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Changes and Driving Mechanism of Microbial Community Structure during Paocai Fermentation

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Abstract: Fermentation of paocai is a dynamic process of the microbial community structure, and the interaction between community structure and physicochemical factors endows paocai with unique taste and flavor. The study of bacterial and fungal community structure changes and the driving mechanism of physicochemical factors induced changes in community structure, showing that *Pseudomonas* belonging to Proteobacteria and *Lactobacillus* belonging to Firmicutes were the dominant bacteria in the process of paocai fermentation. The correlation analysis of physicochemical factors with bacterial community showed that titratable acid was significantly positively correlated with *Lactobacillus* and negatively correlated with *Pseudomonas*, while nitrite was the opposite. Redundancy analysis (RDA) showed that pH was positively correlated with the bacterial community in the early fermentation stage, amino acid nitrogen was positively correlated with the bacterial community in the middle fermentation stage, and titratable acid was positively correlated with the bacterial community in the late fermentation stage. Variance partitioning analysis (VPA) showed that environmental factors, pH and metabolites, were the main driving forces of bacterial community diversity, which jointly explained 32.02% of the bacterial community structure variation. To study the glucolysis and nitrogen metabolism in the process of paocai fermentation, we found that in the early stage of the fermentation, the nitrite reductase enzyme of *Pseudomonas* activity was high, with high nitrite content in the prophase, but by the end of fermentation, lactic acid bacteria rapidly increased, the content of L−lactic acid through the glycolysis pathway, making paocai fermentation environment become acidic, then *Pseudomonas* decreased. Ascomycota and Basidiomycota were the main phylum fungi in the fermentation process. RDA analysis showed that the fungal community was positively correlated with pH, nitrite, and soluble protein at the early fermentation stage, amino acid nitrogen was positively correlated with the fungal community at the middle fermentation stage, titratable acid and reducing sugar were positively correlated with the fungal community at the late fermentation stage. VPA analysis showed that metabolites were the main driving force of fungal community diversity and accounted for 45.58% of fungal community diversity. These results had a certain guiding significance for the production and preservation of naturally fermented paocai.

Keywords: paocai; microbial community; high−throughput sequencing; driving mechanism

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1. Introduction

Paocai is a traditional fermented vegetable in China. Paocai is mainly made of fresh vegetables, with or without supplementary materials, fermented in 3~7% salt water for 15~30 days by microorganisms. The nutritional value of paocai is very rich, containing a large number of vitamins, minerals, dietary fiber, and other functional components. These can meet the nutritional needs of the human body, and also have the function of regulating intestinal flora, reducing serum cholesterol, as well as anti−oxidation, weight loss, and other health effects [1]. Paocai is fermented in the natural environment, and contributes to the growth of microorganisms, so there are some interactions between microorganisms,

microorganisms and environmental factors; for this reason, a complex fermentation system is formed [2]. The composition of the microbial community changes during fermentation and makes the product rich in microbial metabolites such as lactic acid or amino acids [2]. The initial microorganisms are the microorganisms left on vegetables when the salt water is immersed. With the progress of fermentation, the variety and number of microbial communities continue to change, and finally make the paocai form a unique flavor. At the same time, the fermentation process of paocai is affected by raw materials, ingredients, salt, temperature, acidity, oxygen, and other factors. The microbial succession and change are great, so the fermentation system is complex, including a large number of beneficial microorganisms and some harmful microorganisms. In order to determine the safety of fermented food, reduce harmful substances and improve product quality, we must understand the microbial community structure succession and metabolic characteristics of various microorganisms in the fermentation process and their influence on product quality. Therefore, it is very important to clarify the microbial community succession related to the formation of special flavor substances in the process of paocai fermentation, especially the dynamic change and correlation between the microbial community and metabolites.

The composition of the microbial community changes during fermentation and makes the product rich in microbial metabolites such as lactic acid or amino acids [3]. Generally speaking, bacteria are the main microorganisms in fermentation, and their diversity is more abundant than fungi. With the progress of fermentation, bacterial abundance gradually decreased and fungal abundance gradually increased. The growth of microorganisms is largely affected by environmental factors. The physicochemical factors of the fermentation system not only affect the availability of nutrients needed for microbial growth but also affect the growth and survival of other harmful bacteria [4]. Exploring the structural composition and driving mechanisms of microbial communities in paocai has been the focus of traditional microbiological practice.

However, at present, there is a lack of scientific and holistic research and standards on microbial specification and interactions and their related physiological and biochemical reactions in different paocai, which will become an important issue in the process of vegetable fermentation research in the future. The study of microbial kinetic and driving mechanisms is an important direction in the fermentation industry, which provides an important basis for standardizing fermentation performance and producing paocai in a safe and controlled manner. The popularity of next−generation sequencing (NGS) technology has promoted the popularization of microbiome research in products such as fermented vegetables and fermented milk [5,6]. With the development of technology, we can use metatranscriptome, metagenomic, metaproteome, metabolome and other omics technologies to study the community structure succession and interaction of microorganisms in fermented vegetables, as well as the expression of enzymes and metabolites in different microorganisms. This will help to explain the mechanism of paocai fermentation, and then improve the flavor quality of paocai during production.

Given the above information, this paper investigates the dynamic change pattern of physicochemical factors and the change of bacterial diversity and fungal diversity during the fermentation of naturally fermented paocai, and analyses the driving mechanism of physicochemical factors affecting the structural composition of bacterial and fungal communities, aimed at controlling the fermentation conditions and thus the product quality, and providing technical support to guide the control of paocai production.

2. Materials and Methods

2.1. Paocai Production and Sampling

Fresh cabbage was placed in a cool and ventilated place to dry and dehydrate for 72 h, washed, and loaded into the washed and dried paocai altars, respectively. Each altar was filled with 500 g cabbage, 60 g salt each layer of cabbagewith a layer 20 g of salt pressed, the top sealed with salt and pressed with a pressure stone. Then, 2 L of cooled boiled water was poured and the cabbage submerged, covered with a lid, and placed in

a cool (15 °C) and ventilated place and fermented for a month. During fermentation, the brine was sampled every 2 days for the determination of physicochemical indexes. DNA extraction was performed by a destructive sampling method to collect samples, at 3 d, 15 d, and 30 d during the fermentation period. The 9 samples were numbered S1–S9 and the 3 stages were numbered S1–S3.

2.2. Determination of Physicochemical Indexes in Paocai

The pH values of brine samples were measured with a pH meter (S210, Mettler−Toledo, Shanghai, China). The total acid contents (TTA) were determined by the acid–base titration method [7]. The nitrite contents (NIT) were determined by the naphthalene ethylenediamine hydrochloride method [8]. The reducing sugar contents (RS) were determined by the direct titration of the alkaline copper tartrate solution method [9]. The soluble protein content (SP) was determined by the spectrophotometry method [10]. The amino acid nitrogen content (AN) was determined using the acidity meter method [11].

2.3. Metagenomic DNA Extraction

The metagenomic DNA was extracted from 40 mL of each sample using the QIANamp DNA Microbiome Kit according to the manufacturer's instructions (QIANamp DNA Microbiome Kit, TIANGRN Inc, Beijing, China). The purity, concentration, and integrity of the extracted DNA were detected by spectrophotometry and 1.5% agarose gel electrophoresis. Qualified DNA samples were stored in a −20 °C refrigerator for use.

2.4. PCR Amplification and MiSeq Highthroughput Sequencing

In this study, the V3−V4 region of 16S rRNA was amplified using forward primer 338F (5′−ACTCCTACGGGAGGCAGCAG−3′) and reverse primer 806R (5′−GGACTACHVGGG TWTCTAAT−3′). The PCR amplification system included the following: 4 μL 5 × PCR buffer; 2 μL 2.5 mM dNTP mix; 0.8 μL 5 μmol/L forward primer; 0.8 μL 5 μmol/L reverse primers; 0.4 μL 5 U/μL DNA polymerase; 10 ng DNA template; and supplemented to 20 μL with ddH_2O. The PCR amplification conditions were as follows: 95 °C for 3 min; 95 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s; 30 cycles; and 72 °C for 10 min. The 30 qualified DNA amplicons, diluted to a concentration of 100 nmol/L, were sequenced by a MiSeq high−throughput sequencing platform in Shanghai Majorbio Co., Ltd., Shanghai, China.

2.5. Sequencing Data Processing and Bioinformatics Analysis

The extraction of non−repetitive sequences from optimized sequences was performed using operational taxonomic unit (OTU) clustering −according to 97% similarity. The OTUs were flattened by the minimum number of sample sequences for subsequent analysis. Then, each OTU representative sequence was aligned with the database according to the ribosomal database project (RDP) classifier Bayesian algorithm. R software(ver. 4.1.3, Ross Ihaka, Auckland, New Zealand)was used for analysis of similarities (ANOSIM) of bacterial community differences.

Principal coordinate analysis (PCoA) and principal component analysis (PCA) were used to assess the microbiota structure of different samples; redundancy analysis (RDA), canonical correlation analysis (CCA), and variance partitioning analysis (VPA) were appropriate to analyze relationships between microbial communities and physicochemical factors with R software. Spearman correlation analysis was used to estimate the richness correlation between species.

The PICRUSt software (ver.1.1.4, Curtis Huttenhower, Boston, USA) was employed to predict the functional potential of the bacterial communities in paocai samples, and further analysis was carried out in the context of the Cluster of Orthologous Groups (COG) database. FUNGuild was used for functional classification of fungi.

The data were presented as the mean of three independent experiments. Differences between groups were compared using ANOVA. Differences were considered significant when p value was less than 0.05. Statistical data were analyzed by SPSS software.

3. Results

3.1. The Trend of Physicochemical Indexes in Paocai

Physicochemical factors are essential factors to determine the quality of paocai products and are crucial to the formation of taste [12]. Figure 1A,B show the changes of pH and titratable acid in the fermentation process of paocai. After 1–7 days of fermentation, the pH of the fermentation liquid dropped rapidly, from 5.702 to 4.040, and then the pH decreased slowly with the fermentation time and finally stabilized at 3.855. The titratable acid content continued to increase and reached a stable level at 22 days, reaching 8.596 g/L. Figure 1C shows that the nitrite content reached a peak level of up to 23 mg/kg on the 4th day, and the nitrite content was stable at about 3 mg/kg in the later stage of fermentation, indicating that the nitrite content in paocai was far lower than the national standard. The limit standard of nitrite content is 20 mg/L. The soluble protein showed a downward trend (Figure 1D), from 26.21 mg/100 g at the beginning of fermentation to 5.80 mg/100 g. Amino acid nitrogen is an important indicator of the umami taste of paocai, showing a trend of rising first and then decreasing, and the content was relatively little (Figure 1E). The content of amino acid nitrogen at the beginning of fermentation was only 0.010%, and increased to 0.11% on the 17th day and remained stable until the 21st day, and then slowly decreased to 0.026%. In this study, it was found that the content of reducing sugars showed a trend of decreasing in the early stage of fermentation, then rising, and finally stabilizing. In the early stage of fermentation, saccharification was rapid. On the 3rd day of fermentation, the content of reducing sugars was 41.53 mg/mL, and then began to decline slowly, then reached 39.8 mg/mL at the end of fermentation.

3.2. Sample Grouping Analysis

The ANOSIM (Analysis of Similarities) test was followed by a one−way analysis based on one factor under 999 permutations and at 5% of significance level, which was used to test the differences between groups and within groups, to judge whether the experimental grouping was meaningful [13]. As shown in Figure 2, ANOSIM analysis was performed on the samples based on the OTU level. The results showed that the bacterial community of naturally fermented paocai at different fermentation times had significant community distribution differences, and the difference between groups was greater than the difference within groups (R^2 = 0.6296, p = 0.001). Therefore, the naturally fermented paocai can be divided into three stages according to the sampling time: S1 was the initial stage of fermentation, S2 was the middle stage of fermentation, and S3 was the late stage of fermentation.

3.3. Composition of Bacterial and Fungal Communities in Paocai Samples

The bacteria detected from 9 paocai samples belonged to 6 phyla, 9 classes, 29 orders, 47 families, 59 genera, and 83 species. Fungi belong to 7 phyla, 21 classes, 40 orders, 56 families, 72 genera, and 92 species.

The bacterial and fungal community structures of the three groups of samples were analyzed at the phylum and genus levels, respectively. The results showed that at the phylum level (Figure 3A), the dominant bacterial phyla were Firmicutes and Proteobacteria, as well as Bacteroidota and Actinobacteria. In the early stage of fermentation, Proteobacteria was the absolute dominant phylum, with a relative abundance of 84.18%, while the relative abundance of Firmicutes was only 10.61%. In the middle stage of fermentation, the relative abundance of Proteobacteria decreased to 33.19% and Firmicutes gradually increased to 64.83%. Until the late stage of fermentation, the content of Firmicutes further increased to 77.51%, and the relative content of Proteobacteria further decreased to 17.91%. It can be seen that the succession between Proteobacteria and Firmicutes occurred gradually in the early to middle stages of fermentation.

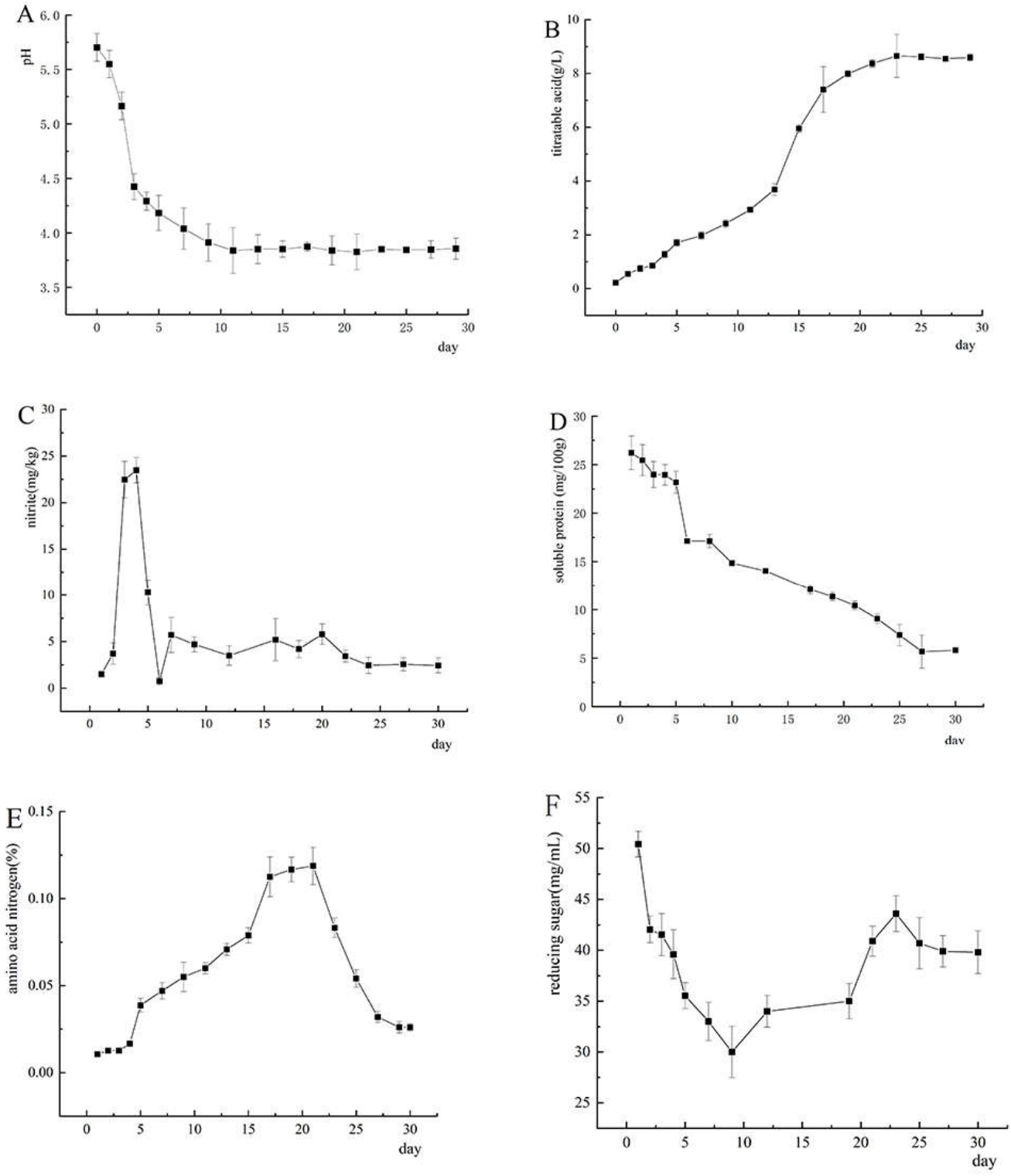


Figure 1. The variation trend of pH (**A**), the titratable acid (**B**), nitrite (**C**), soluble protein (**D**), amino acid nitrogen (**E**), and reducing sugar (**F**) during paocai fermentation.

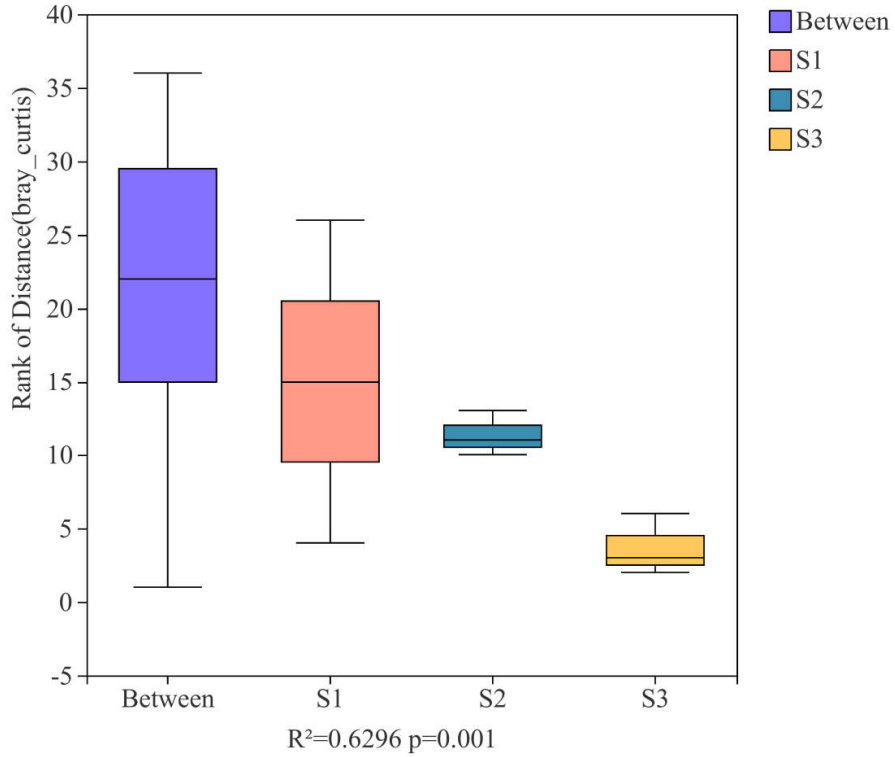


Figure 2. Anosim similarity among groups of samples.

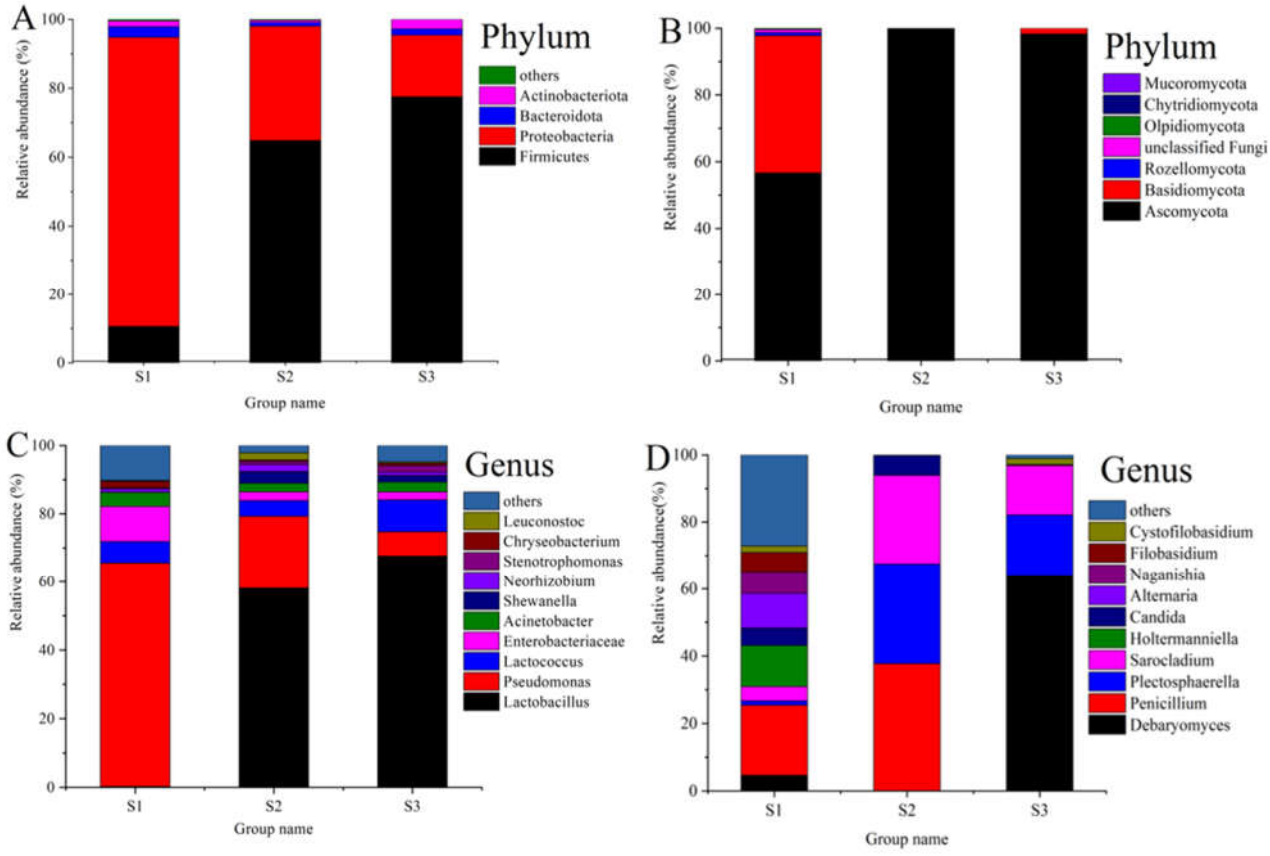


Figure 3. The relative abundance distribution of bacteria at phylum level (**A**) and genus level (**C**) and fungi at phylum level (**B**) and genus level (**D**) in fermented paocai.

At the phylum level, Ascomycota and Basidiomycota were the most dominant Fungi phyla (Figure 3B). In the S1 stage, the relative abundance of Ascomycota was 56.59%, the relative abundance of Basidiomycota was 41.16%, and the relative abundance of Rozellomycota was 0.94%, Olpidiomycota 0.33%, Chytridiomycota 0.06%, and unclassified Fungi 0.93%. In the S2 stage, the naturally fermented paocai only contained Ascomycota and Basidiomycota, and their relative abundance were 99.88% and 0.11%, respectively. In the S3 stage, the relative abundance of Ascomycota and Basidiomycota were 98.22% and 1.75%, respectively, and there was also Mucoromycota with a relative abundance of 0.03%.

At the genus level, as shown in Figure 3B,D in the S1 stage, the relative abundance of *Pseudomonas* was 65.36%, *Enterobacteriaceae* 10.33%, *Lactococcus* 6.27%, *Acinetobacter* 4.10%, *Chryseobacterium* 1.8%, and that of *Lactobacillus* and *Leuconostoc* were less than 1% (0.21%, 0.20%). In the S2 stage, the relative abundance of *Lactobacillus* increased rapidly to 58.06%, and at the same time the abundance of *Pseudomonas* decreased swiftly to 21.21%, *Enterobacteriaceae* 2.51%, *Lactococcus* 4.62%, *Acinetobacter* 2.54%, *Shewanella* 3.40%, *Leuconostoc* 2.08, and the relative abundance of *Stenotrophomonas* and *Chryseobacterium* was less than 1%. At the S3 stage, the relative abundance of *Lactobacillus* increased to 67.65%, *Pseudomonas* decreased to 7.10%, *Enterobacteriaceae* 2.31%, *Lactococcus* 9.33%, *Acinetobacter* 2.92%, *Shewanella* 1.88%, *Leuconostoc*, and the relative abundance of *Chryseobacterium* and *Stenotrophomonas* were less than 1%.

As for fungi at the genus level, as seen in Figure 3D, *Debaryomyces*, *Penicillium*, *Plectosphaerella*, *Sarocladium*, and *Candida* were the dominant genera. In the S1 stage, the relative abundance of *Debaryomyces* was 4.61%, *Penicillium* 20.82%, *Plectosphaerella* 1.32%, *Sarocladium* 4.18%, *Holtermanniella* 12.22%, *Candida* 5.20%, *Alternaria* 10.25%, *Naganishia* 6.46%, *Filobasidium* 5.78%, *Cystofilobasidium* 2.01%, *Kazachstania* 3.63%, *Cladosporium* 2.50%, *Sampaiozyma* 2.80%, *Dipodascaceae* 1.84%, *Pseudogymnoascus* 1.29%, *Aureobasidium* 1.57%, and the relative abundance of others was 10.18%. This indicated that the fungal community structure was more complex in the early stage of fermentation. In the S2 stage, the relative abundance of *Debaryomyces* was only 0.02%, *Penicillium* 37.74%, *Plectosphaerella* 29.82%, *Sarocladium* 26.38%, *Candida* 5.86%, and other genera's relative abundance were less than 0.1%. In the S3 stage, the relative abundance of *Debaryomyces* was 63.92%, *Penicillium* 0.05%, *Plectosphaerella* 18.06%, *Sarocladium* 14.69%, *Candida* 0.31%, *Cystofilobasidium* 1.77%, *Cladosporium* 0.46%, *Pseudogymnoascus* 0.34%.

3.4. Research on Beta Diversity and Driving Factors of Samples

The correlation between microorganisms and fermentation periods was analyzed by Beta diversity analysis. Bacterial PCoA (Principal coordinate analysis) is shown in Figure 4A. PC1 and PC2 were two factors, which accounted for 65.44 and 15.43% of variability, respectively. PC1 had the greatest impact in the early fermentation stage and PC2 in the late fermentation stage. The fungal PCA (Principal component analysis) showed the variability between samples (Figure 4B). PC1 (35.65%) and PC2 (22.84%) explain 68.51% of the variability. PC1 had the largest influence in the early stage of fermentation and PC2 in the later stage of fermentation. As shown in the figure, the bacterial and fungal community structures had a huge difference between the early fermentation and the middle fermentation, while the difference became smaller in the late fermentation, indicating that the microbial community structure of the fermentation system gradually stabilized with the extension of the fermentation time.

The RDA analysis (Redundancy analysis) between physicochemical factors and bacterial community during paocai fermentation is shown in Figure 4C; PC1 and PC2 explained 67.69% and 17.16% of the microbial community variation. The pH, reducing sugar, and nitrite were positively correlated and TTA was negatively correlated with the initial stage of fermentation, and the opposite was observed in the late stage of fermentation. The gradual decrease of the soluble protein with the extension of fermentation time is positively correlated with the late fermentation stage, and the positive correlation between amino acid nitrogen and the middle fermentation stage indicated that the amino acid nitrogen

metabolism reaction mainly occurred in the middle fermentation stage, which also explained the trend of amino acid nitrogen increasing first and then decreasing reaching the maximum in the middle of fermentation.

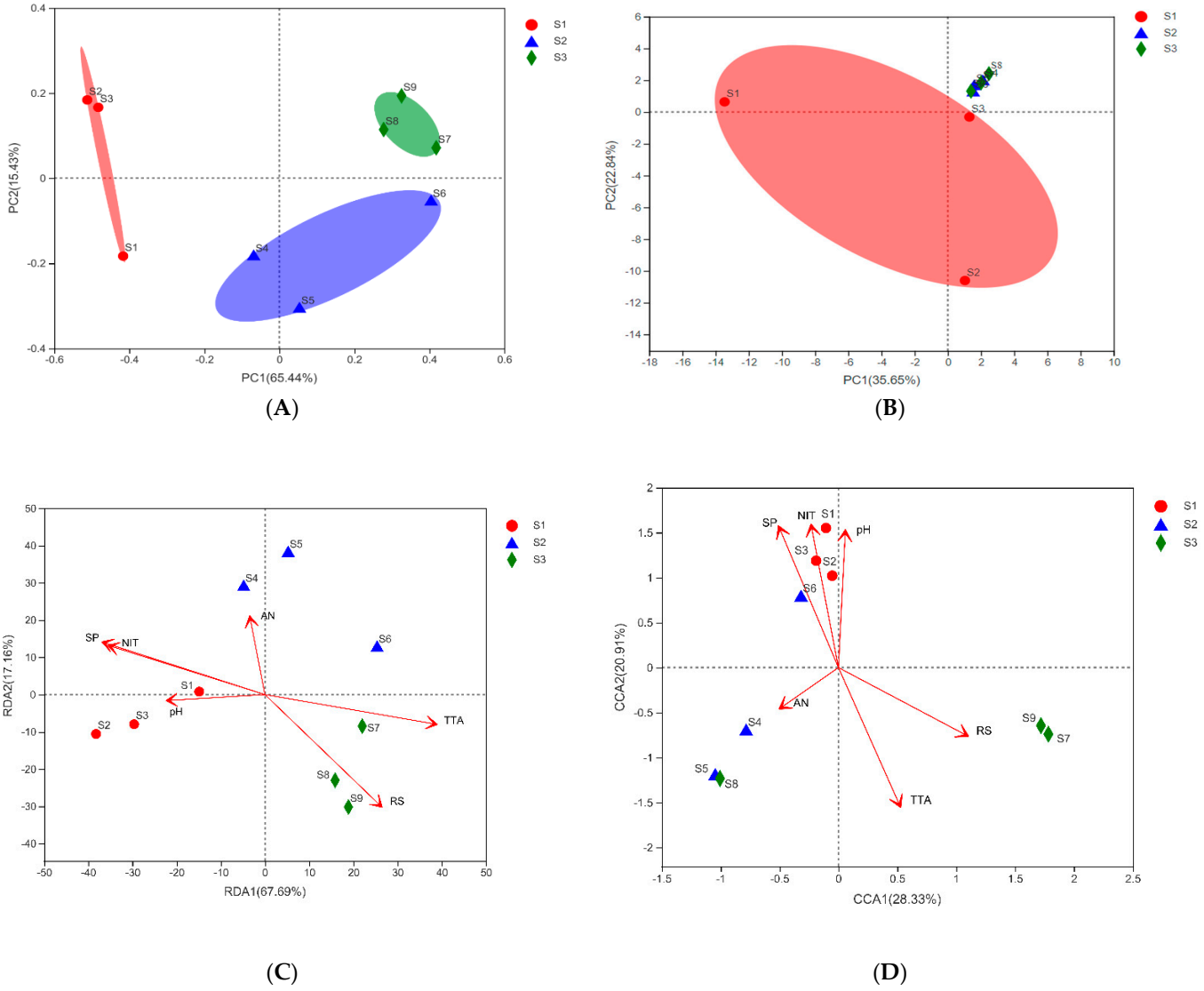
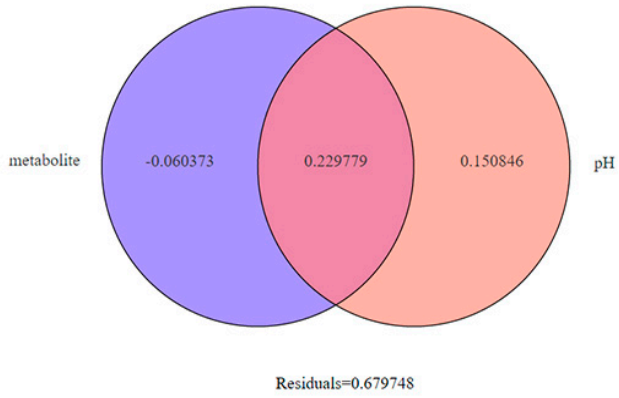


Figure 4. The PCoA of bacteria samples (**A**); the PCA of fungi (**B**) at different fermentation stages. RDA between physicochemical factors and bacteria (**C**); CCA between physicochemical factors and fungi (**D**) in paocai fermentation.

The CCA analysis (Canonical Correlation Analysis) was used to explore the relationship between physicochemical factors and fungal communities in paocai (Figure 4D). PC1 and PC2 explained 28.33% and 20.91% of the microbial community variation. The results of the CCA analysis showed that the effect of physicochemical factors on the fungal community structure was different from that of bacteria. The fungal community structure in the early fermentation stage was positively correlated with amino acid nitrogen (AN), meanwhile, it was positively correlated with pH, nitrite (NIT), and the soluble protein (SP) in the middle fermentation stage, and was positively correlated with titratable acid (TTA) and reducing sugar (RS) in the late fermentation stage. The physicochemical factors (nutrients) in paocai determined the differences in the fungal community structure.

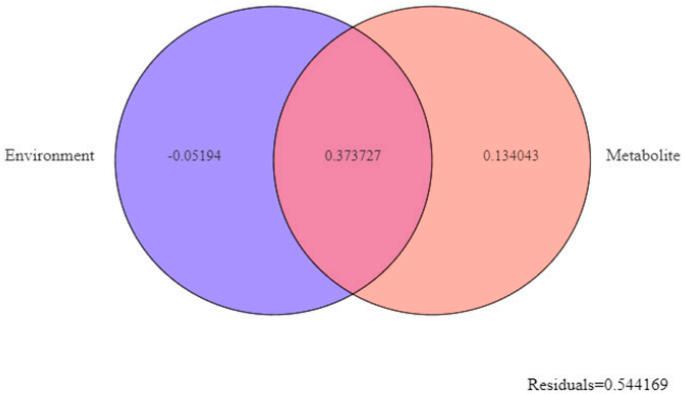
VPA (Variance Partitioning Analysis) of physicochemical factors, the purpose of this analysis was to determine the proportion of specific environmental factors that explained changes in community structure [14].VPA of bacteria is shown in Figure 5A, the physic-

ochemical factors were divided into two groups: pH represented environmental factors, and metabolites represented metabolites including the titratable acids, nitrites, soluble proteins, reducing sugars, and amino acid nitrogen. Among them, pH can explain that the distribution of the community structure of the whole bacteria was 15.06%, the interpretation degree of the interaction between pH and metabolites was 22.98%, and the total interpretation degree was 32.02%. The results showed that pH and metabolites had an important impact on the bacterial community structure in naturally fermented paocai. The adjR^2 of pH, TTA, NIT, RS, and SP was greater than 0, indicating that the interpretation degree of metabolites on bacterial community structure was greater than that of randomly generated normal distribution. The explanatory variables, in general, the environmental factors pH and metabolites were the main drivers of bacterial community diversity during the fermentation of paocai.



Env	Envgroup	AdjR²
pH	pH	0.3806
TTA	metabolite	0.3666
NIT	metabolite	0.372
RS	metabolite	0.0955
AN	metabolite	-0.1107
SP	metabolite	0.3603

(**A**)



Env	Envgroup	AdjR^2
pH	Environment	-0.0198
TTA	Environment	0.0549
NIT	Metabolite	0.0085
RS	Metabolite	0.161
AN	Metabolite	-0.0034
SP	Metabolite	0.0563

(**B**)

Figure 5. Variance partitioning analysis of environmental factors and metabolites explaining bacterial (**A**) and fungal (**B**) community diversity distribution pattern during paocai fermentation.

VPA of fungi is shown in Figure 5B. The physical and chemical factors were divided into two groups: pH, as titratable acid represented environmental factors; while metabolites represented metabolites including nitrite, soluble protein, reducing sugar, amino acid nitrogen, where individual metabolites explain the distribution of the community structure of the whole fungus by 13.40%, the interpretation degree of interaction between environmental factors and metabolites accounted for 37.37%, and the total interpretation degree accounted for 45.58%. This shows that metabolites had an important impact on the community structure of fungi in naturally fermented paocai. The adjR^2 of TTA, NIT, RS, and SP were greater than 0, indicating that the explanatory degree of metabolites on fungal community structure was greater than that of randomly generated normal distribution

explanatory variables. In conclusion, metabolites were the main driving force for changes in the diversity of paocai fungal communities.

3.5. Correlation of Bacterial and Fungal Communities in Fermented Paocai

The correlations between physicochemical factors and species are shown in Figure 6A,B. TTA was significantly positively correlated with *Lactobacillus* and *Stenotrophomonas*, significantly negatively correlated with *Pseudomonas* and *Sacchairbacillus*, and extremely significantly negatively correlated with *Janthinobacterium*. The correlation of NIT between species was just opposite to that of TTA. It was worth noting that the synthesis of AN had a significant positive correlation with *Leuconostoc* and *Shewanella*, the soluble protein had a significant negative correlation with *Lactobacillus* and *Stenotrophomonas* and a significant positive correlation with *Pseudomonas*, *Janthinobacterium*, and *Saccharibacillus*. It also further demonstrated that *Lactobacillus* had a positive correlation with the acidic environment of the paocai fermentation system, and this acidic environment can inhibit the growth and metabolism of some harmful bacteria, such as *Pseudomons* and *Sacchairbacillus*. The *Pseudomonas* were positively correlated with the production of nitrite, which also explained that the nitrate content reached the highest in the early stage of fermentation, the content of *Pseudomonas* decreased sharply and the nitrite content gradually decreased in the later stage of fermentation.

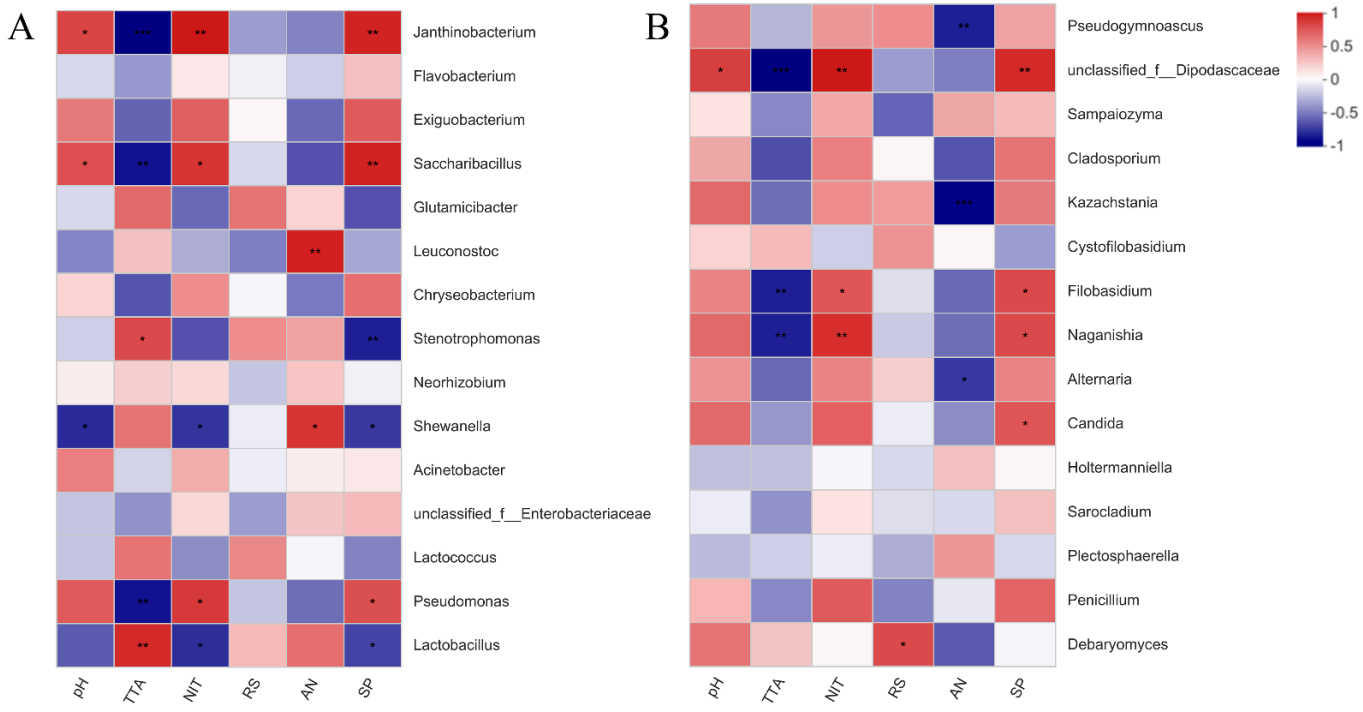


Figure 6. Microbe−environmental factors correlation heatmap showing associations between bacterial and the environmental factors (**A**), fungi and the environmental factors (**B**) based on the Person index. (Red denotes negative correlation, blue denotes positive correlation. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$).

Correlation analysis between environmental factors and the top 15 species was carried out, and the results are shown in Figure 6B. Titratable acid (TTA) was significantly negatively correlated with *Naganishia*, *Filobasidium*, and *Dipodascaceae*, nitrite (NIT) was significantly positively correlated with *Naganishia*, *Filobasidium*, and *Dipodascaceae*, and the amino acid nitrogen (AN) was significantly negatively correlated with *Kazachstania* and *Pseudogymnoascus*. The soluble protein (SP) was significantly positively correlated with *Dipodascaceae*, *Naganishia*, and *Filobasidium*, *Candida*.

3.6. Prediction and Analysis of Bacterial and Fungal Community Functions in Fermented Paocai

To better understand the important role of the microbiota present in samples, the PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) program was used to predict our 16S rRNA based high−throughput sequencing data and further analyze the data in the context of the Cluster of Orthologous Groups (COG) database. Using these methods, we obtained a microbial COG profile and the microbial functional features found in the paocai samples (Figure 7). According to analysis of the relative abundance of the COG, samples in different stages had very similar functional properties, which indicated that the functional composition of microorganisms in fermented pickles was more conservative than the classification composition, indicating that the function composition of the microbial fermentation paocai more conservative than the classification. Amino acid transport and metabolism, energy production and conversion, carbohydrate transport and metabolism, cell motility, inorganic ion transport and metabolism were the most abundant functional attributes in paocai fermentation process, highlighting the importance of these functions for microorganisms to adapt to the ecological niche of the paocai fermentation system.

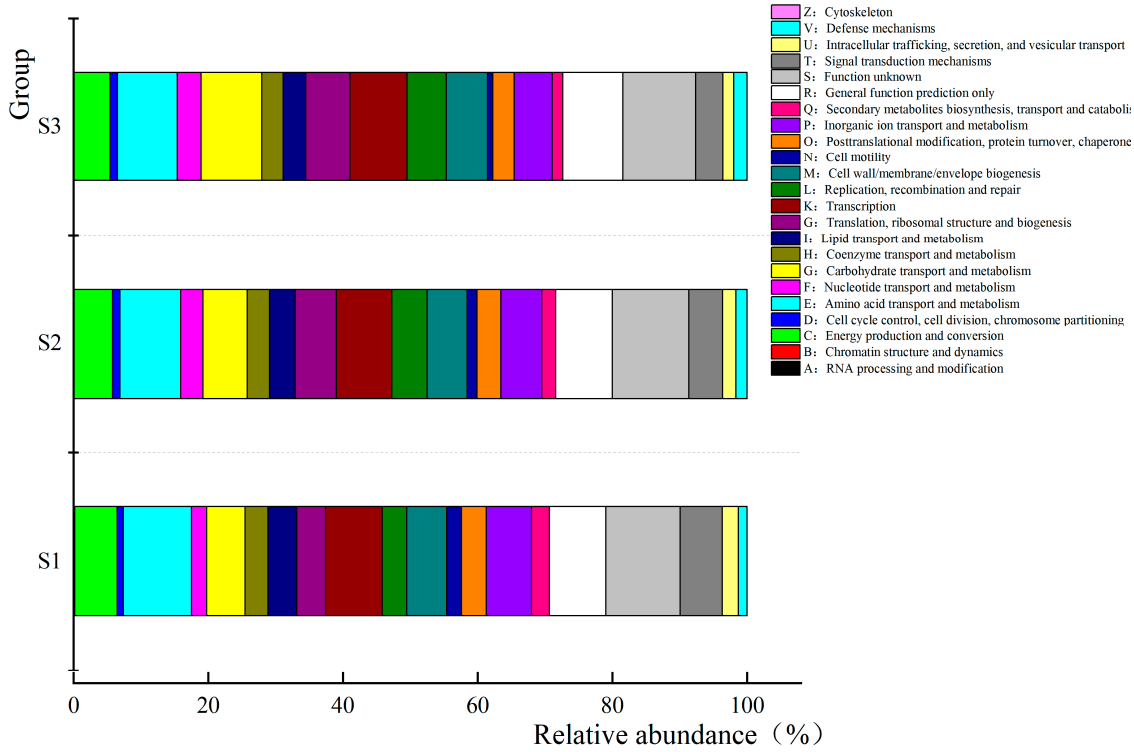


Figure 7. Analysis of bacterial COG functional enrichment.

Since lactic acid bacteria mainly participate in the metabolism of glycolysis, the differences in glycolysis metabolic pathways in the three fermentation stages were further analyzed, and the results are shown in Figure 8A. The modules M0001, M0002, and M0003 involved in glycolysis had significant differences in S1 and S3 fermentation stages ($p < 0.05$). Glucose or glycogen in cells was broken down by glucolysis reaction into pyruvate, pyruvate was broken down by different enzymes into alcohol and carbon dioxide, or into lactic acid. The results of this study revealed that the main types of paocai fermentation were ethanol fermentation and homotype lactic acid fermentation.

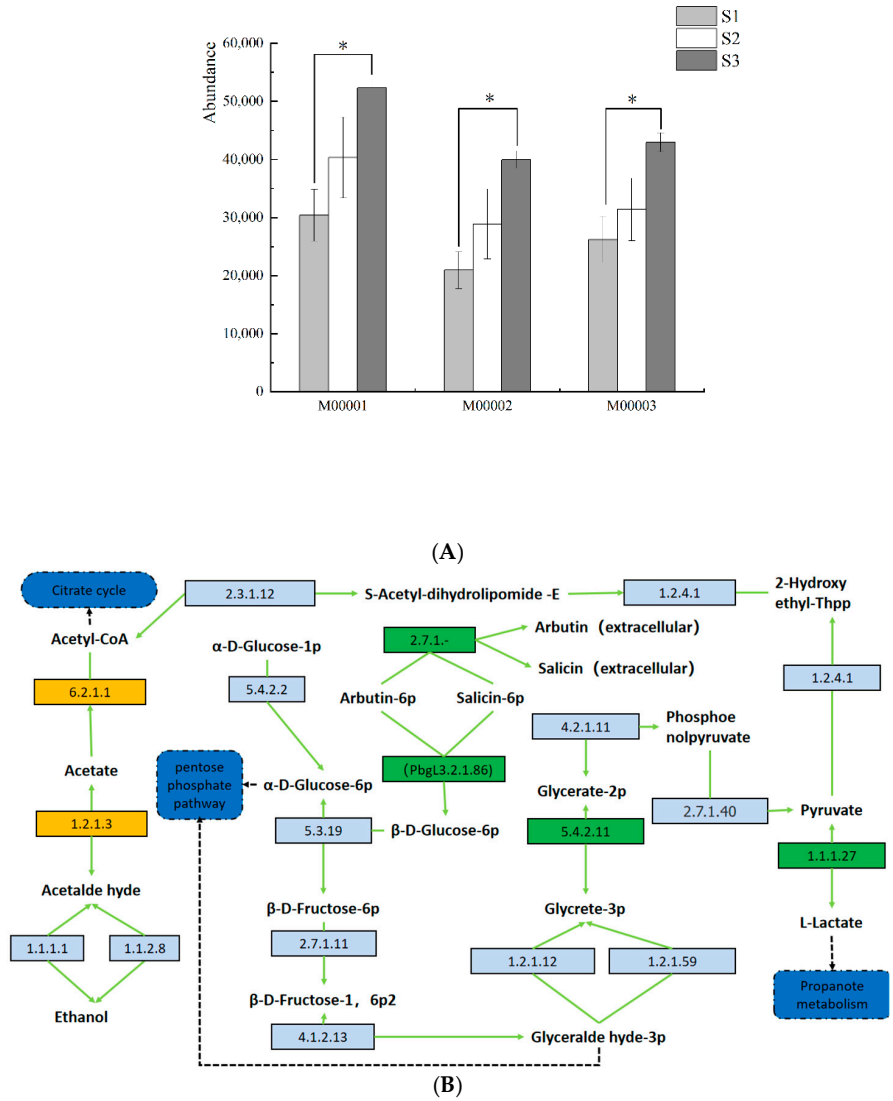


Figure 8. Comparison of glycolysis metabolism at different stages of paocai fermentation (**A**) and metabolic pathway diagram (**B**); (where M0001 is Glycolysis (Embden-Meyerhof Pathway), glucose => Pyruvate, M0002 is Glycolysis, Core Module involving three−Carbon Compound, M0003Gluconeogenesis, Oxaloacetate => Fructose 6P. Green indicates that the enzyme abundance in S3 stage is higher than that in S1 stage, and yellow indicates that the enzyme abundance in the S1 stage is higher than that in S3 stage, * $p < 0.05$).

The process of glycolysis is shown in Figure 8B. Starting from glycogen, it went through the activation stage, oxidation capacity stage, and homotype lactic acid fermentation. In S1, S2, and S3, there were significant differences in L−lactate dehydrogenase (1.1.1.27), β −glucoside (3.2.1.86), phosphoglycerate mutase (5.4.2.11), acetocoA ligase (6.2.1.1), aldehyde dehydrogenase (NAD^+) (1.2.1.3), β−glucoside (3.2.1.86), phosphoglycerate mutase (5.4.2.11), aldehyde dehydrogenase (NAD^+) (1.2.1.3). It was found that L−lactate dehydrogenase mainly exists in all 22 species of the *Lactococcus* genus, *Streptococcus*, *Lactobacillus*, *Weissella*, *Enterococcus*, and *Leuconostoc*. β−glucosidase mainly exists in the late *Lactobacillus* and *Lactococcus*, phosphoglycerate mutase (5.4.2.11) existed in *Escherichia*, *Enterobacter*, *Lelliottia*, *Erwinia*, *Staphylococcus*, *Exiguobacterium*, *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Weissella* and *Enterococcus* also explained that the abundance of these three enzymes in the late fermentation stage was higher than S1 and S2, while acetic−CoA ligase (6.2.1.1) mainly existed in *Enterobacter*, *salmonella*, *Lelliottia*, *Erwinia*, *Xanthomonas*, *Stenotrophomonas*, *Pseudoxanthomonas*, *Acinetobacter Shewanella*, *Pseu-*

doalteromonas, *Alteromonas*, *Janthinobacterium*, *Acetobacter*, *Bacillus*, *Streptococcus*, *Lelliottia*, *Erwinia*, *Bacillus*, Aldehyde dehydrogenase (NAD^+) (1.2.1.3) mainly existed in *Enterobacter*, *Proteus*, *Xenorhabdus*, *Actinobacillus*, *Xanthomonas*, *Stenotrophomonas*, *Pseudoxanthomonas*, *Pseudomonas*, *Alteromonas*, *Legionella*, *Halomonas*, *Burkholderia*, etc., and these two enzymes do not exist in lactic acid bacteria, which also explained that the abundance of these two enzymes in early fermentation was higher than that in middle and late fermentation. That is to say, the alcohol−generation stage mainly occurred in the early fermentation stage, while the homotype lactic acid fermentation was mainly in the late fermentation stage, which was the same as the results of previous studies, and increased the explanation of the mechanism. Combined with the previous correlation analysis and RDA analysis, at the early stage of fermentation, the abundance of lactic acid bacteria was little, and the production of acid in the fermentation environment was low. In the middle and late stages of fermentation, lactic acid bacteria metabolized in an anaerobic environment to produce a large amount of L−lactic acid, which increased TTA content and reduces pH. After lactose was converted into lactic acid, biochemical changes such as increased protein digestibility, the release of fatty acids, and production of bioactive compounds occurred during fermentation, which improved the nutrition and quality of fermented products.

Another finding was that M00615 Nitrate assimilation was significantly higher in the S1 fermentation stage than in S2 ($p = 0.014$), and extremely significantly higher than in the S3 stage ($p = 0.005$). However, M00531Assimilatory nitrate reduction showed no significant difference in different stages, as shown in Figure 9. Nitrate (NO^{3-} was the main form of nitrogen absorbed by most microorganisms. After ingesting nitrate, it needed to reduce nitrate to ammonia, and then participated in the synthesis of related nitrogen−containing organic compounds in the body. The process was called nitrate reduction. The reduction of nitrate can be divided into two major steps: reduction of nitrate to nitrite and reduction of nitrite to ammonia. In the study of the nitrogen metabolism pathway of Ko00910, the main metabolic pathways are shown in Figure 9C. There were four main pathways in the process of reduction to ammonia: (1) reduction of formamide to ammonia by formamidase, (2) nitrile to ammonia by nitrilase, (3) nitrite by nitrite reductase, (NADH) and the iron redox protein nitrite reductase produces ammonia (4); nitrite was reduced to nitric oxide by nitrite reductase, and then reduced to nitrous oxide by nitric oxide reductase (cytochrome *c*), and then by nitrous oxide reductase was reduced to nitrogen, and finally, ammonia was generated by nitrogenase. Among the 4 pathways, (3) was the main pathway, and the abundance of nitrite reductase (NADH) was the highest. Among them, nitrite reductase (1.7.2.1) was widely present in most *Pseudomonas*, such as *Pseudomonas aeruginosa*, *Pseudomonas protegens*, *Shewanella amazonensis Shewanella loihica*, *Idiomarina*, *Nitrosococcus*, *Hahella*, *Neisseria*, *Burkholderia*, *Sinorhizobium*, *Brucella*, *Corynebacterium*. Nitric oxide reductase (cytochrome *c*) (1.7.2.5) was mainly present in *Actinobacillus Actinobacillus succinogenes*, *Pseudoxanthomonas*, most of *Pseudomonas*, *Shewanella*, *Legionella*, *Methylococcus*, *Methylomonas*, *Nitrosococcus*, *Neisseria*, Nitrilase (3.5.5.1) was mainly present in *Pseudomonas veronii*, *Methylomonas*, *Paraburkholderia*, *Achromobacter*, *Campylobacter*, *Zymomonas*, but not in *Pseudomonas aeruginosa*.

In association with the previous correlation analysis and RDA, these enzymes related to nitrate reduction were mainly present in the Proteobacteria, which was mainly present in the early stages of paocai fermentation, also indicated that nitrite content was high in the early stages, while nitrite content was negatively correlated with the late stages of fermentation. It has been shown that pH directly affected the abundance and nitrification rate of bacteria [15], indicating that the growth of the major genus *Lactobacillus* and the acidic environment in the later stages inhibited the growth of microorganisms such as *Pseudomonas*, which reduced nitrite enzyme activity, thus inhibiting nitrite metabolism and reducing nitrite content.

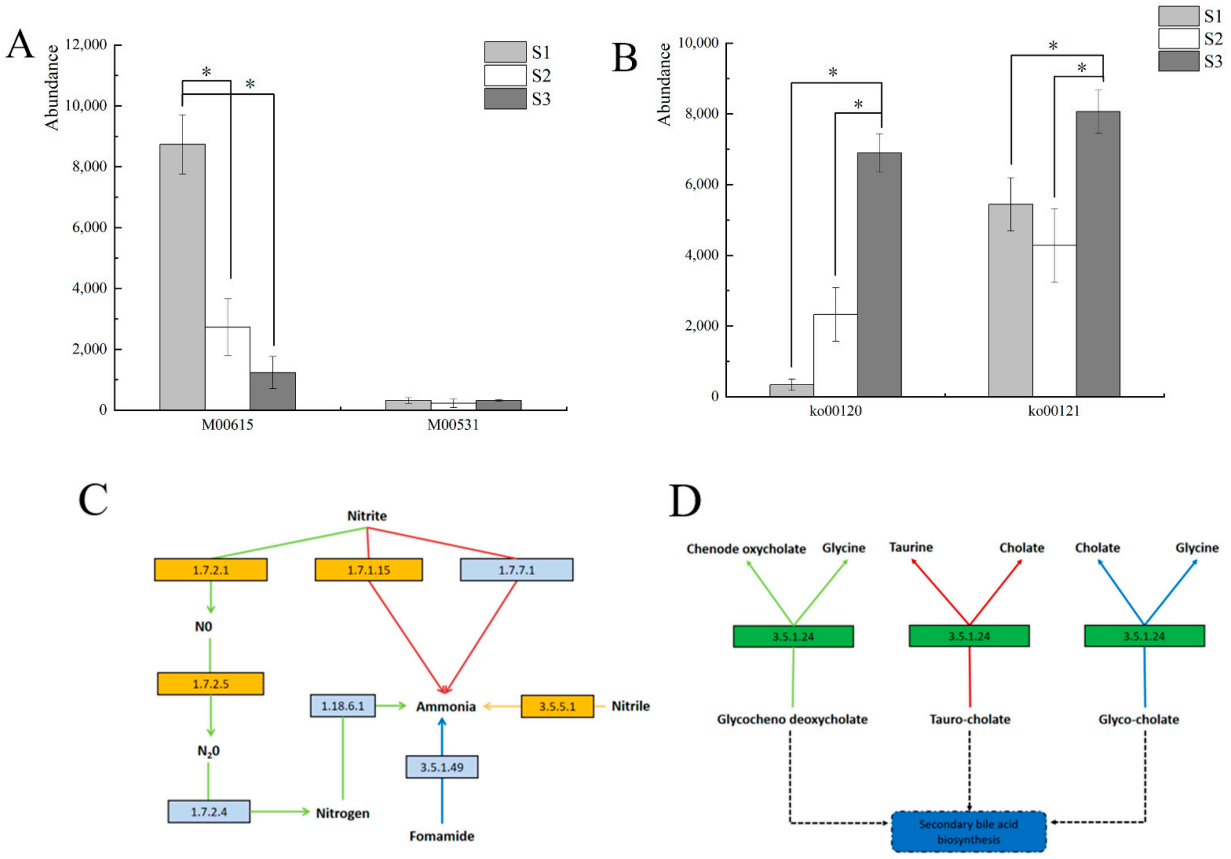


Figure 9. Comparison of nitrite reduction at different fermentation Stages (**A**) and metabolic pathway map (**C**), comparison of primary bile acid bioanabolism at different fermentation stages (**B**) and metabolic pathway map (**D**) (M00615 nitric acid assimilation, Nitrate assimilation Nitrate reduction, M00531 Nitrate reduction Assimilate, Ko00120 Primary bile acid biosynthesis, Ko00121 Secondary bile acid biosynthesis Green indicates that the enzyme abundance in S3 stage is higher than S1 stage, and yellow indicates that the enzyme abundance in S1 stage is higher than S3 stage, * $p < 0.05$).

Another interesting finding was that the abundance of metabolic pathways (Figure 9B), primary bile acid biosynthesis (Ko00120), and secondary bile acid biosynthesis (Ko00121) in phase S3, was higher than phases S1 and S2, and the abundance of choloylglycine hydrolase (3.5.1.24) was also higher in phase S1 than in phases S2 and S3, (1012, 324, 44). Bile salt hydrolase (BSH) mediates the conversion of conjugated bile acid (CBA) to unconjugated bile acid (UCBA) and glycine or taurine, which is a key step in the transformation of primary bile acids to secondary bile acids mediated by intestinal microorganisms—[16]. The metabolic pathway of bile acids in paocai fermentation was mainly bile acyl glycine hydrolase involved in primary bile acid biosynthesis as shown in the Figure 9D, with three main pathways: (1) glycco−cholate hydrolyzed by bile acyl glycine hydrolase to hydroxycholate and glycine; (2) tauro−cholate hydrolyzed by bile acyl glycine hydrolase to taurine and cholate; (3) glycocheno deoxycholate hydrolyzed by bile acyl glycine hydrolase to chenode oxycholate and glycine. It will be involved in the metabolism of secondary bile acids. The bile acyl glycine hydrolase (3.5.1.24) was mainly present in *Lactiplantibacillus* (*Lpb.*) *plantarum* WCFS1, JDM1, ZJ316, etc., and *Enterococcus*, while the expressing microorganisms mainly belong to the Firmicutes, which also indirectly indicated that *Lpb. plantarum* had good bile salt hydrolase activity.

3.7. Ecological Characteristics and Functional Analysis of Fungi in Naturally Fermented Paocai

The ecological function analysis of the fungi showed that 112 OTU mainly consisted of three nutrient types, symbiotroph, saprotroph, and pathotroph, as shown in Figure 10,

and plant pathogens, endophytes, animal pathogens, fungi, leaf saprotroph, undefined saprotroph, and unknown groups. OTU111 was the *Debaryomyces* of *Ascomycota Saccharomycetes*. The main function of OTU111 was undefined saprophytic bacteria, with a relative content of 47.52%, 70.05%, and 79.02% in the early, middle, and late fermentation stages. *Plectosphaerella* was the main function of plant pathogens in the three fermentation stages of the relative content of 2.47%, 29.82%, and 18.08%. However, the colocation group of 25.37% unknown in the S1 stage also indicated the complex function of the fungal community in the early fermentation stage.

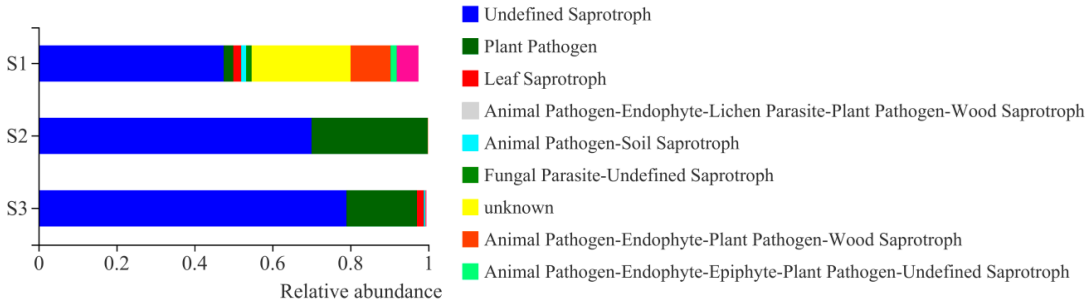


Figure 10. Ecological characteristics of fungi in naturally fermented paocai.

One−way ANOVA was performed on the the main metabolic pathways (Figure 11A) and enzymes (Figure 11B) engaged in fermentation metabolism were PWY−7279, PWY−3781, Glyoxylate−bypass, PWY−5994, PWY−7288, denoting aerobic respiration II (cytochrome *c*) (yeast), aerobic respiration I (cytochrome *c*), glyoxylate cycle, palmitate biosynthesis I (animals and fungi), and fatty acid and beta−oxidation (peroxisome, yeast). The glyoxylate cycle was significantly different ($p < 0.05$) in the early and late stages of fermentation, and the five enzymes with the highest content were 3.6.1.3 Adenosinetriphosphatase, 5.3.1.4 L−arabinose isomerase, 2.7.7.6 DNA−directed RNA polymerase, 2.7.7.7 DNA−directed DNA polymerase, 3.2.1.18 Exo−alpha−sialidase, but no significant differences during fermentation (Figure 11).

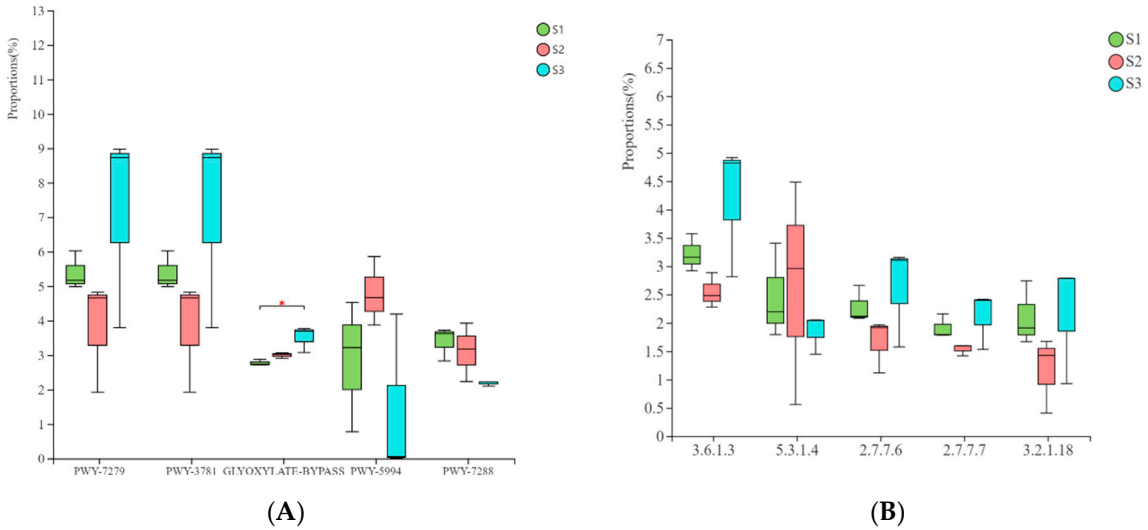


Figure 11. One−way ANOVA of metabolic pathway (**A**) and enzymes (**B**) in naturally fermented paocai, * $p < 0.05$.

The glyoxylate cycle was a reaction process closely related to the conversion of fat to sugar [17]. The microorganisms involved in the glyoxylate cycle were generally aerobic, and among the fungi they were mainly *Saccharomyces*, *Penicillium*, and *Aspergillus niger* [18]. Isocitrate lyase and malate synthase were the key enzymes involved in the glyoxylate

cycle; in the present study, isocitrate lyase were present in the pre−mid and late stages of fermentation, and the glyoxylate cycle achieved the conversion of fat to sugar. This further explains the elevation of reducing sugars in the later stages of fermentation and the high value of paocai with less fat and fewer calories.

4. Discussion

In China, paocai is processed in various ways and its colony structure varies, but the dominant microorganisms causing fermentation are lactic acid bacteria. The differences in the community structure of lactic acid bacteria lead to differences in the growth and development patterns, metabolism, and metabolic products of microorganisms during the fermentation of paocai, which endow the paocai with different styles and tastes, and achieve a variety of paocai in China. At present, rational control of the paocai fermentation process, improving the rate of paocai fermentation, ensuring the fermentation quality and safety of paocai, increasing the scientificity of the paocai fermentation industry and the rationality of the fermentation process are important topics in current vegetable fermentation−related research.

High−throughput sequencing results showed that different bacterial flora existed at the beginning of paocai fermentation, but lactic acid bacteria (*Lpb. plantarum*, *Latilactobacillus* (*Lat.*) *curvatus*) gradually became representative in the paocai samples as the fermentation process progressed. *Lpb. plantarum* increased substantially by the late stage of fermentation, indicating that *Lpb. Plantarum* played a dominant role in the late stage of the fermentation process. *Lactococcus* spp. were also found, and they were widely present in traditional fermented foods in China [19]. *Lactobacillus spp.* played an important role in the fermentation process, and *Lpb. plantarum* had been screened as a fermenting agent, and the metabolism of *Lactobacillus*, *Leuconostoc* contributes to flavor formation closely related to the flavor of paocai [20], while *Lactococcus*, *Enterobacter* is also a common bacterial species in fermented vegetables. Studies had shown that *Lactobacillus*, *Lactococcus*, and *Enterobacter* were associated with amino acids in fermented paocai. Amino acids are produced during food fermentation by primary protein hydrolysis of raw materials by *Lactobacillus* proteases and are the main contributors to the fermentation flavor in paocai. *Enterobacteriaceae* are associated with acetic acid in paocai and *Streptococcaceae* with olefins in paocai [21]. In addition, some *Enterobacteriaceae* bacteria may not be associated with foodborne pathogens, as many of them are symbiotic organisms of plants and humans. *Pseudomonas* has long been detected in paocai fermentation and is considered to be one of the major groups of bacteria during early fermentation [22], which is consistent with the findings of the present experiment. *Pseudomonas* spp. can adapt to a wide range of survival environments, and in the later stages of fermentation, the content of *Pseudomonas* gradually decreased due to the anaerobic acidic environment. Some *Pseudomonas* spp. have been reported to be powerful producers of ketones and also produced esters, hydrocarbons, alcohols, and sulfides that may contribute to flavor formation during industrial paocai fermentation [23]. *Shewanella* was previously detected for the first time in Kaifir pellets by high−throughput sequencing technology [24]. It has been suggested that some low−abundance bacteria are important in the formation of unique flavors of paocai.

Research on the correlation between species, RDA analysis of physicochemical factors and bacterial community structure in the fermentation stage of paocai, and the discovery of the main glycolysis and nitrogen metabolism pathways that occurred during the fermentation of paocai, because proteobacteria such as *Pseudomonas* as the main dominant genera of nitrite reductase enzyme activity were relatively high, leading to high nitrite content in the prophase, but by the end of fermentation, lactic acid bacteria through the glycolytic pathway to L–lactic acid content increased rapidly, making the paocai fermentation environment become acidic; TTA content further increased and pH reduced gradually. With the increase of protein digestion rate, the protein content decreased, and amino acid metabolism mainly occurred in the middle of fermentation.Therefore, there was a very significant negative correlation between *Pseudomonas* and *Lactobacillus*, the titratable acid

was significantly positively correlated with *Lactobacillus* and significantly negatively correlated with *Pseudomonas*, while the correlation between nitrite and species was the direct opposite to that of titratable acid. The soluble protein was significantly negatively correlated with *Lactobacillus* and *Stenotrophomonas*, positively correlated with *Pseudomonas* and *Janthinobacterium*. The amino acid nitrogen synthesis was significantly positively correlated with *Leuconostoc* and *Shewanella*.

Recently, an increasing number of studies have revealed that BSH (bile salt hydrolase) has an important regulatory role on intestinal microbes [25]. On the one hand, BSH contributes to microbial colonization and adhesion in the gut, and on the other hand, BSH influences the host's response to lipid digestion and absorption, cholesterol metabolism, energy metabolism, and inflammation by regulating bile acid metabolism [26]. *Lpb. plantarum* and *Listeria* express multiple BSH genes, and *Lpb. plantarum* BSH tends to hydrolyze glycosamine−conjugated deoxycholic acid, which is consistent with previous findings [27]. Lactic acid bacteria have a tolerance to bile salts [28], and most bacteria are not tolerant to bile salts, which further explains the reason why the number of lactic acid bacteria increased and the number of proteobacteria decreased in the later period. The presence of active BSH enzymes has been considered an important criterion for the selection of bacterial probiotic potential, and the optimal conditions for BSHs to hydrolyze bile salts usually occur at an acidic pH between 4.5 and 6.5 [29]. As a multifunctional signaling molecule, bile acid regulates not only its synthesis and circulation but also triglyceride, cholesterol, glucose, and energy homeostasis by regulating various signaling processes [30]. It also indicates that paocai has potential application value.

Fungi was an important group of microorganisms in the paocai fermentation process, including *Saccharomycetes*, *Aspergillus*, and *Penicillium*, which not only played a role in the fermentation process such as saccharification and liquefaction but are also related to the flavor and quality of paocai [31]. For example, *Saccharomycetes* not only produce volatile flavor substances such as alcohols and esters but also produce the precursor substance of tetramethylpyrazine−ethyl coupling. It is evident that fungi play an important role in the fermentation process [32]. During the fermentation of paocai, with the growth of acid−producing bacteria, the total acid content in paocai rose, so that the relative abundance of acid−intolerant *Bacillus* decreased, the proteins were mainly decomposed by protease−producing fungi in the later stages of fermentation, and the relative abundance decreased in the later stages due to the pressure of the acidic environment that makes it difficult for some fungi to survive. While the correlation between reducing sugars and fungal communities decreased, indicating that there were differences in the influencing factors of fungal and bacterial communities. The correlation between different fungi and physical and chemical factors was quite different, because of the interaction of various fungi, physicochemical factors and fungi interacted with each other. By controlling the fermentation environment, fungal community composition can be affected to improve the flavor of paocai.

In this study, it was found that physical and chemical factors had a certain influence on the succession of the fungal community of paocai, and more physical and chemical factors related to fungal growth, such as oxygen concentration and enzyme activity, could be introduced in the follow−up, which might be helpful to explore the changes of the fungus community of paocai. It has been reported that *Aspergillus* successfully inhibited tumor progression in mice in vivo [33], indicating that some fungi can produce some metabolite−antagonizing tumors, which may provide methods for tumor treatment or have protective effects on organisms, and may reveal and inhibit tumor occurrence and development.

Uncovering the correlation between the species of microorganisms and their role in a particular ecosystem remains one of the main goals of microbial ecology. This study provided profound insights into the microorganisms and contributed to the study of fermentation production of paocai. In the next step, the role of the dominant species of lactic acid bacteria and its influence on the flavor and sensory experience of fermented

vegetables should be discussed, and the influence of the composition of microorganism on the fermentation of paocai should be further studied, as well as the relationship between microbial community, metabolites, and sensory characteristics. In addition, the potentially pathogenic bacteria should be controlled in the fermentation process. This study provided useful guidelines for the standardization and industrialization of high−quality pickled cabbage production.

5. Conclusions

In this study, the changes of community structure and driving mechanism of naturally fermented paocai at different stages were studied. The results showed that *Pseudomonas* was the dominant bacteria in the early fermentation stage, *Lactobacillus* was the dominant bacteria in the late fermentation stage, and Ascomycota and Basidiomycota were the main fungi in the fermentation process. Through the correlation and function prediction analysis of physicochemical factors and community structure, it was confirmed that pH and metabolites were the main driving forces of community structure change. The nitrite content was high in the early stage of fermentation, lactic acid bacteria produced lactic acid through glycolysis, which made the fermentation environment become acidic and inhibited the growth of harmful bacteria. Glyoxalic acid cycle metabolism mainly existed in the late fermentation stage, which realized the transformation from fat to sugar.

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