# Acidity, sugar, and alcohol during the fermentation of Osmanthus-flavored sweet rice wine and microbial community dynamics (#103011)



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# Acidity, sugar, and alcohol during the fermentation of Osmanthus-flavored sweet rice wine and microbial community dynamics

**Ping Tian** [1], **Jiaqiong Wan** [1], **Tuo Yin** [1], **Li Liu** [2], **Hongbing Ren** [2], **Hanbing Cai** [1], **Xiaozhen Liu** [1], **Hanyao Zhang** [Corresp. 1]

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Sweet rice wine is a popular traditional Chinese rice wine that is widely loved by the Chinese people for its high nutritional value. However, the dynamics of the sugar level, acidity, alcohol content, and microbial community during the fermentation of *osmanthus-flavored* sweet rice wine have not been evaluated, which can lead to the unstable quality of *osmanthus* flower sweet rice wine (OFSRW). In this study, the dynamic changes in sugar level, acidity, alcohol content, microbial community composition, and microbial metabolic pathways during traditional fermentation of OFSRW at four-time points-0 h (AG0), 24 h (AG24), 36 h (AG36), and 43 h (AG43)-were analyzed via direct titration, total acid assays, alcoholometry, and high-throughput macrogenomic techniques. First, we found that bacteria were the dominant microorganisms in the early stage of OFSRW fermentation (AG0), fungi were the dominant microorganisms in the middle and late stages of fermentation (AG24 and AG36), and *Rhizopus* was the main fungal genus throughout fermentation. Acidity and total sugars increased with fermentation time, and alcohol was not detected until the end of fermentation. Diversity analysis revealed that the dominant species at the beginning of natural fermentation was *A. johnsonii,* and *R. delemar* became the dominant species as natural fermentation progressed. Metabolic pathway analysis revealed that energy production and conversion, carbohydrate transport, amino acid transport, and metabolic pathways were the most active metabolic pathways in the fermenter. These results can provide a reference basis for changes in the microbial community during the fermentation of cinnamon-flavored sweet rice wine.

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**Abstract:** Sweet rice wine is a popular traditional Chinese rice wine that is widely loved by the Chinese people for its high nutritional value. However, the dynamics of the sugar level, acidity, alcohol content, and microbial community during the fermentation of *osmanthus-flavored* sweet rice wine have not been evaluated, which can lead to the unstable quality of *osmanthus* flower sweet rice wine (OFSRW). In this study, the dynamic changes in sugar level, acidity, alcohol content, microbial community composition, and microbial metabolic pathways during traditional fermentation of OFSRW at four-time points-0 h (AG0), 24 h (AG24), 36 h (AG36), and 43 h (AG43)-were analyzed via direct titration, total acid assays, alcoholometry, and high-throughput macrogenomic techniques. First, we found that bacteria were the dominant microorganisms in the early stage of OFSRW fermentation (AG0), fungi were the dominant microorganisms in the middle and late stages of fermentation (AG24 and AG36), and *Rhizopus* was the main fungal genus throughout fermentation. Acidity and total sugars increased with fermentation time, and alcohol was not detected until the end of fermentation. Diversity analysis revealed that the dominant species at the beginning of natural fermentation was *A. johnsonii,* and *R. delemar* became the dominant species as natural fermentation progressed. Metabolic pathway analysis revealed that energy production and conversion, carbohydrate transport, amino acid transport,

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**Keywords:** sweet rice wine; *Osmanthus*; high-throughput sequencing; sugar content; acidity; alcohol content; microbial communities and differences; dominant strains.

## INTRODUCTION

*Osmanthus* flower sweet rice wine (OFSRW), a low-alcoholic beverage with regional characteristics in China, is mainly made from high-quality glutinous rice and *osmanthus* flowers, which are mixed and fermented in a natural environment with the addition of a fermenting agent (Jiuqu). Many studies have confirmed that glutinous rice is a complex organism composed of various macromolecules with edible and medicinal value (Zheng et al., 2023). For example, it warms the spleen and stomach, stops cold dysentery deficiency, reduces stool, allows spontaneous sweating, and facilitates urination. *Osmanthus fragrans* (Thunb), an evergreen shrub or tree belonging to the Lignaceae (Oleaceae) family, is a valuable and common ornamental aromatic plant with good medicinal value (Huang et al., 2019). Its petals contain many nutrients, such as soluble sugars, soluble proteins, organic acids, vitamin C, flavonoids, free amino acids, and many minerals. It has the effect of strengthening the stomach and resolving phlegm, generating fluids, dispersing blood stasis, and flattening the stomach (Zhou et al., 2013), and it can treat asthma, coughs, toothache, and diarrhea (Wanget al., 2022), as well as having anticancer, antioxidant, and anti-inflammatory effects (Huang et al., 2023). Sweet rice wine is soft, long, and pleasantly aromatic and is an essential part of the food culture of the Chinese people. The nutritional value of rice wine is also of interest. In addition to the nutrients in the raw material itself, the raw material also contains oligosaccharides, peptides, proteins, B vitamins, and minerals; amino acids and other components produced during the traditional brewing process of rice wine are easily digested and absorbed by the human body and can promote appetite;

warm the stomach; strengthen the spleen; benefit qi; stop diarrhea; produce fluids to stop sweating; refresh and relieve fatigue (Yuan et al., 2020; Cai et al., 2012); and have anti-aging effects (Liu et al., 2020; Zhao et al., 2018). Therefore, the OFSRW produced by combining glutinous rice and *osmanthus* flowers through the mixed fermentation of wine curd has the aroma of ordinary sweet rice wine and retains the fresh fragrance of *osmanthus* flowers, which not only increases the color, aromas, and taste of sweet rice wine but also allows the release of nutrients such as oligosaccharides, polypeptides, amino acids, and ethanol in sweet rice wine to increase its nutritional value.

Jiuqu is rich in microorganisms involved in saccharification, fermentation, and the production of flavor-related metabolites. For example, *Rhizopus* breaks the α-1,4 and α-1,6 bonds in the rice starch structure, which is converted more completely into fermentable sugars. *Saccharomyces* plants draw monosaccharides such as glucose, fructose, and mannose into the cell and break them down into carbon dioxide and ethanol under anaerobic conditions and through the action of endoenzymes. *Lactic acid bacteria* (LAB) play a major role in fermentation and the intrinsic properties of fermented products, influencing the development of their aroma, texture, and acidity (Cai et al., 2019). The fungal communities in Jiuqu have also been shown to play an essential role in starch and protein hydrolysis and the production of ethanol, organic acids, higher alcohols, and esters (Medina et al., 2013). Whereas bacterial communities that produce hydrolytic enzymes, glucoamylases, proteases, and esterases are produced by various bacteria during fermentation to degrade the raw material substrates, all of which could lead to the accumulation of aroma-related compounds or secondary metabolites and intermediates (Gammacurta et al., 2018; Simonen et al., 1993). The bacterial and fungal communities varied significantly among the different starters of Hongqu yellow rice wine, and the core microorganisms were positively correlated with specific organic acids and aromatic esters in the starters (Huang et al., 2019). Regional variations in the wild native microbes and environmental conditions of Jiuqu production may influence the microbial community composition and quality of Jiuqu, especially in nonsterile fermentation processes (Zhao et al., 2022). The influence of

different regional environments, raw materials, and additives in sweet rice wines may also lead to differences in SRW fermenters, which may confer different organoleptic characteristics, flavors, and other features of SRW (Su et al., 2014). For example, Chen et al. (Chen et al., 2020) reported that different microbial communities in three different traditional huangjiu fermenters resulted in significant differences in the aroma composition of their fermented rice wines. One study comparing eight CSRW starter samples from different regions of southern China revealed significant high variation in the bacterial and fungal composition, which likely contributed substantially to the final flavor quality of the respective CSRWs (Cai et al., 2018). Sugars, acidity, and alcohol content, which are crucial parameters in the sweet rice wine fermentation process, have a great impact on the quality and flavor of sweet rice wine. For example, insufficient sweetness or oversweetness will make sweet rice wine taste too light or too mushy. Excessive alcohol content will make sweet rice wine bitter and astringent, with a slight off-flavor, a strong taste, or even a distinct white wine taste. Excessive acidity will reduce the taste of sweet rice wine or even cause rancidity.

OFSRW fermentation is a complex process involving maceration, steaming, rinsing, fermentation inoculation, and saccharification. The entire fermentation process includes a range of strains obtained from fermenters, raw materials, and the environment. In addition, in this complex environment, a series of changes in sugar level, acidity, alcohol content, and microflora occur, affecting the unique aroma, flavor, and color of rice wine. Furthermore, OFSRW fermentation follows the traditional technique of an uncontrolled fermentation process that produces inconsistent flavors. However, few studies have evaluated the evolution of sugar, acidity, alcohol, and microbiota during traditional OFSRW fermentation, and the interactions between microbiota and sugar, acidity, and alcohol have not been elucidated. To explain this, it is first necessary to understand the dynamic relationships among the brix, acidity, alcohol, and microorganisms in OFSRW. In this paper, direct titration was used to determine the sugar level in OFSRW. Total acidity determination was used to determine the acidity of OFSRW, and alcoholic strength was used to determine the alcoholic strength of OFSRW. Available methods

include culture-dependent methods (Lv et al., 2012) and culture-independent PCR-denaturing gradient gel electrophoresis (DGGE) techniques (Lv et al., 2015; Lv et al., 2017) were employed to study the microbial composition of OFSRW, but all of the above ones have difficulties distinguishing the species present at population densities below $10^3$ CFU/g or two orders of magnitude lower than the most abundant members of these communities (Cocolin et al., 2011; Prakitchaiwattana et al., 2004). High-throughput sequencing technology, on the other hand, is capable of analyzing the transcriptome and genome data of a species in a detailed and comprehensive manner, also known as deep sequencing or next-generation sequencing (NGS). This technique has quantitative capabilities for determining the abundance of species components in a sample. In addition, the utilization of this technique is simple and cost-effective, the results are feasible (Liu et al., 2019), and it is faster and better than ITS PCR and fluorescent ITS PCR capillary electrophoresis. This technique has been widely used to analyze the microbial community dynamics of various fermented foods and vegetables, such as Sichuan kimchi (Luo et al., 2021), soy sauce (Zhao et al., 2021), kiwifruit (Zhang et al., 2022), grape juice (Zhao et al., 2022), and rice wine (Zou et al., 2023), and the use of macro-genome sequencing in these studies provided a theoretical basis for analyzing the relationships between microbial populations and specific flavors in these fermented foods.

Therefore, in this paper, direct titration, total acid determination, alcoholometry, and high-throughput macrogenomic rDNA (16S rRNA and ITS genes) were used to dynamically monitor the brix, acidity, alcohol content, and microbial community structure of traditionally fermented OFSRW, direct titration was used to determine the sugar level in OFSRW, with total acidity determination used to determine the acidity in OFSRW. Alcoholic strength was used to determine the alcoholic strength of OFSRW. (1) Changes in brix, acidity, alcohol content, and microbial community composition of OFSRW were analyzed. (2) Comparisons were made between the OFSRW in this paper, and direct titration was used to determine the sugar level in OFSRW, total acidity determination was employed to determine the acidity in OFSRW, and alcoholic strength was used to determine the alcoholic strength of OFSRW microbial diversity

changes and differences during fermentation and the correlation between microbial community and sugar level, acidity, and alcohol content. (3) The functional metabolism prediction of OFSRW was followed. This study provides insights into how microorganisms in OFSRW fermentation broth adapt to environmental changes during fermentation and can also be crucial for optimizing fermentation conditions and improving product quality and flavor.

## Materials and Methods

### Sample preparation and sampling

The raw materials used for OFSRW were Angie's Sweet Wine Quartz (Hubei Angie's Yeast), glutinous rice, and *osmanthus* (Guangzhou Zhenyuantang Food Co., Ltd.). The prime operation process of OFSRW is as follows: glutinous rice→washing, soaking→steaming→drinking and cooling→mixing with quartz→mixing with *osmanthus*→bottling→compacting→mashing→placing in a constant temperature box at 30°C for fermentation for 43 h. In the production of TRSW, it is necessary to select glutinous rice that is full of grains, has no yellowing and no mold, and has no insect pests. When making TRSW, glutinous rice with full grains, no yellowing, no mildew, and no insect pests were selected, and the rice was washed into a pottery jar with 2.5-3.0 times the quality of glutinous rice to soak for 9-12 h. The soaked glutinous rice at 108°C was steamed for 40-50 min, and the steamed rice was hard and soft outside and inside, loose but not rotten, with no white heart or uniformity. After the glutinous rice was steamed and cooked, 30% cool white boiled water was used to cool it to 30-35°C, after which 0.4% of the wine and rice was added and stirred evenly. After that, 0.4% of the wine and rice were stirred well, and 0.75% of the dried cinnamon was added again and stirred well. Finally, the mixture was bottled, compacted, mashed, and placed in a constant temperature box at 30°C for 43 h of fermentation. At 0, 24, 36, and 43 h, 20 g of the samples were randomly taken for the determination of sugar, acidity, and alcohol content. At the same time, 380 mL of

fermentation mash was collected for high-throughput sequencing analysis.

## Determination of Brix, Acidity, and Alcohol Content

The direct titration method GB 5009.7-2016 was used to determine the sugar content in *osmanthus* sweet rice wine. The acid was sent to the Laboratory Department of Hongbin Foods Co. Ltd. for testing, and its determination method was GB 12456-2021. The alcohol content was determined using the GB 5009.225-2023 alcoholometer method.

## High-throughput sequencing and bioinformatics analysis DNA extraction and sequencing

Total DNA was extracted from each microbial sample using the CTAB method (Liu et al., 2017). High-throughput sequencing was performed by DynaTech Biotechnology Limited (Yunnan Province, China) for macro-genome sequencing. Metagenomic sequencing was conducted, and ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS2 (GCTGCGTTCTTCATCGATGC) were used for sequencing (Kumar et al., 2020). The sequences had a mean read length of 150 bases and a Q score of 30. The sequencing data were analyzed on the GenesCloud platform (www.genescloud.cn).

## Sequence processing and analysis

Paired-end sequencing of DNA fragments was performed on the Illumina platform. Vsearch (v2.13.4-linux-x86_64) and cutadapt (v2.3) were used to denoise and cluster the sequences (Rognes et al. 2016). After using the QIIME cutadapt trim paired to excise sequence primer fragments, the sequences unmatched with primers were discarded.

The Vsearch module was used for splicing, deduplicating, and dechimericing the sequences, and the UNITE database (Release 8.0, https://unite.ut.ee/) was used to filter the concentrated chimeras to obtain high-quality chimeras. The QIIME2 (classify sklearn algorithm

184 https://github.com/QIIME2/q2-feature-classifer) was used to annotate the characteristic
185 sequences of each operational taxonomic unit (OTU) in the naive Bayes classifiers (Elolimy et al.
186 2020). The QIIME2 qiime feature-table Rarefy function was used to set the leveling depth to 95%
187 of the smallest sample sequence size, and the final OTUs were obtained.

188 **Analysis of changes in species composition**

189 Krona software (https://github.com/marbl/Krona/wiki) was used to analyze the community
190 taxonomic composition of the samples2020 (Ondov, Bergman & Phillippy, 2011). The
191 RGGplot2 package was used to construct a circle stair tree diagram, and the abundance of each
192 group was added to the diagram in the form of a pie chart (Steenwyk & Rokas, 2021). To further
193 compare the species composition differences among the samples and display the distribution
194 trend of the species abundance of each sample, R language and the pheatmap package were used
195 to construct heatmaps for the species composition analysis.
196

197 **Alpha diversity analysis**

198 Alpha diversity refers to the diversity within a sample. Commonly used alpha diversity
199 indices include the Chao1 estimator, Good's coverage index, observed species index, Pielou's
200 evenness index, Shannon index, Simpson index, etc. (Chao & Ricotta 2019; Liu et al. 2020). The
201 Chao 1 index measures species richness and estimates the number of species in a sample. Good's
202 coverage refers to the coverage rate of each sample library, and this index reflects whether the
203 sequencing result represents the situation of the microorganisms in the sample. The observed
204 species index represents the number of species in the samples. Pielou's evenness indicates the
205 uniformity of the community. The Shannon index combines abundance and evenness, giving
206 more weight to rare species. The Simpson index combines abundance and evenness but focuses
207 more on common species.
208 QIIME2, R language, and the ggplot2 package were used for alpha diversity analysis. After

209 using the unleveled OTU table, calling the "qiime diversity alpha-rarefaction" command, and
210 setting the minimum leveling depth to ten and the minimum sequencing depth to 95% of the
211 sample sequence, each depth value was fattened ten times to calculate the alpha diversity index.
212

213 **Beta diversity analysis**

214 Beta diversity refers to the differences between samples or groups and is often used to
215 analyze whether the differences in microbial composition between two groups are significant.
216 Commonly used beta diversity indices include the Jaccard, Bray–Curtis, unweighted UniFrac,
217 and weighted UniFrac indices (Lozupone et al. 2007; Chao & Ricotta 2019). The Jaccard index
218 compares the similarities and differences between limited sample sets. Bray–Curtis dissimilarity
219 is a measure used to analyze differences in species composition in different places. Unweighted
220 UniFrac can detect the presence of variations between samples, while weighted UniFrac can
221 further quantitatively detect the variation between samples of various lineages.
222 Principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS)
223 methods were used to analyze the beta diversity of the samples (Ramette, 2007; Legendre &
224 Legendre, 1998). By default, the UPGMA algorithm was used for cluster analysis of the Bray-
225 Curtis distance matrix (Bray & Curtis, 1957), and a ggtree of R language was used to analyze the
226 relationships between different samples for visualization.
227

228 **Functional prediction**

229 Functional annotation information was obtained by comparing the nonredundant genes with
230 each functional database using DIAMOND software, taking the annotations with $e<1e^{-5}$, and
231 filtering the proteins with the most abundant sequences. For each sequence comparison result,
232 the comparison result with the highest SCORE (oneHSP>60 bits) was selected for subsequent
233 analysis (Backhed, Roswall & Peng, 2015). Using PICRUSt2 software

(https://github.com/picrust/picrust2/wiki), the abundance values of metabolic pathways were obtained. The generated data were entered into the KEGG biological metabolic pathway analysis database (KEGG Pathway Database, http://www.genome.jp/kegg/pathway.html), the eggNOG database, and metabolic pathways for different sample statistics (Qin, Li &Cai., 2012). Using R language and the MetaGenomeseq package, the Fit Feature Model function was employed, and the distribution of each pathway/group was analyzed using a zero-log-normal model. The results were used to calculate the significance of the metabolic differences between each natural fermentation sample and the CK control group. According to the data selected in the metabolic pathway abundance table, a bar chart was drawn to analyze which species affect the metabolic pathways.

## Results

### Brix, acidity, and alcohol analysis

During OFSRW fermentation, the time before fermentation (AG0h) was 0 h, the brix of OFSRW was undetectable, the acidity of OFSRW was 0.03, and the alcoholic strength of OFSRW was undetectable, indicating that fermentation had not yet begun, that no fermentable sugar existed in the raw material and that the acidity was low, which might be related to the activity of *L. plantarum*. In the middle of fermentation (AG24h and AG36h), the sugar level of AG24h was 7.11, the acidity was 0.14, and the alcoholic strength was not detected, while the sugar level of AG36h was 14.05, the acidity was 0.25, and the alcoholic strength was not detected. The increase in brix and acidity during the mid-fermentation period also confirms the association of enhanced microbial activity, especially related to the activity of LAB and *Rhizopus*, which can produce acid through the metabolism of sugars during the fermentation process. An increase in acidity helps to prevent the growth of other microorganisms, thus controlling the structure of the microbial community to some extent during fermentation, and an

increase in acidity causes a decrease in the viability of LAB. The sugar level after fermentation (AG43h) was 16.55, the acidity was 0.35, and the alcohol content was 2%Vol, which indicated that the fermentation process had begun to enter the alcoholic fermentation stage (Table 1). The fact that alcohol was not detected until the end of fermentation may be due to the inhibition of *Saccharomyces* and *Rhizopus* activity by *L. plantarum* during the pre-fermentation period.

Table 1 Physicochemical properties of sweet rice wine produced from cinnamon *osmanthus* flower.

## Analysis of species composition

In this study, the dynamics of fungal and bacterial communities in OFSRW fermenters at different time intervals were detected using Krona analysis (Fig. 1). According to the results of the study, it was concluded that in the AG0 samples, bacteria were mainly dominated by *Gammaproteobacteria* and *Bacilli,* with 76% and 18%, respectively. Fungi were dominated by *Mucoromycetes* and *Magnoliopsida* at 51% and 40%, respectively. In the AG24 sample, the bacteria were the same as those in the AG0 stage. The fungi were dominated by *Saccharomycetes* and *Mucoromycetes* at 86% and 8%, respectively. In the AG36 samples, fungi decreased, and bacteria increased, but fungi remained the dominant species. The bacteria were dominated by 11% *Enterobacterales*. Fungi were dominated by *Mucorales* and *Saccharomycetales* at 66% and 15%, respectively. In the AG43 samples, *Mucorales* was the dominant order of fungi, accounting for 83%. The bacteria were dominated by *6%* and 3% *Enterobacterales* and *Moraxellales*, respectively.

Figure 1 Classification level and abundance information of Krona diagram of sample species. From the inside to the outside, the Krona circle represents the seven taxonomic levels of domain, phylum, class, order, family, genus, and species. The sector's size reflected the relative abundance of different taxa, and there were specific

values.

The 30 most abundant species for each sample were plotted as a bar graph (Fig. 2). The dominant species in AG0 were *A. johnsonii* (abundance value= 25328.43406), *P. pentosaceus* (abundance value= 19416.53625) and *L. plantarum* (abundance value= 13204.09352). The significant activity of *P. pentosaceus* and *L. plantarum* also explains the low acidity at this stage. However, as fermentation continued, A. *johnsonii* and *P. pentosaceus* disappeared. After 24 h of fermentation, *K. ascorbata* decreased. The dominant fungi were *C. lusitaniae* (abundance = 347248.9604) and *R. delemar* (abundance = 20415.18217). Subsequently, the abundance of bacteria increased, but fungi remained dominant, and the abundance of *C. lusitaniae* decreased or even disappeared. The significant activity of *Rhizopus* indicates the beginning of fermentation of starch in rice, thus converting it into fermentable sugars. *C. lusitaniae* is a non-*Saccharomyces* yeast species capable of utilizing sugars for fermentation, resulting in turbidity and precipitation of OFSRW and alcohol production (Cao et al., 2014). After 36 h of fermentation, *Rhizopus* accounted for the greatest percentage, with *R. delemar* (abundance = 221286.5492) being the dominant strain, followed by *R. microsporus* (abundance = 42293.8848). *W. anomalus* (abundance = 74557.64045) was dominant among the yeasts. The continuous activity of *Rhizopus* at this stage indicates an inextricable relationship with the sustained increase in brix. *W. anomalus* is a yeast of the non-*Saccharomyces* genus with certain aroma-producing, ester-producing, and alcohol-producing abilities that can significantly enhance the sensory quality of wine, and it is an important functional microorganism in the fermentation of wine grains (Xie et al., 2022). The significant activity of *Rhizopus*, *C. lusitaniae* and *W. anomalus* in the middle and late stages of fermentation paved the way for the alcoholic fermentation stage (AG43) to monitor the alcohol content. As fermentation continued, the yeasts decreased or even disappeared. At 43 h of fermentation, *Rhizopus* became dominant, especially *R. delemar* (abundance value = 284014.9015), which became the dominant strain, and *R. microsporus* (abundance value = 52561.42163) accounted for a greater percentage. At this stage, the alcohol content was tested at

2%Vol, but the *Saccharomyces* decreased or even disappeared, indicating that the elevated alcohol content inhibited the yeast activity. However, *Rhizopus* was still the dominant fungus, which stated that the continuous increase in both the detected alcohol content and the sugar content was related to *Rhizopus* because *Rhizopus* has abundant amylase and certain liquefaction enzymes, which can chain OFSRW with saccharification and fermentation throughout the whole fermentation process from the beginning to the end of the fermentation process, and the fermentation effect was more thorough; thus, the starch yield further improved.

Figure 2 Column diagram of the horizontal species composition of each sample species. The abscissa is the name of each group of the grouping scheme, and the ordinate is the relative abundance of each taxon at a specific taxonomic level.

Heatmaps were generated based on the average abundance of the top 50 strains, reflecting the correlation of colonies between samples and showing the trend of the distribution of strains in each sample. The results are shown in Figure 3. The relationship between each sample and each colony can be seen in the heatmap. Species diversity was highest at AG36. The diversity gradually increased as fermentation progressed. The results of the heatmap analysis were consistent with the results of the species composition analysis.

Figure 3 Horizontal distribution heatmap of each sample species.

**Analysis of alpha diversity index**

The Shannon, Simpson, and invsimpson indices were calculated to characterize the alpha diversity of the microbiota in each of the starting samples (Fig. 4). The Shannon and Simpson indices reflect the diversity of the microbial community, with higher Shannon scores and lower Simpson scores indicating greater diversity of the microbial community, and there were

differences between bacteria and fungi. During OFSRW fermentation, the highest species diversity was found in sample AG0, and the lowest in AG24. In the four fermentation broth samples of AG0 (CK), AG24, AG36, and AG43, the Shannon index first decreased and then increased and then decreased, and the Simpson index also showed the same trend as the Shannon index, which indicated that the species diversity first reduced and then increased and then decreased with the continuation of natural fermentation.

Figure 4 Alpha diversity indices among the samples.

**Beta diversity analysis**

The results of the beta diversity analysis are shown in Figure 5. Using AG0 as the sample control (CK), the species composition of the AG0 samples was far from the species composition of the AG24, AG36, and AG43 samples, and the difference in species composition was large, which indicated that the number of strains in the CK samples was not large, and the colony structure was more different from that in the AG24, AG36, and AG43 samples.

Figure 5 PCoA and NMDS diagram. Each dot in the figure represents a sample, and different colored dots indicate different samples (groups).

The similarity between the samples is shown in the form of a hierarchical tree (Fig. 6), with the AG36 and AG43 samples having the closest species composition distances, suggesting that the two samples are most similar. Taken together, these results indicate that the microbial communities of OFSRW fermenters from different periods showed variability and similarity. The clustered hierarchical tree showed that the proportion of R. *delemar* was greater in samples AG36 and AG43, but the largest proportion of C. *lusitaniae* was found in sample AG24. On the

other hand, the species composition of sample AG0 was the farthest from that of the other samples, indicating that the species composition of AG0 was different from that of the other samples. The difference between them was significant. This result is consistent with the results of the PCoA and NMDS analyses.

Figure 6 Hierarchical cluster analysis among samples. The upper panel shows a hierarchical clustering tree diagram in which the samples were clustered according to similarity. The shorter the branch length between the samples was, the more similar the samples were. The lower panel is a stacked histogram of the 30 most abundant species.

## Prediction of microbial function

The functional metabolic capacity of the microbial community was inferred from the composition of 16S rRNA genes in the macro-genomic data of different fermenters(Chen et al., 2020). Among the first-order KEGG metabolic pathways, the predicted functional genes enriched in the OFSRW fermenters were related to cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems. A comparison of the abundances of the four samples is shown in Figure 7. Metabolism-related pathways were significantly enriched in most of the samples, especially AG0, while the abundance of predicted genes related to organismal systems was relatively low in the AG0 samples, but the metabolic pathways declined and then stabilized with increasing fermentation time, while the number of organismal systems metabolic pathways was greater than that in the AG0 fermentation samples in AG24, AG36, and AG43.

Figure 7 Comparison of KEGG primary metabolic pathways. The vertical coordinates are the mean values of the abundance of functional pathways in the selected samples, and the horizontal coordinates are the sample groupings. Different colors represent different metabolic pathways.

To explore the reasons for the changes in functional pathways, we counted the secondary pathways involved in the metabolism of OFSRW, which involved a total of 45 secondary metabolic pathways, and the abundance values corresponding to each secondary pathway are shown in Table S1. The secondary metabolic pathways with increased expression were subjected to heatmap analysis, as shown in Figure 8. The most abundant predicted metabolism in the category of level 2 KEGG pathways was energy production and conversion, followed by inorganic ion transport and metabolism, carbohydrate transport, amino acid transport and metabolism, nucleotide transport and metabolism, and lipid transport and metabolism. Inferred carbohydrate transport, amino acid transport, and metabolism were prominent in sample AG0; energy generation and conversion metabolic pathways were prominent in sample AG24; posttranslational modification, protein turnover, and chaperone metabolic pathways were prominent in sample AG36; and chromatin structure and dynamics were prominent in sample AG43, which may have contributed to the observed variations in volatile compound profiles between these samples.

Figure 8 Heatmap of the horizontal distribution of KEGG secondary metabolic pathways for each sample.

## Discussion

Brix, acidity, and alcohol content are the most important parameters applied to monitor the fermentation process of *osmanthus* flower sweet rice wine. Glucose, sucrose, and maltose are three major fermentable sugars in the fermentation process of yellow rice wine because the starch in rice/wheat is predominantly degraded by α-amylase and glucosidase from Jiuqu (M. Kim et al., 2021; Yu et al., 2015). During fermentation, some low-molecular-weight sugars are mainly consumed by microorganisms, contributing to an increase in the organic acid content (Huang et al., 2019). In this study, the time before fermentation (AG0h) was 0 h, the sugar content was not detected, the acidity was 0.03, and the alcohol content was not detected, which

indicated that fermentation had not yet begun, that basically, no fermentable sugar existed in the raw material and that the acidity was low, which might be related to the activity of *L. plantarum*. The sugar content and acidity gradually increased with fermentation time. This may be related to the gradual decomposition of other sugars and indicates that the microorganisms became active and consumed sugars to produce energy and the gradual increase in acidity (from 0.03 to 0.35) also confirms the intensification of microbial activity, especially the activity of acid-producing bacteria such as *P. pentosaceus* and *L. plantarum*, which are capable of producing acid through the metabolism of sugars during the fermentation process. The sustained increase in acidity is consistent with previous findings (Tian et al., 2022), and the increase in acidity may be related to the decrease in *L. plantarum*, which lowers the pH of OFSRW and inhibits the growth of spoilage microorganisms that are sensitive to acidic conditions (Perpetuini et al., 2020), thereby controlling, to some extent, the fermentation microbial community structure during the process. *Rhizopus*, as the main fungal genus throughout the fermentation process, could more completely convert the starch in glutinous rice into fermentable sugars, indicating that the presence of *Rhizopus* was inextricably linked to the increase in sugar content.

In this study, by the end stage of fermentation (AG43h), the first brix of 2%Vol was detected, which indicated that the fermentation process had started to enter the alcoholic fermentation stage. The lack of alcohol detection in the first three fermentation stages could also be related to *L. plantarum*, since LAB has strong inhibitory activity against *E. coli*, *Saccharomyces,* and *Mucor* under low pH conditions (Russo et al., 2017). Alcohol production marks the beginning of the conversion of sugars into alcohol and carbon dioxide by *Saccharomyces,* typical of the traditional alcoholic fermentation phase (Fugelsang et al., 2007; Fleet et al., 1993). The detection of alcohol at the end stage of fermentation (AG43h) may be related to the significant activity of *Saccharomyces* and *Rhizopus* in the middle and late stages of fermentation (AG24 and AG36), where *Rhizopus* is capable of hydrolyzing starch to obtain sugars, and *Saccharomyces* are capable of fermenting sugars to produce alcohol, especially *C. lusitaniae* and *W. anomalus*, because *C. lusitaniae* can utilize sugars in the fermentation of

alcohol, and *W. anomalus* can secrete a variety of glycosidases, such as β-D-glucosidase, β-D-xylosidase, and α-L-rhamnosidase, which can promote the formation of aroma and flavor substances, and it can produce high amounts of ethyl acetate and 2-phenylethanol, which can significantly improve the sensory quality of the wine body (Padilla et al., 2018; Sabel et al., 2014; Sun et al., 2022). However, the trend of alcohol content in this study was opposite to that of total sugar, possibly because sugar has not yet been converted to ethanol and L. *plantarum* during the continuous fermentation process.

It has been shown that microorganisms play a crucial role in the formation of Chinese rice wine, including the synthesis of many flavor, texture, and color metabolites (Englezos et al., 2022; Huang et al., 2019). Krona analysis revealed that the microbial communities differed significantly at different fermentation stages, which may be closely related to changes in nutrients, pH, temperature, and other biotic and abiotic factors in the fermentation environment. At the early stage of fermentation (AG0), bacteria were the dominant microorganisms and were dominated by *Gammaproteobacteria* and *Bacilli.* The high proportion of *Gammaproteobacteria* may be related to their stronger metabolic activity in the sugar-rich environment. After 24 h of fermentation (AG24), fungi, especially the yeast group *C. lusitaniae*, began to dominate, showing high adaptability to the environment and efficient conversion of substrates during fermentation. The rapid growth of the yeast species may be related to their ability to grow under low oxygen or anaerobic conditions, which are common during hermetic fermentation. After 36 hours (AG36), a decrease in the proportion of fungi and an increase in the proportion of bacteria were observed, which may be attributed to a reduction in fungal activity due to the depletion of available sugars in the fermentation substrate, while the bacteria adapted to this change and began to utilize the products of fungal metabolism or other nonsugar organic acids. By 43 hours (AG43), *Rhizopus* became dominant, and its growth may have been due to increased acidity and certain nutrients (e.g., proteins and fats) becoming more available later in the fermentation process. This change reflects the adaptive strategy of the microbial community to survive and thrive under nutrient competition and environmental stresses during the fermentation process.

*Pediococcus* is widely distributed in Jiuqu, which helps improve food taste and nutrition. The occurrence of *Pediococcus pentosaceus* in the AG0 samples is in line with the results of a previous study (Liang et al., 2020). The presence of *P. pentosaceus* improved the flavor of fermented food by increasing the levels of short-chain fatty acids (SCFAs) (Jiang et al., 2021). In samples AG24 and AG36, the presence of *C. lusitaniae* and *W. anomalus* can decompose sugar into alcohol, carbon dioxide, and other secondary products, which exert a far-reaching effect on the flavor and aroma of Chinese rice wine (Zhang et al., 2022). In AG43h, *Rhizopus* became dominant because *Rhizopus* can utilize macromolecular ingredients in the raw materials and consume nutrients that promote the growth and reproduction of the strain (Wu et al., 2022).

In this study, the trends in metabolic pathway changes during OFSRW fermentation were analyzed in depth, which may be closely related to the dynamics of microbial communities and metabolic activities. By comparing the abundance of metabolic pathways at different fermentation stages, this study revealed the importance and change patterns of specific metabolic pathways during fermentation. First, the abundance of organismal systemic metabolic pathways was generally greater in the AG24, AG36, and AG43 samples during fermentation than in the initial fermentation stage (AG0), which may reflect the mechanism by which fermenting microorganisms respond to environmental stresses. The activation of these metabolic pathways may be related to the microbial response to oxidative stress, nutrient limitation, and other biotic stress conditions in the fermentation environment. In particular, the activation of these pathways may be related to cellular protective mechanisms such as antioxidant and damage repair functions. Second, the gradual decline in metabolic pathways with increasing fermentation time suggested that as the fermentation process proceeded, nutrient sources such as carbohydrates initially utilized were gradually depleted, and the microorganisms had to adjust their metabolic strategies to adapt to less nutritious environments. Finally, functional analysis revealed significant carbohydrate transport, lipid transport and metabolism, and amino acid transport and metabolism pathway activity in the AG0 fermentation broth, implying that flavor formation in these samples was likely linked to protein and starch metabolism (Xiao et al., 2021; Chen et al.,

2020), thus contributing to the increase in sugar content and detectable alcohol content during the middle and late stages of fermentation until the end of fermentation. This finding suggested that carbohydrate metabolism is the main pathway for microbial growth and energy production during the early stages of fermentation. The energy production of microbial flora depends mainly on the phosphorylation of substrates through sugar fermentation to acetate, while energy production and conversion) can drive the energy demand of bacterial flora, which explains the increase in bacteria in this AG24 sample. The significant activation of the metabolic pathway of energy production and conversion in the fermentation broths of AG24 suggested that *C. lusitaniae* and *R. delemar* are active during this fermentation phase and thus contribute to the increase in sugar level and acidity. The significant activation of posttranslational modifications, protein turnover, chaperones in AG36 fermentation broth, and signal transduction mechanisms in AG43 fermentation broth suggested enhanced microbially driven nutrient-seeking activity in samples from this phase, which may have increased microbial signal perception through a complex signaling network, enabling better nutrient utilization (Zhao et al., 2022), especially in the case of *W. anomalus* and *R. delemar, and* significant activity may contribute to the increase in alcohol and sugar levels through posttranslational modifications, protein turnover, chaperones and signal transduction mechanisms. The high expression of these metabolic pathways may, therefore, be inextricably linked to increased sugar, acidity, and alcohol content, suggesting that some of the differences in the functional metabolic abundance of the samples from different periods may be related to the composition of the microbial community at various periods, which may help to observe changes in the volatile compound profiles between these samples.

## 4 Conclusion

It is the first report on employing high-throughput sequencing to study the predicted dynamic changes in microbial communities and metabolic pathways in OFSRW natural fermentation broth. By comparing the bacterial strains under natural fermentation conditions at different time points, it was found that the number of strains detected in the samples first

increased and then decreased with increasing fermentation time. However, bacteria decreased, and fungi became the dominant microorganisms over time, with *Rhizopus* being the dominant fungal genus throughout the fermentation process. The increase in acidity and total sugar content with fermentation time was associated with *L. plantarum* and *Mucor*, and the addition in alcohol content was not detected until the end of fermentation because *Saccharomyces* and *Mucor* were inhibited by *L. plantarum* at 0 h, 24 h, and 36 h of fermentation. The diversity analysis results showed that the species composition of the AG0 samples was very different from that of the AG24, AG36, and AG43 samples, and the species diversity showed a decreasing trend followed by an increasing and then decreasing trend. Energy production and conversion, carbohydrate transport, amino acid transport, and metabolic pathways are the most active metabolic pathways in fermenters. The results of this study not only provide insights into how microorganisms adapt to environmental changes during fermentation but can also be crucial for optimizing fermentation conditions and improving product quality and flavor.

## Authorship Contribution Statement

Ping Tian performed the experiments, analyzed the data, prepared figures and/or tables, and authored or reviewed drafts of the paper.

Jiaqiong Wan performed the experiments and reviewed drafts of the paper.

Tuo Yin: Methodology, formal analysis.

Li Liu: Providing and experimental equipment, guiding the production process.

Hongbing Ren: Providing production site and experimental equipment, funding acquisition.

Hanbing Cai: Image modification.

Xiaozhen Liu: Review and editing, supervision, funding acquisition.

Hanyao Zhang: Methodology, writing-review and editing, supervision, funding acquisition.

All the authors have read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Ren Hongbing is the chairman of Honghe Hongbin Foods Co Ltd. Liu Li is employed by Honghe Hongbin Foods Co., Ltd.

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## Table 1(on next page)

Table 1 Physicochemical properties of sweet rice wine produced from cinnamon *osmanthus* flower.

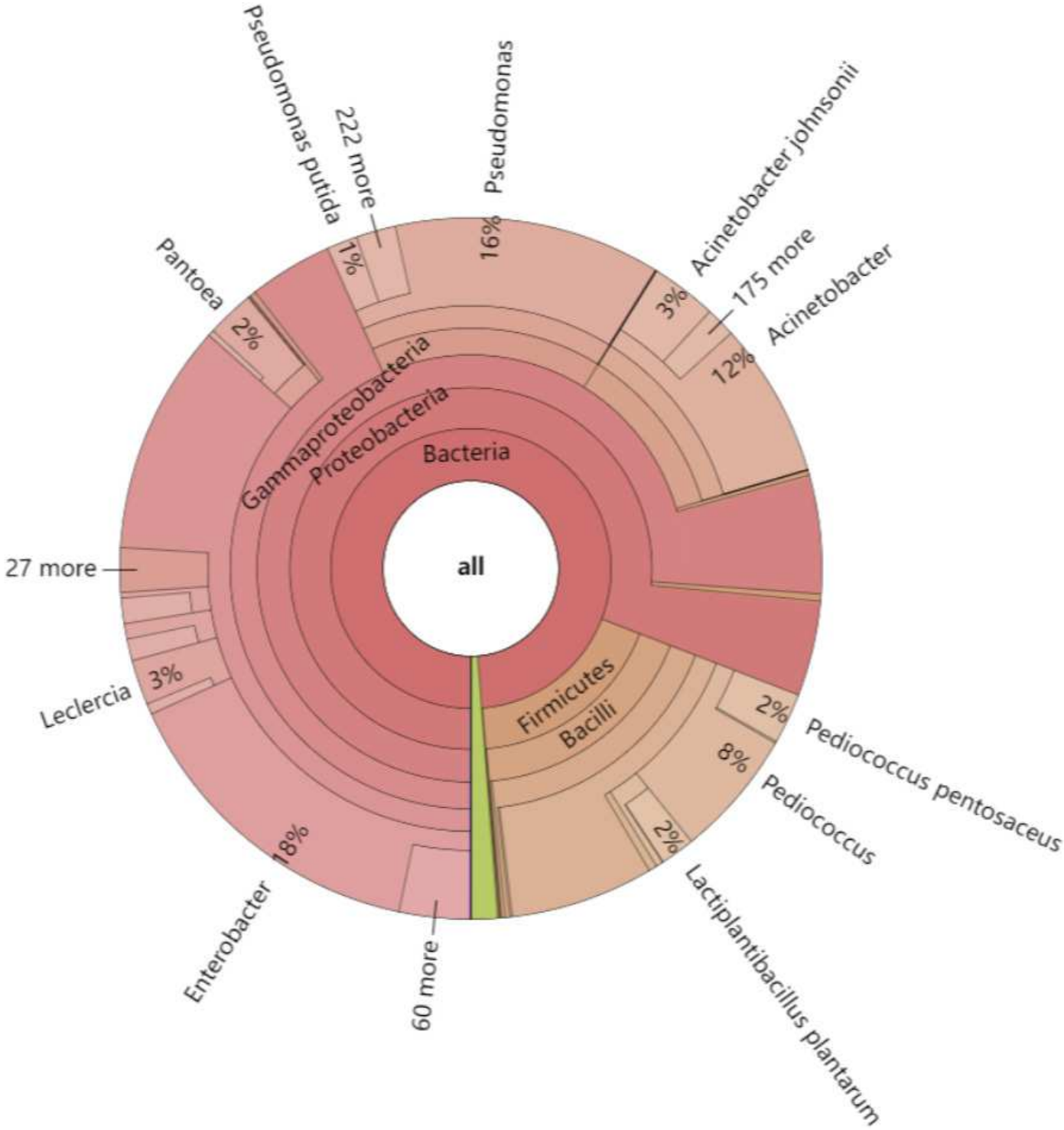
1 Table 1 Physicochemical properties of sweet rice wine produced from cinnamon *osmanthus* flower.

| Local flavor fermentation cycle / Physical and chemical indicator | OFSRW | | | |
|---|---|---|---|---|
| | 0h | 24h | 36h | 43h |
| acidity | 0.03 | 0.14 | 0.25 | 0.35 |
| total sugar （g/250ml） | not detected | 7.11 | 14.05 | 16.55 |
| alcoholic strength （v/vol,%） | not detected | not detected | not detected | 2 |

# Figure 1

Fig. 1. Classification level and abundance information of Krona diagram of sample species.

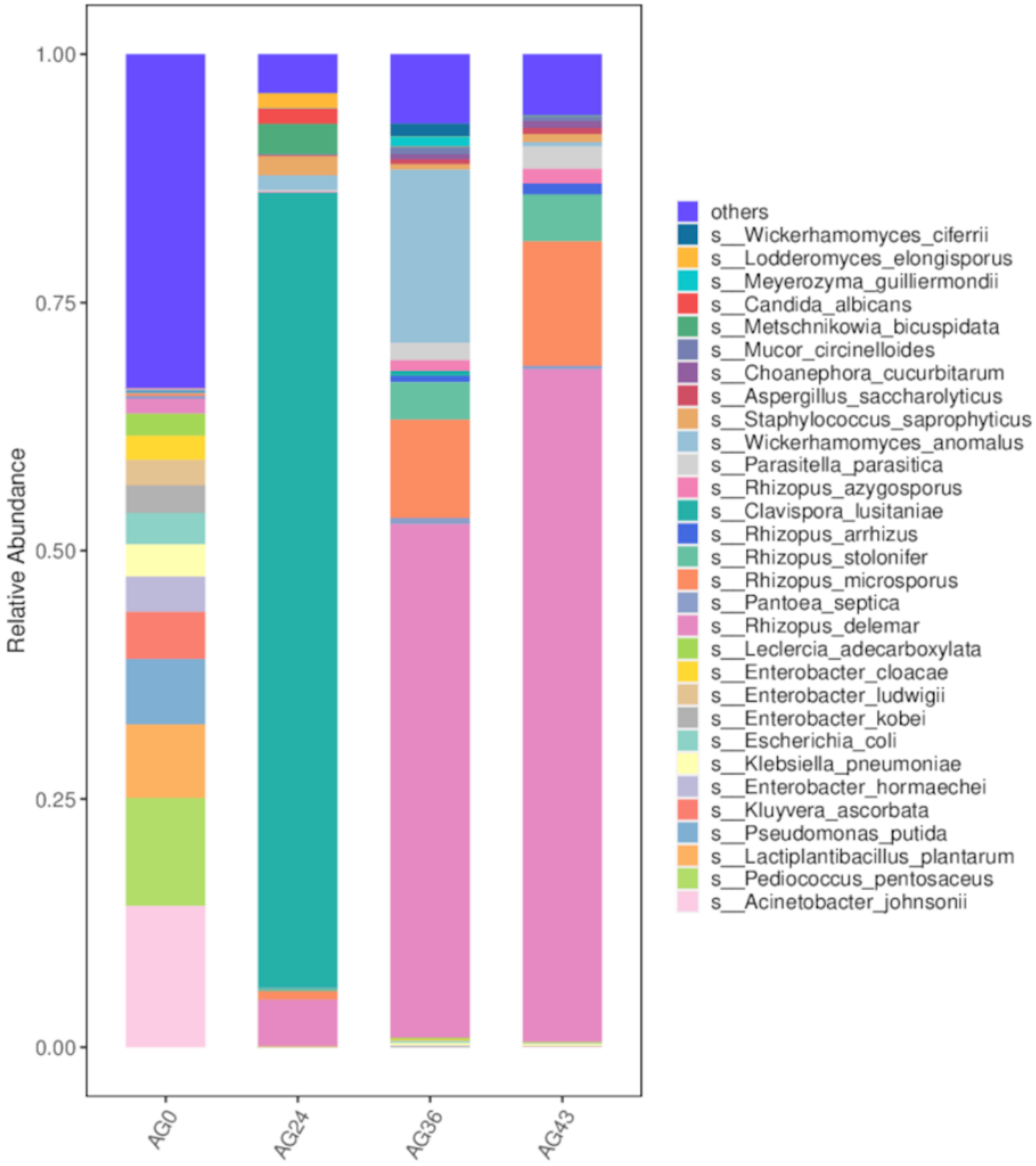
From the inside to the outside, the Krona circle represents the seven taxonomic levels of domain, phylum, class, order, family, genus, and species. The sector’s size reflected the relative abundance of different taxa, and there were specific values.


all
Bacteria
Proteobacteria
Gammaproteobacteria
Firmicutes
Bacilli
Pseudomonas
16%
Pseudomonas putida
1%
222 more
Acinetobacter johnsonii
3%
175 more
Acinetobacter
12%
Pantoea
2%
27 more
Leclercia
3%
Enterobacter
18%
60 more
Pediococcus pentosaceus
2%
Pediococcus
8%
Lactiplantibacillus plantarum
2%

# Figure 2

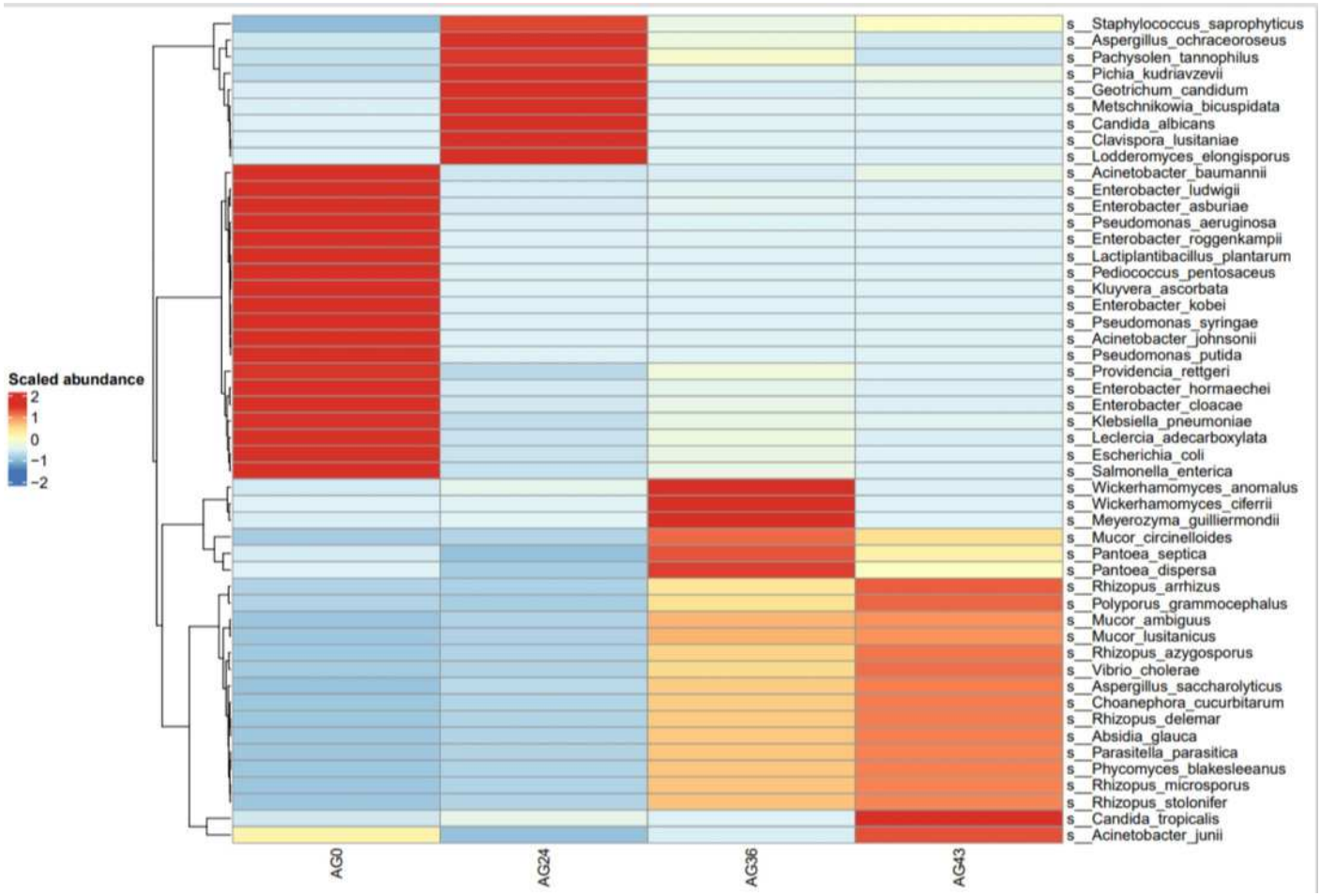
Fig. 2. Column diagram of the horizontal species composition of each sample species.

The abscissa is the name of each group of the grouping scheme, and the ordinate is the relative abundance of each taxon at a specific taxonomic level.


Relative Abundance
1.00
0.75
0.50
0.25
0.00
AG0
AG24
AG36
AG43
others
s__Wickerhamomyces_ciferrii
s__Lodderomyces_elongisporus
s__Meyerozyma_guilliermondii
s__Candida_albicans
s__Metschnikowia_bicuspidata
s__Mucor_circinelloides
s__Choanephora_cucurbitarum
s__Aspergillus_saccharolyticus
s__Staphylococcus_saprophyticus
s__Wickerhamomyces_anomalus
s__Parasitella_parasitica
s__Rhizopus_azygosporus
s__Clavispora_lusitaniae
s__Rhizopus_arrhizus
s__Rhizopus_stolonifer
s__Rhizopus_microsporus
s__Pantoea_septica
s__Rhizopus_delemar
s__Leclercia_adecarboxylata
s__Enterobacter_cloacae
s__Enterobacter_ludwigii
s__Enterobacter_kobei
s__Escherichia_coli
s__Klebsiella_pneumoniae
s__Enterobacter_hormaechei
s__Kluyvera_ascorbata
s__Pseudomonas_putida
s__Lactiplantibacillus_plantarum
s__Pediococcus_pentosaceus
s__Acinetobacter_johnsonii

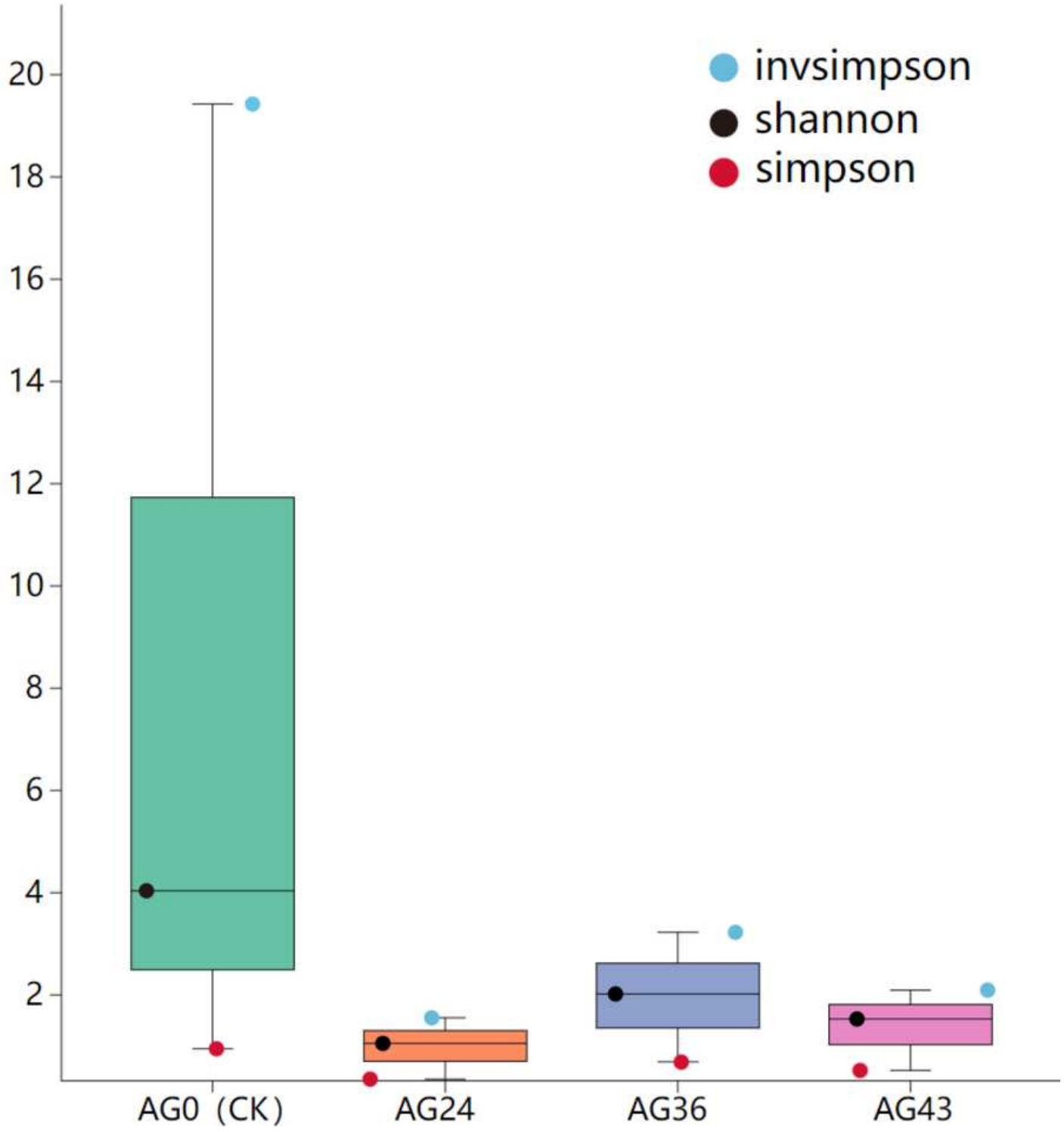
# Figure 3

Fig. 3. Horizontal distribution heatmap of each sample species.



# Figure 4

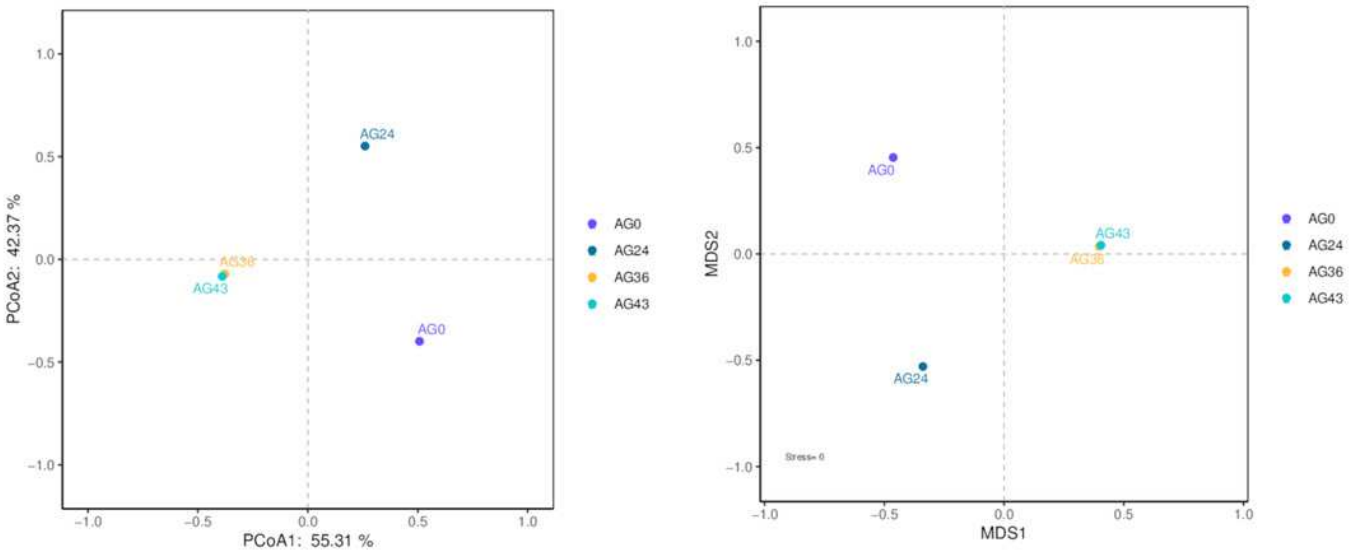
Fig. 4. Alpha diversity indices among the samples.



# Figure 5

Fig. 5. PCoA and NMDS diagram.

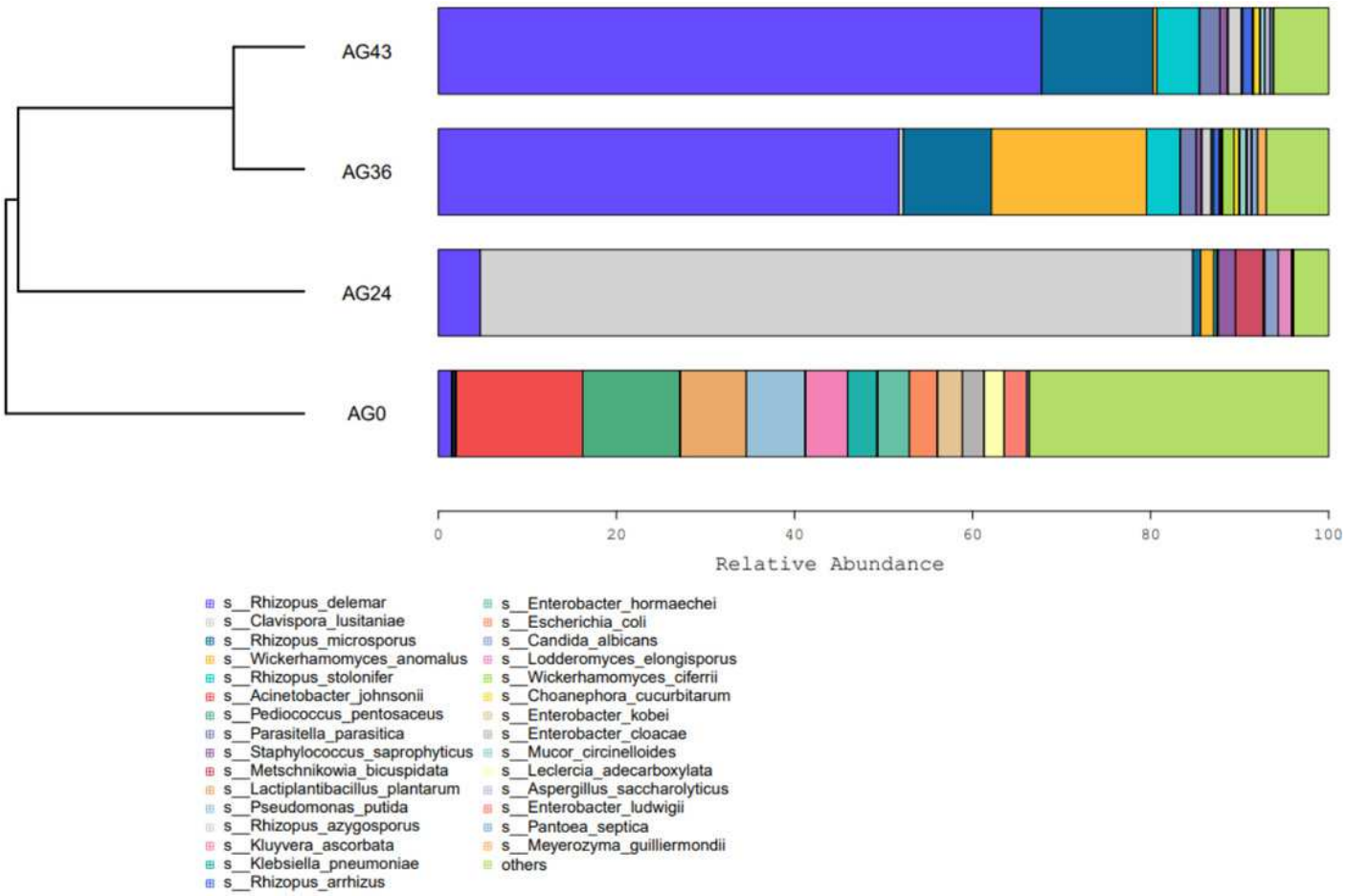
Each dot in the figure represents a sample, and different colored dots indicate different samples (groups).


PCoA2: 42.37 %
PCoA1: 55.31 %
AG0
AG24
AG36
AG43
MDS2
MDS1
Stress= 0

# Figure 6

Fig. 6. Hierarchical cluster analysis among samples.

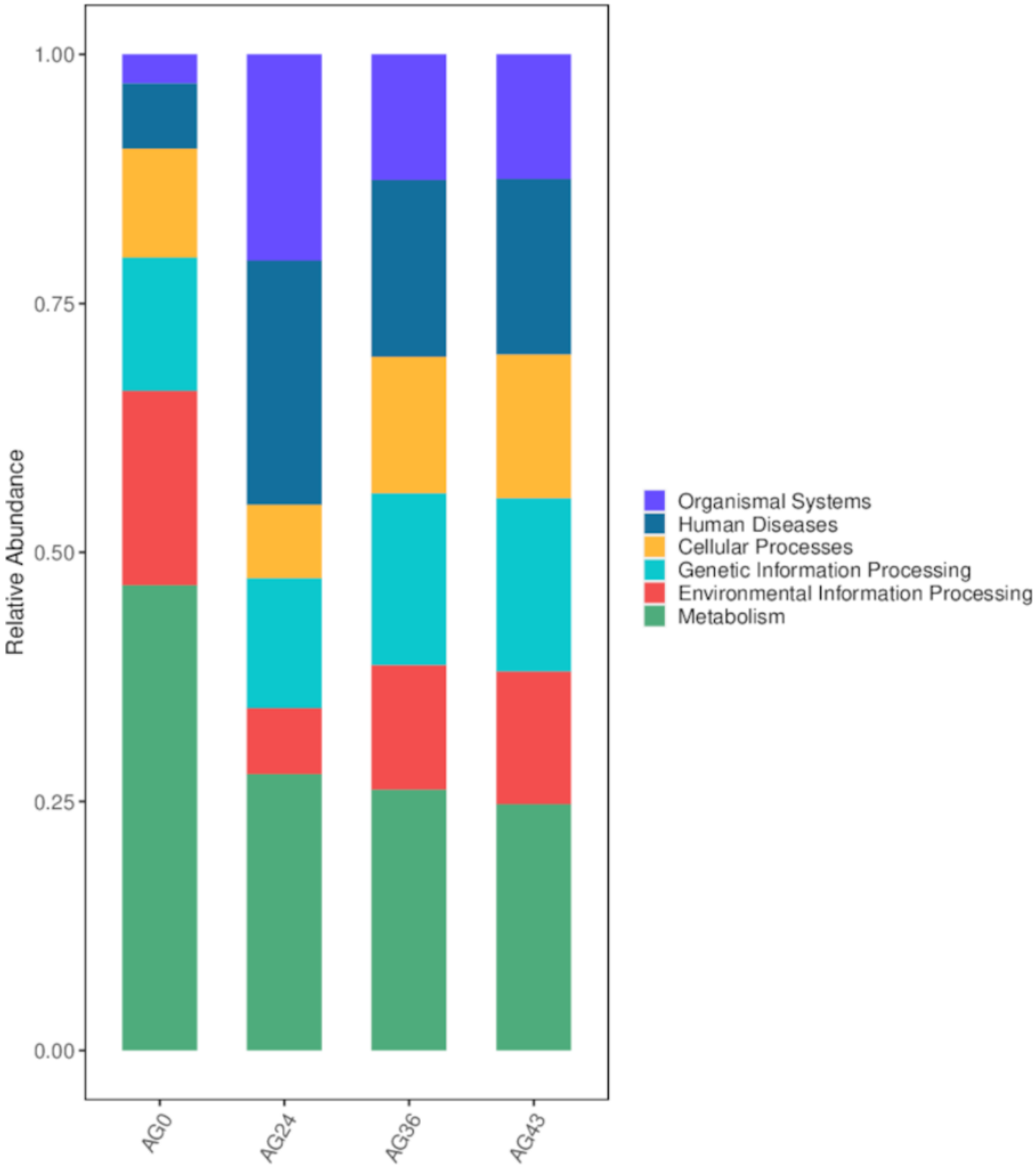
The upper panel shows a hierarchical clustering tree diagram in which the samples were clustered according to similarity. The shorter the branch length between the samples was, the more similar the samples were. The lower panel is a stacked histogram of the 30 most abundant species.



# Figure 7

Fig. 7. Comparison of KEGG primary metabolic pathways.

The vertical coordinates are the mean values of the abundance of functional pathways in the selected samples, and the horizontal coordinates are the sample groupings. Different colors represent different metabolic pathways.


1.00
0.75
0.50
0.25
0.00
Relative Abundance
AG0
AG24
AG36
AG43
Organismal Systems
Human Diseases
Cellular Processes
Genetic Information Processing
Environmental Information Processing
Metabolism

# Figure 8

Fig. 8. Heatmap of the horizontal distribution of KEGG secondary metabolic pathways for each sample.