can produce enzymes such as lipase and peptidase (Leroy et al., 2017; Olesen and Stahnke, 2003), and may contribute to fatty acid catabolism in Mexican ripened cheese (Escobarzepeda et al., 2016). However, there have been no scientific studies about the function of *Saccharopolyspora* during huangjiu fermentation.

Amplicon sequencing and metagenomic sequencing have been widely used to study various microbial communities (An et al., 2013; Costea et al., 2017; Liu et al., 2017; Yao et al., 2017). Compared with amplicon sequencing, metagenomic sequencing offers deeper insight into the metabolic potential of a microbial community (Walsh et al., 2017). Metagenomic sequencing has been used extensively in studying microbiota and their function in fermented foods (Escobarzepeda et al., 2016; Ferrocino et al., 2017; Illeghems et al., 2015; Lu et al., 2016). These studies provided the rationale to analyze the relationships between the microbiota and specific flavors in huangjiu by using metagenomic sequencing.

In this study, the MHJFM in which *Saccharopolyspora* has been reported as the dominant microorganisms (Hong et al., 2016; Wang et al., 2014), was used to reveal the microbiota participating in the flavor metabolic network. Genes encoding the enzymes involved in main flavor component formation from *Saccharopolyspora*-dominated MHJFM were predicted by taxonomic and functional annotation.

## 2. Materials and methods

### 2.1. Sample collection

Samples of fermenting huangjiu mash were collected at 0, 24, 72, 120 and 312 h from the workshop of mechanized huangjiu at Shaoxing city, Zhejiang province. Sterile sampling bags (LabPLUS) were used to collect samples (about 1000 mL) from fermentation tanks and were immediately stored at −80 °C pending further analysis.

### 2.2. Analysis of non-volatile compounds

After centrifuged at 12000 rpm for 10 min, the supernatants were analyzed using a Waters e2695 high-performance liquid chromatography (HPLC) system equipped with an Athena C18-WP column (ANPEL no. 462572; 250 × 4.6 mm, 5 μm) and a UV detector. For the analysis of amino acids, supernatants were derivatized with phenyl isothiocyanate (PITC) and analyzed according to previous report (Wang et al., 2014).

For the analysis of organic acids, supernatants were precipitated with an equal volume of trichloroacetic acid (10%, w/v) for 4 h at 4 °C. The precipitates were removed by centrifugation at 12000 rpm for 10 min, and the supernatants were filtrated by 0.22 μm microporous membrane for HPLC analysis. The mobile phase was phosphate buffer (0.025 M $KH_2PO_4$, pH 3.1) with a flow rate of 0.8 mL/min at 30 °C and the detection wavelength was 210 nm.

Poly-Sery HLB SPE cartridges (CNW, 500 mg/6 mL) were used to concentrate the monophenols in the huangjiu supernatants (8 mL). The loaded cartridges were then washed with 24 mL water and the monophenols were eluted with methanol (4 mL). The eluate was concentrated to 1 mL using a stream of nitrogen gas and then subjected to HPLC analysis. Chromatographic conditions were based on the previous report (Favre et al., 2014).

### 2.3. Analysis of volatile compounds

The volatile flavor compounds were extracted through headspace solid phase microextraction (HS-SPME) and analyzed using gas chromatography–mass spectrometry (GC–MS) (Liu et al., 2015) with a Thermo Fisher Trace GC Ultra equipped with a TG-Wax column (30 m × 0.25 mm, 0.25 μm film thickness) and a TSQ Quantum XLS mass selective detector. Helium (0.8 mL/min) was used as the carrier gas. The injector was used in splitless mode at 250 °C. The oven temperature was held at 40 °C for 2 min, and then increased to 230 °C at 4 °C/min and kept at 230 °C for 5 min. All mass spectra were acquired in electron impact (EI) mode at 70 eV, using full scan ranging from 33 to 400 amu. Main compounds were quantified using external standards, and 2-octanol (220 mg/L, 20 μL) was used as the internal standard to eliminate variations in extraction efficiency.

### 2.4. Genomic DNA extraction

DNA was extracted using the method below. MHJFM samples (30 mL) were centrifuged at 7500 rpm for 10 min at 4 °C, and the sediments were quickly ground in liquid nitrogen. Then the sediments were resuspended in 10 mL of DNA extraction buffer (100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 100 mM $Na_3PO_4$, and 1.5 M NaCl) and shaken for 5 min at room temperature using a vortex oscillometer instrument. At this point, the suspensions were centrifuged at 2000 rpm for 5 min at 4 °C and the supernatants were collected. Lysozyme (50 mg/mL, 30 μL) and lywallzyme (50 mg/mL, 10 μL) were added to the supernatants, and the mixtures were incubated in water bath at 37 °C for 30 min. SDS (10%, 1.25 mL) and proteinase K (20 mg/mL; 50 μL) were added next, and the supernatants were incubated in water bath at 37 °C for 1 h. Then, an equal volume of CTAB (2%) was added and the supernatants were incubated in water bath at 65 °C for 1 h. It is important that the supernatants were inverted end-over-end every 10 min when in the water bath. After incubation, the supernatants were extracted three times with an equal volume of chloroform-isoamyl alcohol (24:1, v/v), and the aqueous phase was recovered after centrifugation at 12000 rpm for 10 min at 4 °C. DNA was precipitated from the aqueous phase by adding 0.6 times its volume of isopropanol, and then incubating the mixture for 1 h at −20 °C. The crude nucleic acids were obtained by centrifugation at 1200 rpm for 15 min at 4 °C, washed with pre-chilled 70% ethanol, and then resuspended in sterile water. Finally, the crude nucleic acids were purified using a magnetic bead purification kit (Biocanal Scientific, Inc). DNA purity was assessed using the A260/A280, and DNA integrity was verified with 1% agarose gel electrophoresis under ultraviolet light. The DNA was stored at −20 °C pending further processing.

### 2.5. Library construction and metagenomic sequencing

DNA libraries were constructed on the Illumina platform according to the manufacturer's instructions (Li et al., 2017). A total amount of 1 μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The DNA sample was fragmented by sonication to a size of 350 bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified using real-time PCR. Sequencing was performed with an Illumina Hiseq 4000 at the Novogene Bioinformatics Technology Co., Ltd.

### 2.6. Quality control and assembly

To acquire clean data for subsequent analysis, raw reads were subjected to the following treatments: (1) removal of reads with > 40 low-quality bases (quality threshold value ≤38); (2) removal of reads with > 10 ambiguous N bases; (3) removal of reads that shared an overlap of > 15 bases with the adapter.

The clean data was assembled and analyzed with SOAP denovo software (Luo et al., 2012) using k-mer 55 to acquire scaffolds. The assembled scaffolds were then interrupted from N (ambiguous base) connections to acquire scaftigs without N. The clean data from all samples were compared to each scaftig, respectively, with SoapAligner (version 2.21) software to acquire the reads not used, which were combined and then were processed as described above for mixed assembly. Finally, scaftigs shorter than 500 bp were filtered out.

### 2.7. Gene prediction and gene catalogue construction

The open reading frames (ORFs) within all scaftigs (≥500 bp) were predicted using MetaGeneMark software, and ORFs shorter than 100 nt were filtered out. CD-HIT software (Fu et al., 2012; Li and Godzik, 2006) was used to remove redundancy. Sequences with ≥95% identity and 90% coverage were thought to be redundant and the longer one was retained. Then, sequences which mapped to < 2 reads were removed, and the remaining gene catalogue (unigenes) was used for subsequently analysis.

The absolute abundance and relative abundance of unigenes were calculated using the formula below, in which r represents the number of reads mapped to the unigenes and L represents the unigenes length (Villar et al., 2015; Zeller et al., 2015).

$$G_k = (r_k/L_k)/\bullet \sum_{i}^{n} (r_i/L_i)$$

$$\text{Relative abundance} = \frac{G_k}{\sum_i^n G_i}$$

### 2.8. Taxonomic assignment and function annotation

All genes in our catalogue were aligned to the NR database and KEGG database using DIAMOND (Buchfink et al., 2015; Stadlmayr et al., 2015). Taxonomic assignment of predicted genes was carried out using BLASTP alignment against the integrated NR database. BLASTP alignment hits with e-values larger than $1 \times 10^{-5}$ were filtered, and for each gene the significant matches which were defined by e-values ≤ 10 × e-value of the top hit were retained to distinguish taxonomic groups. We used BLASTP to search the protein sequences of the predicted genes in the KEGG database with e-value $\leq 1 \times 10^{-5}$ (Qin et al., 2010).

### 2.9. Construction of the flavor metabolic network

Using the KEGG database or information from the literature (Albi and Serrano, 2015; Lukasheva et al., 2014; Pires et al., 2014), enzymes associated with enzymatic reactions or metabolic pathways related to the main flavor components were sorted out. Based on the results of taxonomic assignment and function annotation, these enzymes were connected with the microbiota of huangjiu fermentation mash by gene ID. When a gene ID from a microorganism was simultaneously annotated as an enzyme coding gene, then the connection of enzyme and microbiota was built. For example, when a gene ID 120h_4897 in unigenes was annotated from *Saccharopolyspora* by NR database, and was also annotated to code the enzyme EC 1.2.1.3 by KEGG database, than a connection between EC 1.2.1.3 and *Saccharopolyspora* was built. The taxonomic distribution and enzyme reads for substrate breakdown and flavor formation in microbial community were connected accord to previous reports (Qin et al., 2010; Qin et al., 2012; Stewart et al., 2018; Turnbaugh et al., 2009; Wu et al., 2017).

## 3. Results and discussion

### 3.1. Analyses of flavor compounds

The compositions of the organic acids, amino acids, monophenols and volatile compounds found in MHJFM are shown in Fig. 1. The concentrations of the flavor components grew most rapidly at 24 h, and the concentration of a variety of components (such as amino acids and higher alcohols, volatile phenols, esters) decreased after 312 h (Chen et al., 2018). The 72 h and 120 h time points, which represent the end of pre-fermentation and the beginning of post-fermentation respectively, were the time points before and after the fermentation temperature changes. Although some flavor compounds existed at 0 h, they were mainly from the raw materials like wheat qu and rice, not the microorganisms. The microorganisms at 0 h were mainly from wheat qu and the existence form of these microorganisms is mainly hypopus like hypnospores of fungi and bacilli which lacked or had little metabolic activity (Frisvad et al., 2019; He et al., 2015). Amino acid concentration increased significantly from 24 h to 312 h. Arginine, tryptophan, alanine, proline and glutamic acid, accounted for 53.09% to 61.39% of the 18 free amino acids. Lactic acid was the main organic acid, comprising 65.79% to 75.07% of the eight organic acids. Protocatechuic acid was the dominant monophenols at 0 h, while catechin became the dominant monophenols (> 50%) at 24 h and 72 h and then concentrations of epicatechin increased significantly at 120 h and 312 h. A total of 53 volatile compounds were identified. The volatile compounds accumulated primarily during the first 24 h. The 20 dominant volatile compounds, comprising 98.74% to 99.97% of the volatile compounds are shown in Fig. 1D. Ethyl lactate and ethyl acetate were the most abundant esters; β-phenylethanol, isoamyl alcohol and isobutanol were the most abundant higher alcohols; and 4-vinylguaiacol, 4-ethylguaiacol and 4-ethylphenol were the most abundant volatile phenols. Collectively, these main flavor components were similar to those identified in other reports (Chen et al., 2013). Therefore, these dominant flavor compounds, or their precursors, were used for predicting the relevant biosynthetic networks of the huangjiu microbiota in the following study.

### 3.2. Overview of metagenomic data

To identify the genetic information of the microbiota present during the huangjiu fermentation process, MHJFM samples at different fermentation stages were analyzed using metagenomic sequencing. This process generated 42.53 Gbp of raw reads, of which 42.22 Gbp remained after quality control. > 95% of the reads had sequencing errors of < 1% (Q20, Table 1), showing the high quality of the sequencing procedure. After gene prediction, 376,868 ORFs were acquired, and the gene catalogue contained 120,258 non-redundant unigenes. Of these, 93,935 unigenes accounting for 88.8% of the unigenes in the gene catalogue were shared among the 4 samples (Fig. 2). The 24 h sample

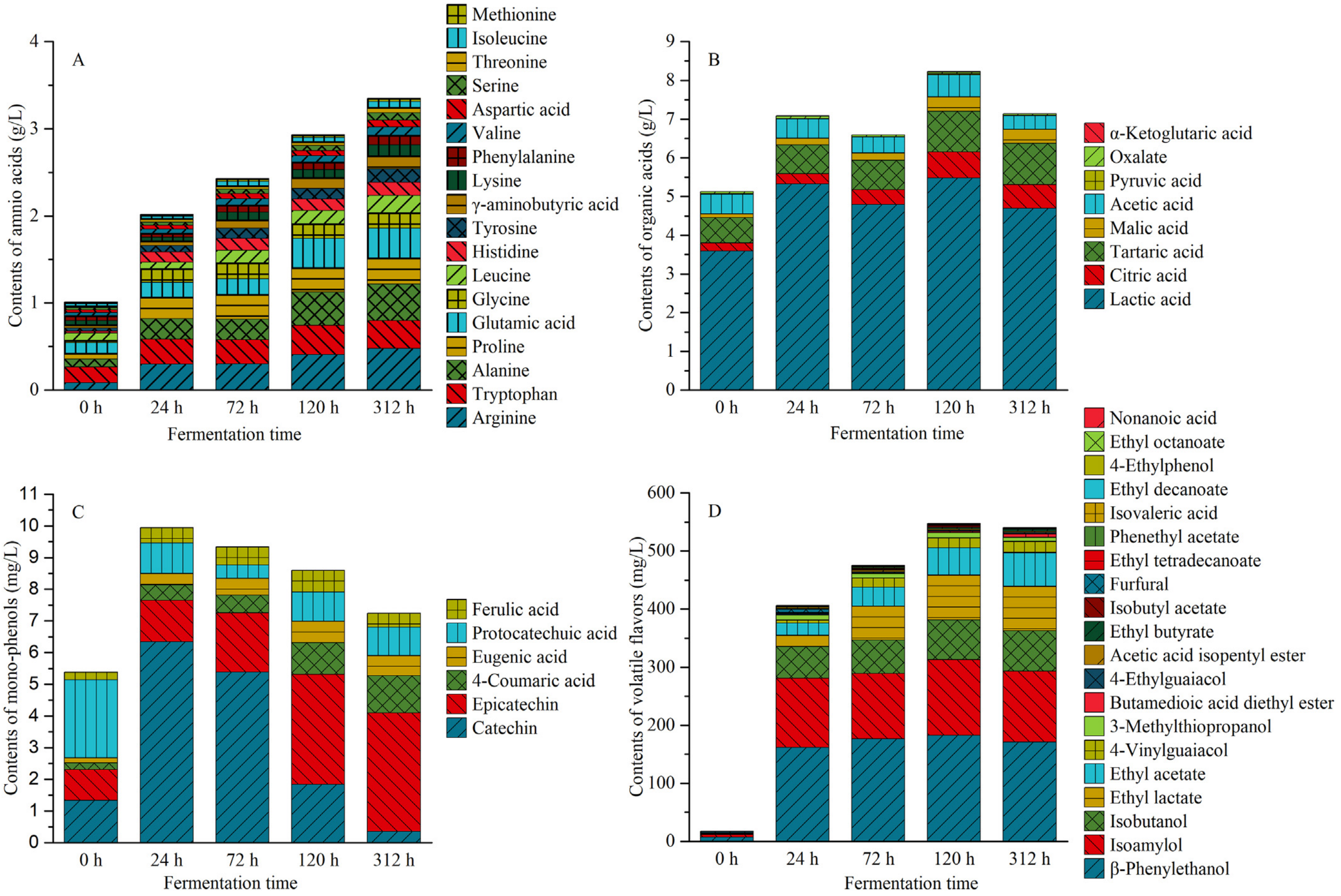


**Fig. 1.** Compositions of the amino acids (A), organic acids (B), monophenols (C), and dominant volatile compounds (D) in MHJFM.

**Table 1**
Statistics of sequencing and bioinformatics analysis.

| | 24 h | 72 h | 120 h | 312 h | Mixed assembly |
|---|---|---|---|---|---|
| Clean data (Mbp) | 11,194.23 | 10,966.56 | 10,020.29 | 10,033.97 | |
| Clean Q20 (%) | 95.31 | 95.23 | 96.37 | 96.06 | |
| Clean Q30 (%) | 89.11 | 89.00 | 91.08 | 90.49 | |
| Clean GC (%) | 49.69 | 50.72 | 54.59 | 55.86 | |
| Coverage (%) | 97.36 | 97.82 | 97.78 | 97.90 | |
| Depth | 19.79 | 29.00 | 33.77 | 33.77 | |
| Scatigs number | 40,123 | 37,225 | 52,186 | 52,337 | 14,166 |
| Scatigs average length (bp) | 2325.25 | 2338.26 | 1598.75 | 1623.04 | 831.53 |
| Scatigs N50 length (bp) | 6461 | 4614 | 2158 | 2129 | 824 |
| Number of ORFs | 93,553 | 84,524 | 89,686 | 89,867 | 19,238 |
| Number of unigenes | 101,718 | 99,781 | 101,391 | 101,838 | |

had largest unique unigenes (350). So, the 24 h sample had more unique gene information than any other sample. The unique genes in 24 h might be mainly from the microorganisms present in the raw materials and environment which would be eliminated by the high ethanol concentration and low pH in the fermentation process.

### 3.3. Taxonomic analysis

The brewing of huangjiu was an open fermentation process with lots of unculturable and unstudied microorganisms. In this study, there are 87.61% unigenes in gene catalogue could be annotated at genus level, while only 54.94% unigenes could be annotated at species level, so the scientific description at genus level were more comprehensive to reflect the microbiota characterization. Microbial taxonomic assignment showed that there were 20 bacterial genera and 3 fungal genera present in the huangjiu fermentation at abundances ≥0.01% (Fig. 3). The dominant bacterial genera (> 1% abundance) were *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, *Streptomyces*, *Actinopolyspora* and *Amycolatopsis*, which belonged to the *Actinobacteria* and *Firmicutes*. Members of the genus *Saccharopolyspora* increased continuously from 38.26% to 54.77% during the fermentation. In most huangjiu fermentation meshes

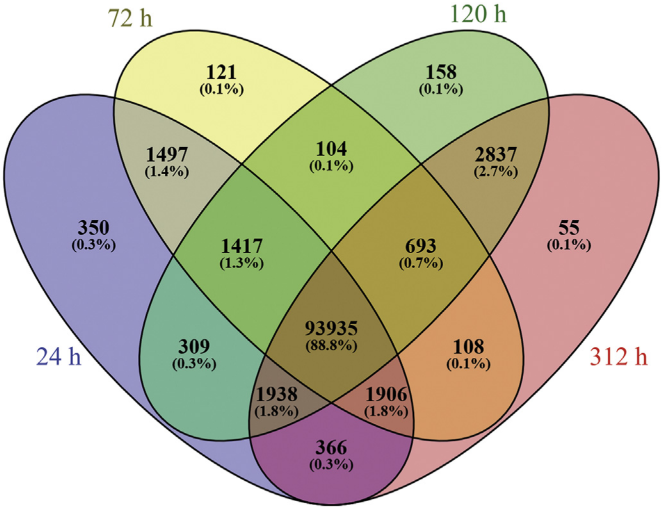


**Fig. 2.** Venn diagram of the distribution of unigenes in MHJFM.

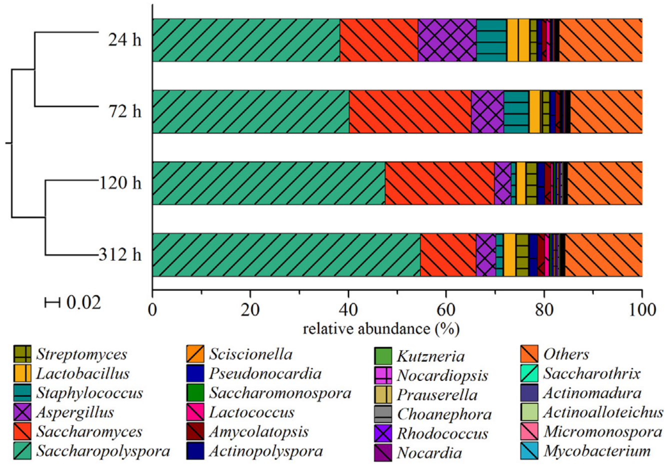


**Fig. 3.** Composition of the microbial community (above 0.01%) present in MHJFM. The cluster tree is based on the Brae-Curtis distance matrix of gene abundance, showing the dissimilarity of microbiota.

(Hong et al., 2016; Wang et al., 2014), members of the genus *Lactobacillus* were the dominant bacteria; the same as sake (Bokulich et al., 2014), and kimchi (Jung et al., 2011). Recent studies had shown that *Saccharopolyspora* could be a dominant genus in some Shaoxing huangjiu fermentation meshes (Hong et al., 2016; Liu et al., 2015) and might derived from wheat qu (Xie et al., 2013). The dominant fungi (> 1% abundance) were *Saccharomyces* and *Aspergillus*, which belong to the *Ascomycota*. *Saccharomyces*, which plays an important role in alcohol fermentation especially during the pre-fermentation, accounts for 11.34% to 25.01% of the microbiota, surpassed only by *Saccharopolyspora*. The highest relative abundance of *Aspergillus*, which plays a major role in liquefaction and saccharification during the pre-fermentation, was 11.93% at 24 h. During the pre-fermentation, the nutrition present in the in raw materials and the oxygen from aeration favor the growth of microorganisms. *Choanephora*, a fungal genus known as plant pathogens, was only found during the post-fermentation period. Cluster analysis showed that the microbiota present during the same fermentation stage were more similar (Fig. 3).

### *3.4. Distribution of genes associated with KEGG pathways in MHJFM*

There were 25,189 (23.81%) unigenes in the gene catalogue that were annotated within KEGG pathways. Among the level 1 KEGG pathways (Table 2), the number of unigenes belonging to metabolism was the most, while the number of unigenes belonging to organismal systems was the smallest. Furthermore, it was found that the unigenes belonging to metabolism increased during the process of huangjiu fermentation, while the genes associated with human diseases rose during the pre-fermentation period and decreased during the post-fermentation period (Fig. 4). This suggested that the genes related to human diseases belonged to microorganisms present in the raw materials or production environment, and that these microorganisms would be suppressed by ethanol (Phongphakdee and Nitisinprasert, 2015), yeast (Foster and Talbot, 2000), *Saccharopolyspora* (Vara and Hutchinson, 1988), *Aspergillus* (Flewelling et al., 2015) and other factors in the fermentation environment. In the multispecies fermentation food like vinegar (Wu et al., 2017) and yoghourt (Bolotin et al., 1999), the genes associated with human diseases metagenome had been found. However, the existing of these genes did not mean the pathogenicity of these food, some bacteria in MHJFM like *Weissella confuse* (Olano et al., 2001)*,* and *Lactococcus lactis* (Bolotin et al., 1999) without clinical pathogenicity contained genes associated with human diseases. Besides, huangjiu as one of the oldest alcohol beverages have been safely used for > 9000 years (Patrick et al., 2004). Among the level 2 KEGG pathways (Fig. 4), the relative abundances of amino acid metabolism and carbohydrate metabolism occupied the highest level, except the relative abundances of global and overview maps, which were similar to fermented sausages (Ferrocino et al., 2017). The higher abundance of genes belonging to amino acid and carbohydrate metabolism indicated that the material basis for flavor formation was mainly protein and starch.

**Table 2**
Relative abundance (%) of genes associated with KEGG pathways (at level 1) in MHJFM.

| KEGG pathway | 24 h | 72 h | 120 h | 312 h |
|---|---|---|---|---|
| Metabolism | 10.11 | 10.42 | 10.71 | 11.25 |
| Genetic information processing | 3.08 | 3.48 | 3.28 | 2.76 |
| Environmental information processing | 2.84 | 2.69 | 2.59 | 2.77 |
| Human diseases | 1.58 | 1.70 | 1.51 | 1.29 |
| Cellular processes | 1.35 | 1.45 | 1.42 | 1.33 |
| Organismal systems | 1.04 | 1.15 | 1.08 | 0.93 |

### *3.5. Metabolic pathways associated with huangjiu flavor*

After referring to the KEGG database and the literature (Albi and Serrano, 2015; Lukasheva et al., 2014; Pires et al., 2014), 216 enzymes related to enzymatic reactions involved in the metabolism of dominant huangjiu flavor components were identified (Supplementary Table S1). The predicted metabolic network for these flavor components was shown in Fig. 5. Rice and wheat qu contained starch and cellulose, which were major substrates for flavor formation, and various extracellular proteins from wheat qu contributed to the fermentation process (Zhang et al., 2012). The degradation of starch and cellulose was related to carbohydrate metabolism, as were the biosynthesis of ethanol, lactic acid, acetic acid, 2,3-butanediol and butanoate. The amino acids in huangjiu come from the raw materials and microbial synthesis, and some amino acids were precursors of higher alcohols. Accordingly, the biosynthesis of amino acids and higher alcohols was related to carbohydrate metabolism and amino acid metabolism. Furthermore, fatty acid biosynthesis was related to carbohydrate metabolism, amino acid metabolism and lipid metabolism. The biosynthesis of monophenols was related to amino acid metabolism and the biosynthesis of other secondary metabolites. However, through the taxonomic annotation of enzymes participating in the formation of monophenols, it was found that 4-coumaric acid, ferulic acid and protocatechuic acid might mainly come from the raw materials, rather than arising through microbial metabolism in the process of huangjiu fermentation. The main volatile phenols in huangjiu, 4-ethylenol, 4-ethylprocrefol and 4-ethylphenol, were degradation products from 4-coumaric acid and ferulic acid, so biosynthesis of these three volatile phenols was correlated with biosynthesis of other secondary metabolites. Although the biosynthesis of esters lacked a definite pathway map in the KEGG database, the production of ester precursors (alcohols and acids), could be interpreted. To summarize, functional annotation of the genes identified in the huangjiu samples led to the conclusion that carbohydrate metabolism and amino acid metabolism played important roles in huangjiu flavor formation.

### *3.6. Distribution of microbes in different flavor biosynthesis pathways*

The relationship between microorganisms and enzymes in different metabolic pathways was shown in Fig. 6. The results showed that six genera were the most closely related to flavor formation: *Saccharomyces*, *Aspergillus*, *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, and *Lactococcus*. Although 77 enzymes were annotated in *Aspergillus*, the abundances of these enzymes in *Aspergillus* were at a low level.

Distribution of microbes in different flavor biosynthesis pathways will be described following flavor formation process in MHJFM from raw material degradation, precursor synthesis to flavor compound

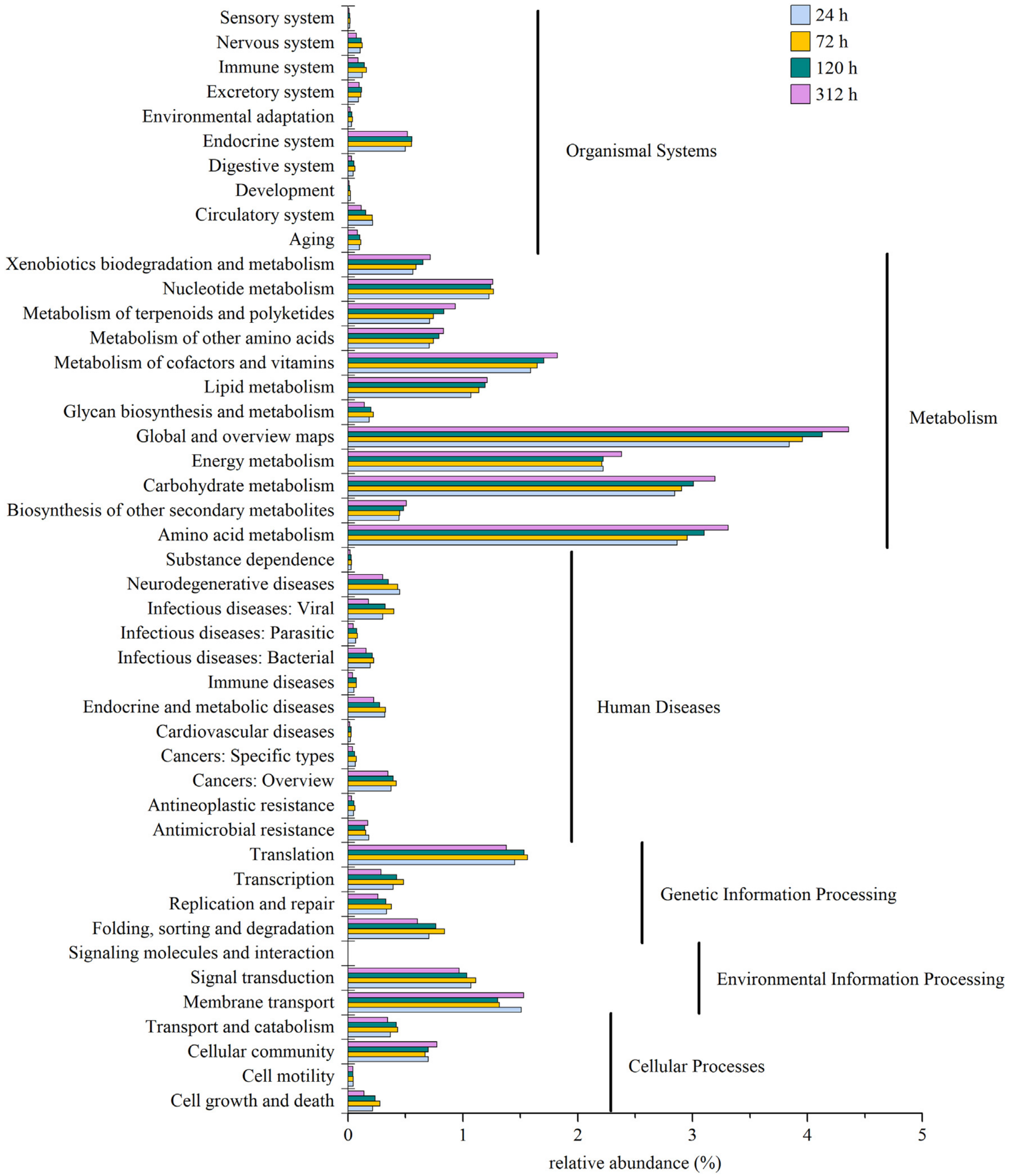


**Fig. 4.** Relative abundance of functional genes predicted in KEGG pathways within microbial community for MHJFM.

formation. *Actinopolyspora*, *Saccharopolyspora*, *Lactococcus*, and *Aspergillus* participated in the hydrolysis of starch, maltose and dextrin, and *Lactobacillus* also participated in starch metabolism. *Aspergillus* should be irreplaceable for the degradation of cellulose, as it was the sole genus annotated with cellulose 1,4-beta-cellobiosidase (EC 3.2.1.91) and cellulase (EC 3.2.1.4), while *Actinobacteria* and *Clostridia* were the main microorganisms of cellulose degradation in the solid-state acetic acid fermentation of traditional vinegar (Wu et al., 2017).

Threonine, methionine, valine, leucine, isoleucine, and phenylalanine were flavor amino acids and the precursors of higher alcohols in huangjiu. *Saccharopolyspora*, *Gluconobacter*, *Aspergillus*, *Saccharomyces*, *Stagonospora* and *Choanephora* might transform oxaloacetic acid to aspartic acid, which could be further transformed into threonine or methionine by *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Aspergillus* and *Saccharomyces*. These six genera may be involved in the synthesis of phenylalanine from prephenic acid. Members of the genus *Lactobacillus* contained genes involved in the metabolism of methionine during the cocoa bean fermentation process (Illeghems et al., 2015). *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, *Aspergillus* and *Saccharomyces* might produce valine and isoleucine using pyruvate as the substrate, while *Saccharopolyspora* and *Staphylococcus* might produce leucine from 2-oxolsovalerate, the α-keto acid corresponding to valine.

γ-Aminobutyric acid is synthesized from glutamate via glutamate decarboxylase (EC 4.1.1.15). Some strains of *Lactobacillus* (Sanchart et al., 2017; Wu and Shah, 2016), *Lactococcus* (Franciosi et al., 2015; Hagi et al., 2016), *Aspergillus* (Ab et al., 2016) and *Saccharomyces* (Correa et al., 1997) had been reported as high-producers of γ-aminobutyric acid, members of the genera *Actinopolyspora* and *Saccharopolyspora* were also found to be capable of producing γ-aminobutyric

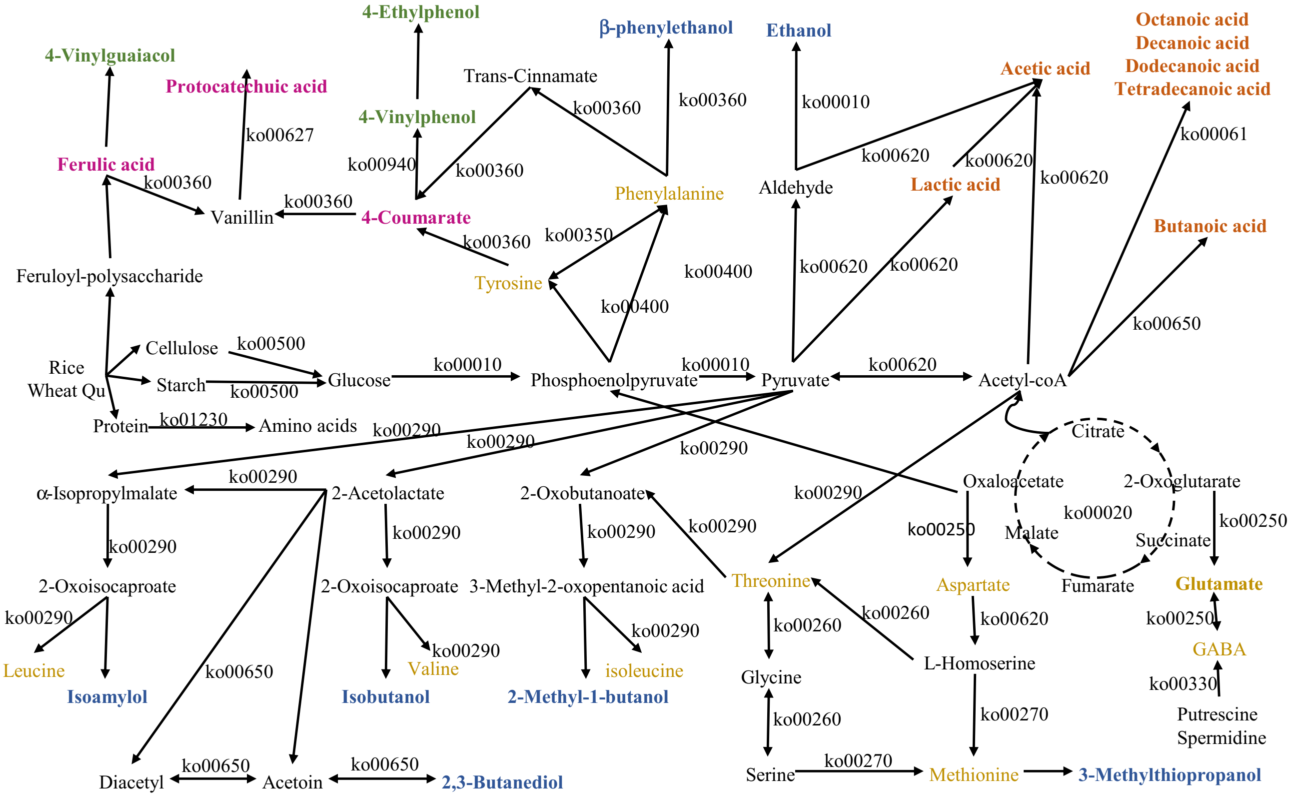


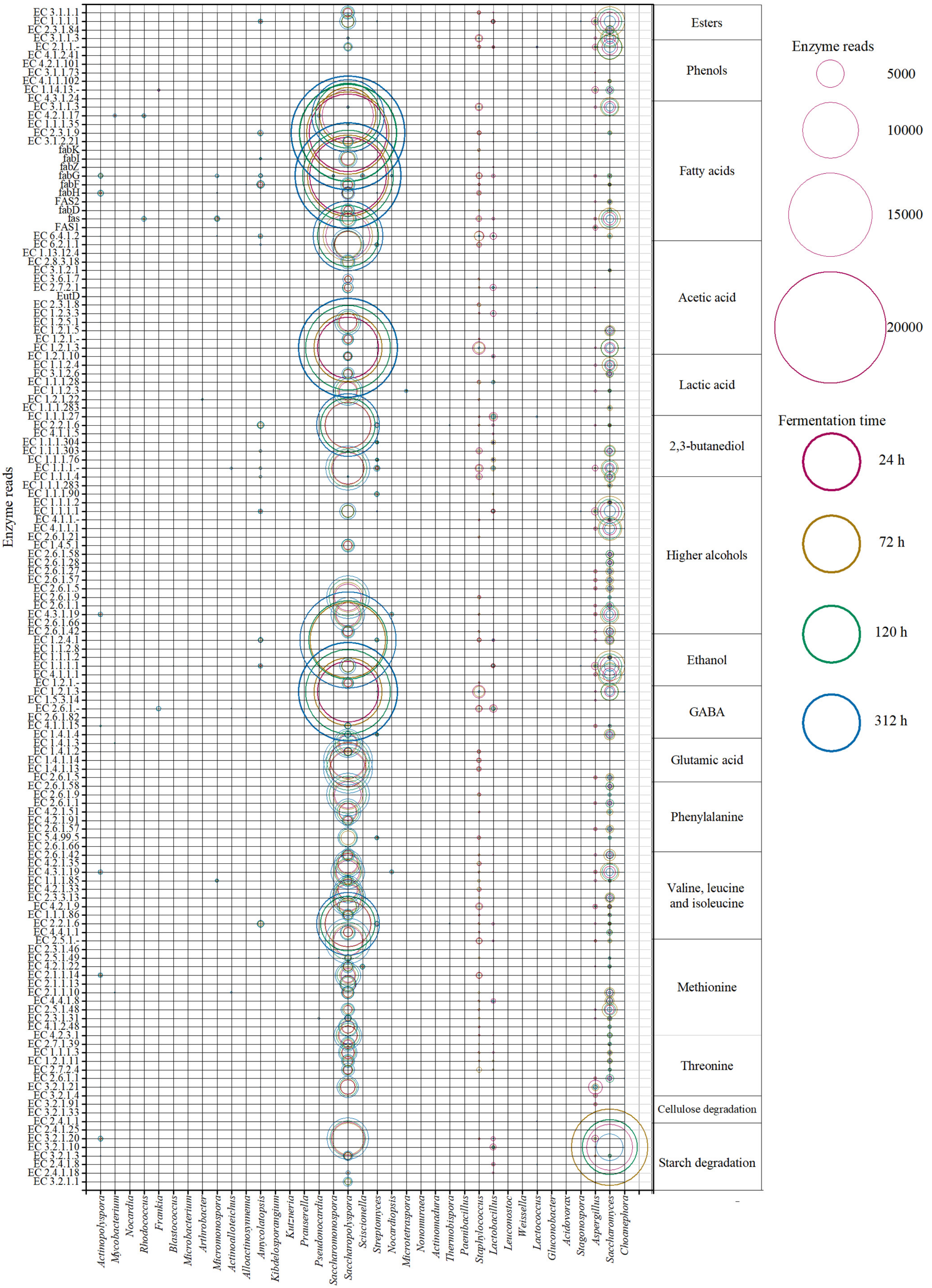
**Fig. 5.** Predicted metabolic network for flavor formation in MHJFM. Yellow, blue, orange, green and pink represent amino acids, higher alcohols, acids, volatile phenols and monophenols, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acid. The genus *Aspergillus* were found to produce γ-aminobutyric acid from spermidine, which provides a new direction for reducing biogenic amines, which were by-products of huangjiu fermentation.

Yeast is usually considered to be the main producer of alcohols in alcoholic fermentation, and higher alcohols and polyols are known as by-products of alcoholic fermentation. Ethanol is the main component of huangjiu, and *Saccharomyces* is the most important ethanol producer. However, *Aspergillus* might also contribute to the formation of ethanol, which had been reported in autolysis process of *Aspergillus oryzae* (Xu et al., 2016). The main higher alcohols in mechanized huangjiu (β-phenylethanol, isoamyl alcohol, isobutanol, 3-methylthiopropanol and so on), were produced from the corresponding α-keto acids by decarboxylation and reduction reactions. The α-keto acids were produced via the de novo biosynthesis of amino acids through carbohydrate metabolism, or the degradation of amino acids (Ehrlich pathway) (Pires et al., 2014). These results indicated that regulation of the higher alcohols concentration in huangjiu must consider the concentration of amino acids in the raw material. In addition to *Saccharomyces*, this study indicated that *Streptomyces*, *Staphylococcus*, *Lactobacillus*, *Aspergillus* and *Choanephora* were potential producers of higher alcohols. Decarboxylase is essential for the formation of higher alcohols, and it was not annotated in *Saccharopolyspora*, although *Saccharopolyspora* was an important functional microorganism in the biosynthesis of amino acids. 2,3-Butanediol, a normal constituent of wine, is one of the rare aroma-producing polyols. It might be synthesized from pyruvate by *Amycolatopsis*, *Saccharopolyspora*, *Streptomyces*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Saccharomyces*. It may also be derived from diacetyl and acetoin in *Leuconostoc* (Escobarzepeda et al., 2016); however, the *Leuconostoc* present during huangjiu fermentation might not participate in this metabolic pathway.

*Staphylococcus* and *Lactobacillus* could produce lactic acid, and *Saccharopolyspora* might utilize lactic acid, while *Saccharomyces* and *Aspergillus* were involved in both the production and utilization lactic acid. The interplay among these organisms was likely the reason for the oscillation of the lactic acid concentration during the fermentation process. Some fatty acids originating from the raw materials and from microbial metabolism were the precursors of aromatic ethyl esters. Butanoic acid, octanoic acid, decanoic acid, dodecanoic acid, and tetradecanoic acid were the precursors of ethyl esters that were present at relatively high levels in mechanized huangjiu. No microbial genera involved in the production of butanoic acid were found in this study, although seven genera (*Lactobacillus*, *Lactococcus*, *Saccharopolyspora*, *Micromonospora*, *Staphylococcus*, *Saccharomyces*, and *Aspergillus*) were found to produce the other four fatty acids from acetyl coenzyme A. The last step of fatty acid synthesis, releasing the fatty acid from acyl carrier protein, was catalyzed by hydrolases such as dodecanoyl-[acyl-carrier-protein] hydrolase (EC 3.1.2.21) and acyl-[acyl-carrier-protein] hydrolase (EC 3.1.2.14). The EC 3.1.2.21 hydrolases related to octanoic acid, decanoic acid, dodecanoic acid was detected in *Lactobacillus*, *Lactococcus*, and *Saccharopolyspora*, while no EC 3.1.2.14 hydrolase related to tetradecanoic acid was annotated. Another pathway for the synthesis of fatty acids in huangjiu, was their synthesis from the triglycerides in rice by triacylglycerol lipase (EC 3.1.1.3), which was found in *Staphylococcus*, *Saccharopolyspora*, *Saccharomyces*, and *Aspergillus*. The EC 3.1.1.3 lipase in yeasts and molds had also been implicated in the formation of cheese sensory properties (Lessard et al., 2014). *Saccharomyces* may contribute to fatty acids during huangjiu fermentation through the hydrolysis of triglycerides in the raw material, rather than through the de novo synthesis of fatty acids, although it had been reported that fatty acids were mainly derived from yeast fermentation instead of raw materials (Troton et al., 1989).

Monophenols contribute to the antioxidant activity of huangjiu. In this study, some reactions in the biosynthesis of 4-coumaric acid, ferulic acid and protocatechuic acid were predicted. Enzymes that catalyzed the release of monophenols from raw materials, like feruloyl esterase (EC 3.1.1.73) in *Aspergillus*, were also found. The monophenols 4-



(caption on next page)

**Fig. 6.** Taxonomic distribution and enzyme reads for substrate breakdown and flavor formation in microbial community of MHJFM. Microorganisms which were predicted with more than one enzyme are shown, and the diameter of circle correlates to the absolute abundance of enzyme. Gene names were used for partial enzymes instead of EC number, for the consistency of KEGG map.

coumaric acid and ferulic acid might be converted to 4-vinylphenol and 4-vinylguaiacol by ferulic acid decarboxylase (EC 4.1.1.102) from *Saccharomyces* and *Aspergillus*, respectively. These compounds might be further reduced to 4-ethylphenol and 4-ethylguaiacol. These results indicate the raw materials and microorganisms in huangjiu both contributed to its complicated monophenols composition.

Esters were trace constituents, compared with other flavor components, but they are vital to huangjiu. Esters were mainly formed by the enzymatic condensation of organic acids and alcohols during fermentation (Pires et al., 2014). The triacylglycerol lipase (EC 3.1.1.3), carboxylesterase (EC 3.1.1.1) and alcohol *O*-acetyltransferase (EC 2.3.1.84) present in *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, *Aspergillus* and *Saccharomyces* could catalyze the formation of acetate esters. Addition of *Lactobacillus* increased the levels of esters in the fermented dairy beverage kefir (Walsh et al., 2016). Broad-specificity alcohol dehydrogenase (EC 1.1.1.1), which could participate in the formation of acetate esters, was predicted in 15 genera (supplementary Table 1) found. Overall, *Saccharopolyspora*, *Staphylococcus*, *Aspergillus*, and *Saccharomyces* were important for the synthesis of esters, since they participated in the formation of ester, acids and alcohols.

Six genera (*Saccharomyces*, *Aspergillus*, *Saccharopolyspora*, *Staphylococcus*, *Lactobacillus*, and *Lactococcus*) were most closely related to the flavor of mechanized huangjiu both because of their abundance and their involvement in flavor biosynthesis during fermentation. Based on the metagenomic analysis, *Saccharopolyspora* might be important for amino acid synthesis, fatty acid synthesis and triglyceride hydrolysis, but *Saccharopolyspora* had a little connection with the formation of ethanol, higher alcohols and phenols. However, the role of *Saccharopolyspora* should be confirmed at species or strain level in the further studies and application.

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