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Exploration of Microbial Community Diversity and Bioactive Substances during Fermentation of Mulberry Jiaosu, an Edible Naturally Fermented Mulberry Product

Jing Zhang [1,†], Minhui Zhao [2,3,†], Yuan Yi [3,†], Yifen Huang [2], Qianqian Yin [2] and Yong Zuo [2,3,*]

[1] College of Food and Biological Engineering, Chengdu University, Chengdu 610106, China; zhangjing2022@cdu.edu.cn
[2] College of Life Science, Sichuan Normal University, Chengdu 610101, China; 18583204060@163.com (M.Z.); 20211501042@stu.sicnu.edu.cn (Y.H.); 18095019301@163.com (Q.Y.)
[3] College of Bioengineering, Sichuan University of Science and Engineering, Yibin 644000, China; yiyuan0602@163.com
* Correspondence: sgzuoyong@tom.com; Tel./Fax: +86-28-84480650
† These authors contributed equally to this work.

Abstract: Mulberry Jiaosu, derived from natural fermentation using fresh mulberry fruit as a raw material, refers to an edible product containing specific bioactive substances. However, the dynamic changes in the bioactive substances of organic acids, amino acids and polyphenols as well as the species and function of microorganisms in mulberry Jiaosu are still not clear. Herein, the whole fermentation process of mulberry Jiaosu was comprehensively researched by analyzing the microbial community structure and bioactive substances. The results showed that the change in physicochemical parameters mainly happened within 30 days of fermentation. The total organic acids and total polyphenols presented upward trends. Total amino acids were partly consumed during the fermentation. A total of 173 fungal genera and 295 bacterial genera were detected in mulberry Jiaosu, mainly including *Torulaspora*, *Zygosaccharomyces* and *Lactobacillus*, whose abundance can be influenced by changes in the fermentation environment. During the fermentation of mulberry Jiaosu, 8 organic acids, 17 amino acids and 9 polyphenols were observed, which could be regulated by the metabolism of microorganisms. *Zygosaccharomyces* exhibited positive correlations with the majority of the organic acids, amino acids and polyphenols, presenting a great influence on the formation of bioactive substances. Compared with fungi, bacteria contributed more to the synthesis of organic acids, free amino acids and polyphenols. This study revealed the bioactive substances and microbial diversity during the fermentation of mulberry Jiaosu, which are findings that will contribute to the precise regulation of the fermentation process and improvement of the product quality.

Keywords: mulberry Jiaosu; microbial community diversity; bioactive substances; high-throughput sequencing; correlation analysis

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1. Introduction

Mulberry, a species of deciduous flora widely distributed in temperate, subtropical and tropical regions, is cultured abundantly in the Yunnan, Shanxi and Sichuan provinces of China. Mulberry fruit is rich in nutrients, including vitamins, proteins, amino acids, polysaccharides, polyphenols and anthocyanins [1], possessing anti-aging powers as well as being able to reduce the risk of both cancer and cardiovascular disease [2–4]. Due to the fruit being particularly susceptible to spoilage and difficult to transport and store, mulberries are commonly processed into Jiaosu, juice and jam [5]. Mulberry Jiaosu, which is naturally fermented from mulberry fruit in barrels or sealed jars for approximately two months up to several years and is full of specific bioactive substances, not only preserves the nutrients of the mulberry fruit but also provides probiotics [6]. The technological process

of production mainly entails raw material pretreatment, composition adjustment, main fermentation and post-treatment. During the fermentation of mulberry Jiaosu, bioactive substances gradually accumulate, which is a phenomenon that is mainly influenced by microorganisms and fermentation substrate.

The bioactive substances in organic acids, amino acids and polyphenols largely persist in mulberry Jiaosu [7,8]. Organic acids not only impart a unique flavor and taste but also inhibit the growth of spoilage microorganisms and pathogenic bacteria [9,10]. Amino acids are important nutrients which may improve the quality of mulberry Jiaosu [11]. Polyphenols are common bioactive substances in fermented foods, holding anti-cancer, anti-inflammatory, anti-oxidation and anti-atherosclerosis powers related to the antioxidant activity of products [12]. These bioactive substances synergistically contribute to the nutritional value of fermented foods. Microorganisms play critical roles in the formation of bioactive substances and contribute to the nutritional value of mulberry Jiaosu. However, the preparation of mulberry Jiaosu mainly relies on natural fermentation, which is a mixed leaven system. The fermentation of mulberry Jiaosu is analogous to a "black box", with both the structure and function of microorganisms remaining unclear and preventing the product quality from being assured. Hence, a comprehensive understanding of the microbial community structure as well as its relationship with bioactive substances is needed for the consistent production of Jiaosu with high nutrients.

Currently, research on the microorganisms in mulberry Jiaosu mainly focuses on the separation, purification and identification of strains. Only a fraction of strains has been obtained, owing to the fact that most microorganisms are difficult to culture and owing to the limitations of traditional methods. Hence, the available literature fails to generate a comprehensive picture of the microbial diversity and functions of mulberry Jiaosu. High-throughput sequencing, a next-generation sequencing technology which allows for comprehensive analysis of microbial community structure, has been broadly implemented for the analysis of microbial diversity in fermented foods, including Baijiu [13], doubanjiang [14] and vinegar [15]. High-throughput sequencing, with the advantages of high efficiency, great accuracy and time efficiency, enables the analysis of microbial diversity in mulberry Jiaosu.

In this study, the bioactive substances of organic acids, amino acids and polyphenols during the fermentation of mulberry Jiaosu were determined by HPLC, and the microbial community structure was explored using high-throughput sequencing. The dynamics of the main physicochemical parameters, bioactive substances and microorganisms were comprehensively analyzed. Notably, the interaction of microorganisms and correlations of microorganisms and bioactive substances were also revealed, providing a theoretical basis for in-depth research on the microorganisms' metabolic mechanisms and the development of new products of mulberry Jiaosu.

2. Materials and Methods

2.1. Chemicals

Standard substances of organic acids, amino acids and polyphenols were purchased from Shanghai Yuanye Biotechnology (Shanghai, China). Other analytical pure reagents were obtained from Sinopharm Chemical Reagent (Shanghai, China).

2.2. Sample Preparation and Collection

Fresh mulberry fruit was purchased from Nanchong, Sichuan Province, China. Mulberry Jiaosu was prepared by mimicking traditional techniques using the following procedures: (1) 10 kg fresh mulberries were selected and rinsed with sterile water; (2) the mulberries were crushed with a juicer; (3) the mulberries were transferred into a sterile fermentor and fermented for 80 days at 28 °C.

Mulberry Jiaosu was sampled on days 0, 5, 10, 15, 30 and 80, respectively, and then filtered with four layers of gauze on a clean bench. The solution was centrifuged at

10,000 rpm for 10 min. The supernatant was taken to determine the physicochemical properties, and the precipitation was used for high-throughput sequencing.

2.3. Determination of Physicochemical Properties

pH was measured using a pH meter (Mettler-Toledo, Greifensee, Switzerland). Reducing sugar was detected using the 3,5-dinitrosalicylic acid (DNS) method [16]. Total acids were tested using acid–base titration [17].

2.4. Determination of Organic Acids Content

High-performance liquid chromatography (1260 infinity II, Agilent, Santa Clara, CA, USA) equipped with an Agilent ZORBAX SB-Aq column (4.6 mm × 250 mm, 5 μm, Agilent, USA) was used to analyze organic acids. A standard solution of oxalic acid, tartaric acid, pyruvate, malic acid, lactic acid, acetic acid, citric acid and succinic acid was accurately prepared and applied for the qualitative and quantitative of organic acids in mulberry Jiaosu.

After diluting with the mobile phase, samples were filtered using a 0.22 μm nylon filter membrane.

The mobile phase was composed of 95% KH_2PO_4 (pH = 2.54) (mobile phase A) and 5% methanol (mobile phase B), and the flow rate was 0.4 mL/min. The injection volume was 10 μL. The column temperature was 30 °C. Organic acids were detected at a wavelength of 210 nm.

2.5. Determination of Amino Acids Content

According to the method described by DUAN [18], amino acids in mulberry Jiaosu were determined using pre-column derivatization with a phenyl isothiocyanate derivative.

Sample derivation: First, 250 μL acetonitrile solution of phenyl isothiocyanate and 250 μL triethylamine-acetonitrile solution were added to 500 μL of the diluted Jiaosu sample. After one hour of incubation, 50 μL acetic acid was further added.

Extraction and purification: First, 1 mL n-hexane was added to the derivative solution. After static stratification, the lower solution was filtered with a 0.45 μm filter membrane and collected for the detection of amino acids.

High-performance liquid chromatography (1260 II infinity, Agilent, Santa Clara, CA, USA). Chromatographic column: C18 column (4.6 mm × 250 mm, 5 μm, Agilent, USA). Mobile phase A: 0.02 M sodium acetate (containing 0.05% triethylamine, pH 6.20); mobile phase B: 80% acetonitrile. The flow rate was 1.0 mL/min. The injection volume was 10 μL. The column temperature was 40 °C. The following elution gradients were used: 0.0–11.0 min, 5% B; 11.0–13.9 min, 10% B; 13.9.0–15.0 min, 15% B; 15.0–29.0 min, 20% B; 29.0–30.0 min, 30%B. Amino acids were detected at a wavelength of 254 nm.

2.6. Determination of Polyphenols Content

Sample preparation: First, 10 mL mulberry Jiaosu was added into 10 mL ethyl acetate. After 5 min of extraction, the supernatant was collected. The extracted solution was evaporated at 35 °C and then dissolved in 2 mL methanol solution, which was followed by filtering with a 0.22 μm filter membrane.

Polyphenols standard solution was freshly prepared with methanol, which was further applied for the detection of polyphenols in mulberry Jiaosu.

Polyphenols were determined by high-performance liquid chromatography (1260 II infinity, Agilent, Santa Clara, CA, USA) according to the method of Valero-Cases [19]. Some improvements were made for the binary gradient elution system. The chromatographic column was an Agilent ZORBAX SB-Aq (4.6 mm × 250 mm, 5 μm, Agilent, USA). The mobile phase was composed of 1% formic acid water (phase A) and 100% acetonitrile (phase B), and the flow rate was 1 mL/min. The injection volume was 10 μL. The column temperature was 30 °C. The following elution gradients were used: 0.0–5.0 min, 5% B; 5.0–15.0 min, 15% B; 15.0–20.0 min, 20% B; 20.0–23.0 min, 20% B; 23.0–28.0 min, 30% B;

28.0–31.0 min, 30% B; 31.0–35.0 min, 45% B; 35.0–40.0 min, 55% B; 40.0–43.0 min, 55% B; 43.0–48.0 min, 65% B; 48.0–60.0 min, 5% B. Polyphenols were detected at wavelengths of 280 nm and 310 nm.

2.7. Microbial Analysis of Mulberry Jiaosu

The community structure of bacteria and fungi in mulberry Jiaosu was identified using high-throughput sequencing. For the bacteria, the V3–V4 region was amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTW TCTAA-3′). The ITS1 region was amplified for fungi, and the primers were ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′). The PCR products were pooled in equimolar and paired-end sequenced using an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.8. Data Analysis

All experiments were repeated three times, and the results were expressed as mean ± standard deviations. Analysis of variance (ANOVA) was performed using SPSS 20.0 to evaluate the significance level.

3. Results and Discussions

3.1. Dynamics of Physicochemical Parameters

The main physicochemical parameters during the fermentation of mulberry Jiaosu were measured. As the results show in Figure 1a, the change in reducing sugar, total acids and pH mainly happened in the early stage of the fermentation (0–30 days). Reducing sugar exhibited a continuous downward trend and remained relatively stable after 30 days of the fermentation. Total acids significantly increased to 28.69 g/L on day 30. On the contrary, pH decreased from 4.58 to 3.26. The results of the organic acids, amino acids and polyphenols are exhibited in Figure 1b. The total organic acids dramatically increased in the early stage of the fermentation and reached a peak of 39.91 g/L on day 30. The total polyphenols rapidly increased within 15 days, which may be related to the conversion of the compound phenols into the free form as well as the depolymerization of high-molecular phenols [20]. At the later stage of the fermentation, total polyphenols mildly decreased owing to the non-enzymatic reaction [21], which was similar to the results of Emmanuel [22]. The total amino acids decreased during 0–15 days, which resulted from the consumption and transformation of amino acids [23].

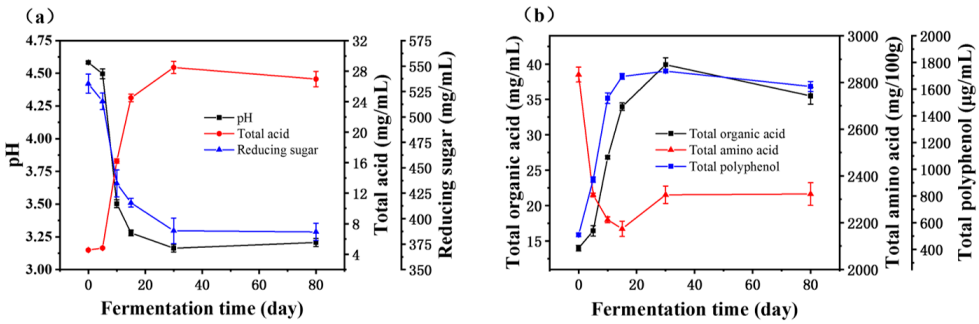

Figure 1. Dynamic changes of physicochemical parameters (**a**) and bioactive substances (**b**) during the fermentation of mulberry Jiaosu. The content of total amino acids was expressed as mg/100 g dry weight. All experiments were repeated three times, and the results were expressed as mean ± standard deviations.

To evaluate the nutrition and function of mulberry Jiaosu, the details of organic acids, amino acids and polyphenols were further determined. In the fermentation process, organic acids were largely produced, which is conducive to maintain the acidity of products and prevent the pollution of harmful microorganisms, ensuring product safety [24]. As the

results show in Table S1, eight kinds of organic acids were detected in mulberry Jiaosu. At the beginning of the fermentation, the contents of tartaric acid, lactic acid, acetic acid, citric acid and succinic acid were relatively high, among which tartaric acid presented the highest concentration. As the fermentation proceeded, lactic acid and acetic acid first increased and then decreased, reaching content values of 11.10 and 7.05 g/L at the end of fermentation, which may be related to the metabolism of *lactic acid bacteria* and *acetic acid bacteria*. The change in citric acid was observed during the fermentation, which is probably because citric acid is involved in the tricarboxylic acid cycle [25]. Tartaric acid, the most common organic acid in mulberry raw materials, experienced a process of first increasing and then decreasing. The increase in tartaric acid may be caused by the dissolution of raw materials and the sharp decrease in pH, while its decrease could be attributed to oxidative degradation or precipitation [26]. At the end of the fermentation, tartaric acid and lactic acid became the main organic acids.

Amino acids are key taste substances and flavor precursors [27] (Wang et al., 2020). As the results show in Table S2, 17 kinds of common amino acids were detected in mulberry Jiaosu. Serine, glycin and tyrosine were main amino acids at the early stage of the fermentation, whose content gradually decreased during the fermentation, accounting for 12.7%, 7.6% and 8.9% of the total amino acids at the end of the fermentation. It was worth mentioning that arginine was the highest amino acid (530.81 mg/100 g) in mulberry Jiaosu, which significantly increased during the process of the fermentation. Scientific studies in vivo and in vitro have shown that arginine possesses the functions of antioxidation, promoting cell proliferation, anti-inflammation, anti-apoptosis and regulating lipid metabolism [27].

Polyphenols, the common bioactive substance in mulberry Jiaosu, could improve the antioxidant activity of the products, exhibiting functions of free-radical scavenging, immune modulation and cancer risk reduction [28,29]. Rutin and syringic acid were relatively high at the beginning of the fermentation, which was consistent with the previous findings of KwawE (Table S3) [30]. The content of polyphenols increased in different degrees except for syringic acid and p-coumaric acid. Vanillic acid and rutin occupied the main position at the end of the fermentation, accounting for 34.9% and 26.1%, respectively. Gallic acid, chlorogenic acid, vanillic acid and caffeic acid increased at first and then decreased. Protocatechuic acid continuously increased and reached a maximum of 202.23 mg/L on day 80. Polyphenols were closely related to lactic acid fermentation, from which *Lactic acid bacteria* metabolize catechin and gallic acid into protocatechuic acid [19]. In addition, phenolic acid decarboxylase produced by *Lactobacillus plantarum* can convert phenols to each other, endowing mulberry Jiaosu with antioxidant activity [31].

3.2. Microbial Community Diversity

After quality control and filtration, 91,857 and 1,129,773 high-quality sequences were obtained from the bacteria and fungi, respectively. At a 97% similarity level, the sequences were clustered. Totals of 321 and 439 OTUs were obtained from the fungi and bacteria, respectively. As the results show in Table S4, the abundance and diversity of bacteria and fungi fluctuated during the fermentation of mulberry Jiaosu. The similarity of the microbial community at different stages of the fermentation was low, and the changes of fungi and bacteria presented the opposite trends (Figure S1). The change in microbial community structure in mulberry Jiaosu is constant adaption and balance to the external environment, including the fermentation substrate and the interactions of microorganisms [32].

Principal coordinate analysis (PCoA) was further performed to evaluate the similarity of the microbial community structure. The distance not only represents the repeatability of samples from the same group but also reflects the difference of samples from different groups. As the analysis of fungi illustrated in Figure 2a, three parallel samples on day 0 were close to each other, and all were located in the second quadrant, presenting significant difference from other samples. The samples from 5 to 15 days formed a tight group, indicating that a small difference existed in these samples. The rest of the samples (days 30 and 80) were located in the third quadrant and were far away from other samples. As for

the bacteria, the short distance of samples on days 0 and 5 suggested a similar bacterial community (Figure 2b). The samples of days 10, 15 and 30 exhibited a tight cluster in the first quadrant, demonstrating that there was almost no difference in bacterial community. The results above indicated that the succession of microorganisms mainly concentrated within 30 days of the fermentation, in which period fermentation substrates were abundant for microorganisms' growth and metabolism.

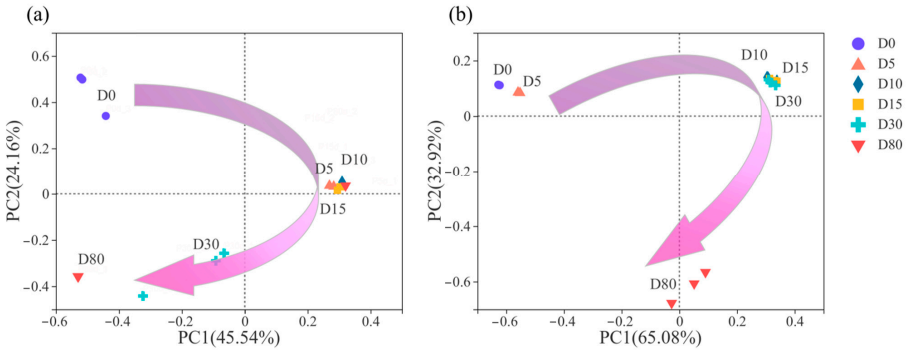

Figure 2. PCA plot of fungi (**a**) and bacteria (**b**). All experiments were repeated three times.

3.3. Succession of Microbial Community Structure

At the genus level, a total of 173 fungal genera were observed, among which 11 genera presented the relative abundance of more than 0.1%. *Torulaspora*, *Hanseniaspora*, *Unclassified_k_Fungi* and *Zygosaccharomyces* were the main fungi during the fermentation of mulberry Jiaosu (Figures 3a and S2). At the beginning of the fermentation, *Unclassified_k_Fungi* and *Cladosporium* were relatively abundant. On day 5, *Torulaspora* sharply increased and became the dominant fungus, which was followed by *Hanseniaspora* and *Citeromyces*. The relative abundance of *Torulaspora* further rose and reached its maximum of 95.2% on the 10th day, occupying the dominant position. After that, *Torulaspora* gradually decreased, while *Saccharomyces*, *Zygosaccharomyces* and *Hanseniaspora* increased in different degrees. At the later stage of the fermentation, the diverse of the fungi declined owing to the consumption of nutrients. *Torulaspora* has been reported to influence malolactic fermentation (MLF), which could promote the production of phenols [33] and improve the quality of products [34]. *Hanseniaspora*, originally isolated from grape and grape juice, plays a vital role in enhancing the flavor of products [35]. *Zygosaccharomyces* were essential microorganisms in food fermentation owing to its secondary metabolites [36].

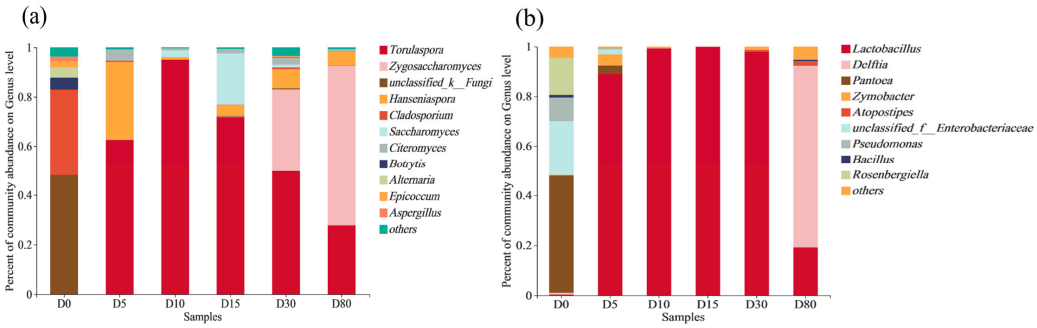

Figure 3. Composition of dominant fungi (**a**) and bacteria (**b**) genera during fermentation of Mulberry Jiaosu. All experiments were repeated three times.

For bacteria, a total of 295 genera were observed, among which 12 genera presented the relative abundance of more than 0.1% (Figures 3b and S3). On day 0, *Pantoea* was the dominant genus, which was followed by *unclassified_Enterobacteriaceae*, *Rosenbergiella*, *Pseudomonas*, *Bacillus* and *Zymobacter*. After 5 days of the fermentation, *Lactobacillus* became the absolutely dominant genus, meaning that the lactic acid fermentation began. *Lactobacillus* plays an important role in the formation of bioactive substances, which can generate

bioactive molecules of bioactive peptides, short-chain fatty acids and polysaccharides, and it can convert phenolic compounds into molecules with additional biological value, improving the antioxidant activity of the products [6]. The abundance of other genera significantly decreased, leading to the decline of abundance and diversity.

3.4. The Interaction Relationship among Microbes

As the microbial community structure is influenced by the interactions of microorganisms [7], the beneficial and antagonistic relationships among the top 20 genera was illustrated. As the results of fungi show in Figure 4a, 19 effective connection nodes and 158 edges were observed, of which 149 edges had positive correlation and 9 edges had negative correlation. The strong connection nodes (≥10 edges) were mostly distributed in *unclassified_k_Fungi*, *Botrytis*, *Alterneria*, *Phoma*, *Cladosporium* and *Epicoccum*. The max positive correlation between *unclassified_k_Fungi* and *Aspergillus* (r = 0.9557) and the max negative correlation between *Torulapora* and *Aspergillus* (r = −0.7215) were observed, respectively.

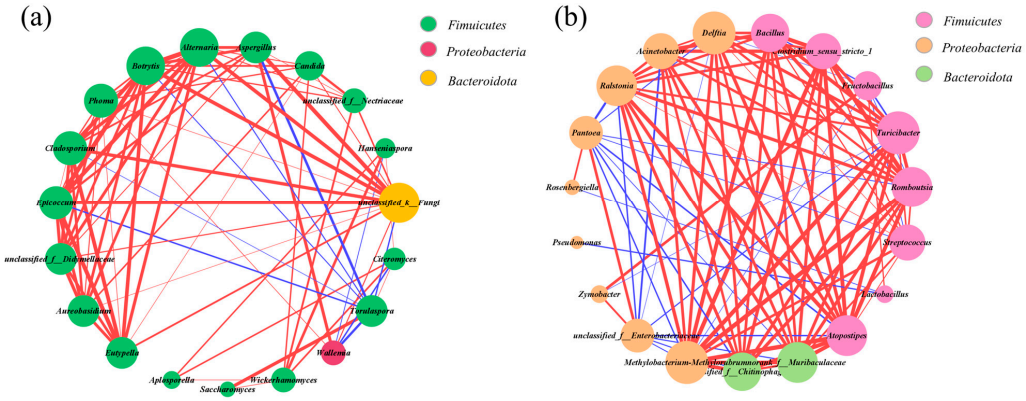

Figure 4. Co-occurrence network of fungi (**a**) and bacteria (**b**). Different classes were represented by different colors. The red and blue lines refer to positive correlations (r > 0.5 and $p < 0.05$) and negative correlations (r < −0.5 and $p < 0.05$), respectively. The size of each node is proportional to the number of connections.

For bacteria, 19 connection nodes and 188 edges were exhibited, of which 163 edges had positive correlation and 25 edges had negative correlation (Figure 4b). The strong connection nodes (≥10 edges) were mostly distributed in *Methylobacterium-Methylorubrum*, *Turicibacter*, *Delftia*, *Atopostipes*, *Ralstonia*, *norank_f_Muribaculaceae*, *Romboutsia*, *Bacillus*, *unclassified_f_Chitinophagaceae*, *Acinetobacter*, *Streptococcus*, *Clostridium_sensu_stricto_1*, *unclassified_f__Enterobacteriaceae* and *Pantoea*. *Romboutsia* presented a max positive correlation with *Methylobacterium-Methylorubrum* (r = 0.9348), and *Ralstonia* exhibited a max negative correlation with *Pantoea* (r = −0.6944). The results demonstrated that microorganisms could be mutually coordinated and constrained during the fermentation of mulberry Jiaosu.

3.5. Correlation Analysis of Microorganisms with Physicochemical Parameters and Bioactive Substances

At the genus level, Spearman's correlation analysis ($|r| \geq 0.5$, $p < 0.05$) of physicochemical parameters and top 20 microorganism genera was performed. For fungi, eleven genera (including *Torulaspora*, *Unclassified_f__Didymellacae*, *Zygosaccharomyces*, *Cladosporium*, *Stemphylium*, *Eutypella*, *Wickerhamomyces*, *Epicoccum*, *Gibberella*, *Saccharomyces* and *Penicillium*) of *Ascomycota* and one genus (*Apiotrichum*) of *Basidiomycota* were correlated with physicochemical parameters (Figure 5a). For bacteria, seven genera (including *Bacillus*, *Fructobacillus*, *Lactobacillus*, *Romboutsia*, *Atopostipes*, *Clostridium_sensu_stricto 1* and *Turicibacter*) of *Fimicutes*, eight genera (including *Pseudomonas*, *Rosenbergiella*, *Delftia*, *Acinetobacter*, *unclassified_f__Enterabacteriaceac*, *Ralstonia*, *norank_f__Mitochondria* and *Pantoea*) of *Proteobacteria* and one genus (*norank_f__norank_o__Chloroplast*) of *Cyanobacteria* exhibited correlations

with physicochemical parameters (Figure 5b). Seven positive and nine negative correlations between microorganisms and pH were identified, indicating that pH exhibited a selective effect on microorganisms. Contrary to pH, the increase in total acids could change the diversity and abundance of microorganisms [37]. Reducing sugar was positively correlated with five bacterial genera and six fungal genera, mainly including *Cladosporium*, *Stemphylium* and *Pantoea*. Three fungal genera and seven bacterial genera presented positive correlations with total organic acids, demonstrating that microorganisms possess the ability to produce organic acids, such as *Zygosaccharomyces*, *Ralstonia* and *Atopostipes* [38]. In addition, the total number of polyphenols was positively correlated with three fungal genera and three bacterial genera, and it was negatively correlated with four bacterial genera. Furthermore, seven positive and four negative correlations between the microorganisms and total amino acids were also displayed. The results suggested that the metabolism of microorganisms can promote the accumulation of bioactive substances. At the same time, the change in physicochemical parameters could regulate the diversity of the microbial community.

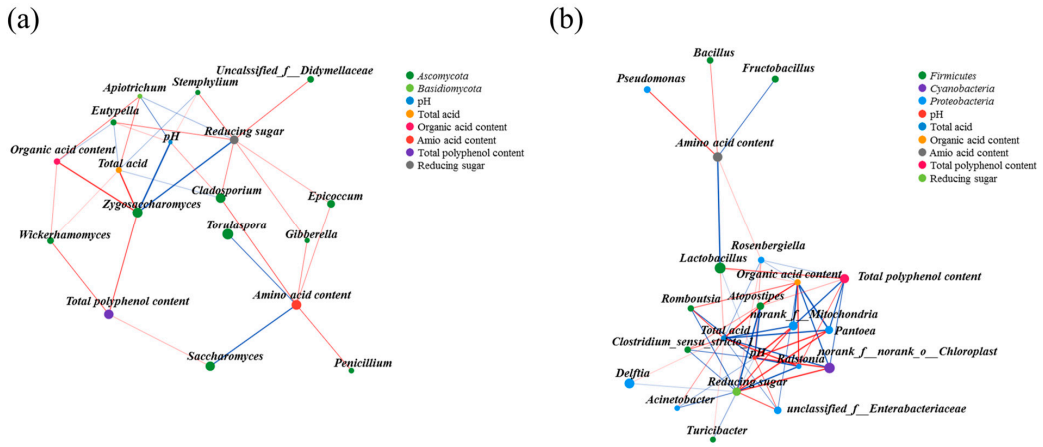

Figure 5. Correlation analysis between the dominant fungal (**a**) and bacterial (**b**) genera with physiochemical characteristics. Red represents a positive correlation and blue represents a negative correlation. The thicker the lines, the stronger the correlation.

To explore the roles of microorganisms in the formation of bioactive substances during the fermentation of mulberry Jiaosu, correlation analysis between microorganisms and bioactive substances was conducted (Figure S4).

Zygosaccharomyces exhibited positive correlations with most of the organic acids. *Wickerhamomyces* could promote the synthesis of tartaric acid, lactic acid, acetic acid and oxalic acid. Compared with fungi, bacteria contributed more to the synthesis of organic acids. *Atopostipes*, *Ralstonia* and *norank_f__Muribaculaceae* showed positive correlations with the majority of organic acids, demonstrating these microorganisms were adaptive to the acidic environment, while *Rosenbergiclla*, *Pantoea* and *unclassified_f__Enterobacteriaceac* were negatively correlated with all the organic acids except for oxalic acid and citric acid. *Lactobacillus*, the most dominant bacteria during the fermentation of mulberry Jiaosu, with the ability to form acid environment through the rapid synthesis of organic acids, was positively correlated with lactic acid and acetic acid [39].

Overall, fungi were negatively correlated with the majority of amino acids. *Zygosaccharomyces* presented positive correlations with aspartic acid, glutamic acid, arginine, alanine, valine, cystine, isoleucine, leucine and phenylalanine, and it presented negative correlations with serine, proline, tyrosine and methionine, which was consistent with the findings of Ke [40]. *Torulaspora* and *Saccharomyces* exhibited negative effects on the formation of glycine and histidine. Close relationships also existed between amino acids and bacteria. Glycine and histidine were positively correlated with *Pseudomonas*, *Rosenbergiclla* and *Pantoea*, and they were negatively correlated with *Lactobacillus* and *Fructobacillus*. The synthesis of lysine was mainly attributed to the metabolism of *Zymobacter* and *Fructobacillus*. Further-

more, aspartic acid, glutamic acid, arginine, threonine, alanine, valine, cystine, isoleucine, leucine and phenylalanine were positively correlated with *Romboutsia*, *Atopostipes*, *Ralstonia*, *Turicibacter* and *Clostridium_sensu_stricto_1*.

Zygosaccharomyces could promote the synthesis of the majority of polyphenols [41]. Syringic acid and p-coumaric acid showed positive correlations with most of the fungi. Bacteria exhibited positive correlations with polyphenols except for p-coumaric acid and syringic acid. Among them, *Lactobacillus* was positively correlated with chlorogenic acid, vanillic acid, ferulic acid, gallic acid and rutin. Furthermore, *Rosenbergiclla*, *Pantoea* and *unclassified_f__Enterobacteriaceae* were negatively correlated with gallic acid, protocatechuic acid and caffeic acid.

4. Conclusions

In this study, the microbial community structure and their correlation with physicochemical parameters and bioactive substances during the fermentation of mulberry Jiaosu were comprehensively analyzed. The change in physicochemical parameters was largely concentrated within 30 days of fermentation. Bioactive substances of lactic acid, arginine, vanillic acid and rutin significantly increased, endowing Jiaosu with abundant nutritional value. High-throughput sequencing results showed that the diversity of fungi exhibited opposite variation trends to that of bacteria. *Torulaspora*, *Hanseniaspora* and *Zygosaccharomyces* were the main fungi, and *Lactobacillus* was the dominant bacteria during the fermentation of mulberry Jiaosu, among which *Zygosaccharomyces* and *Lactobacillus* were the most important microorganisms. *Zygosaccharomyces* was positively correlated with most of the bioactive substances. Compared with fungi, bacteria contributed more to the production of bioactive substances. *Lactobacillus* were positively correlated with lactic acid, acetic acid, chlorogenic acid, vanillic acid, ferulic acid and rutin. Tartaric acid, pyruvate, malic acid, succinic acid, aspartic acid, glutamic acid, arginine, threonine, alanine, valine, cystine, isoleucine, leucine, phenylalanine, gallic acid, protocatechuic acid and caffeic acid were positively correlated with most of the bacteria, including *Romboutsia*, *Atopostipes*, *Ralstonia*, *Turicibacter*, *Clostridium_sensu_stricto_1* and *unclassified_f_Chitinophagaceae*. This work lays the foundation for further researching and understanding of the nutritional value of mulberry Jiaosu as well as the roles and functions of microorganisms, providing a scientific basis for the quality control of mulberry Jiaosu.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation9100910/s1.

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