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Analysis of the Microbial Community Structure and Volatile Metabolites of JIUYAO in Fangxian, China

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Abstract: JIUYAO is an important saccharification starter in the production of huangjiu and is also an important source of flavor. In this study, the microbial community structure of JIUYAO from Fangxian was studied by high-throughput sequencing (HTS) technology for the first time. The volatile flavor compounds of the JIUYAO metabolites were also analyzed by headspace solid-phase microextraction combined with full two-dimensional gas chromatography-mass spectrometry (HS-SPME-GC×GC/MS) for the first time. The results showed that there were 15 dominant bacterial genera, including *Weissella*, *Pediococcus*, *unclasssified_k_norank_d_Bacteria*, *Lactobacillus*, *Leuconostoc*, etc. Thirteen species of dominant fungi included *Wickerhamomyces*, *Saccharomycopsis*, *Rhizopus*, etc. The different samples of JIUYAO were similar in their microbial species, but the number of species was significantly different. A total of 191 volatile flavor compounds (VFCs) were detected, among which esters, alcohols, acids, and alkenes were the main flavor compounds, and 21 terpenoids were also detected. In addition, the functional prediction of micro-organisms in JIUYAO revealed that global and overview maps, amino acid metabolism, and carbohydrate metabolism were the dominant categories. Through correlation analysis, 538 potential correlations between the dominant micro-organisms and the different flavor compounds were obtained. This study revealed the interactions between the micro-organisms and the volatile metabolites in JIUYAO, which provided reliable data for the analysis of the microbial community structure of Fangxian JIUYAO and provided theoretical support for the quality evaluation of JIUYAO.

Keywords: JIUYAO; microbial community structure; volatile flavor compound; correlation analysis; microbial metabolic

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1. Introduction

Qu is an important microbial starter in the brewing process of huangjiu [1]. The quality of Qu has a great influence on the production of huangjiu. Due to the differences in the raw materials, technology, and environment, the varieties of Qu are very different. Qu is divided into Wheat Qu and Rice Qu due to the different raw materials. No matter whether Chinese herbal medicine is added to Jiuqu or not, it is divided into medicine Qu and white Qu. Medicine Qu is usually also called JIUYAO. According to the different shape characteristics, it can be divided into cake, lump, bolus, and powder. Located in Fangxian, Shiyan City, Hubei Province, the production of fuzhi huangjiu is a local specialty. The region has long winters and short summers and abundant water resources, making it suitable for winemaking. Therefore, the region produces large amounts of Qu with a lump or bolus shape, also named rice Qu or JIUYAO. It is made of rice, the unique local polygonum, with the JIUYAO seed made in previous years and using a certain amount of water. Polygonum can not only integrate its own micro-organisms but also promote the growth of yeast and mold [2] and inhibit harmful bacteria [3]. It may also loosen JIUYAO, enabling microbes to

make better use of nutrients and oxygen in the raw material [4]. Compared with daqu and wheat Qu, the cultivation time of JIUYAO is shorter, and the main fermentation time of traditional JIUYAO is only 24–48 h. JIUYAO also has the advantages of simple production, less use, ease of storage, and so on.

The quality of the micro-organisms in JIUYAO has an important influence on the physicochemical, sensory, and flavor of huangjiu [5,6]. The composition of the microbial community in JIUYAO mainly comes from raw materials, polygonum grass, and the production environment [4]. The micro-organisms in JIUYAO are mainly divided into four categories: mold, yeast, bacteria, and actinomycetes. The main function of mold is saccharification and metabolism to produce ester compounds [7]. The most common mold in JIUYAO is *Rhizome*, which also includes *Aspergillus*, *Mucor*, etc. *Rhizopus oryzae* has often been reported in Qu for the breakdown of starch in raw materials [8]. Although the proportion of other molds is lower, they still secrete lipase, protease, and cellulase to solve the low utilization rate of the ingredient. The process of both the saccharification and fermentation of huangjiu was realized by the simultaneous action of *Saccharomyces cerevisiae* and *Rhizopus* [1]. Several researchers have also reported the ability of *Saccharomyces cerevisiae* to produce 2-phenylethanol, a key aroma compound in rice wine [9]. The common aromatic yeasts in JIUYAO are *Hansenula* sp., *Wickham* sp., *Endomycopsis* sp., *Candida* sp., *Pichia* sp., and *Rhodotorula* sp. et al. The main bacteria are *Weissella*, *lactic acid bacteria*, *acetic acid bacteria*, *Bacillus*, etc. [1,10]. The diversity of bacteria is more abundant than fungi, and bacteria can also secrete the enzymes needed for wine making, especially Bacillus, which create a greater contribution to amylase activity [11]. *Lactobacillis*, such as *Pediococcus*, *Lactobacillus*, and *Lactococcus* [12,13] can regulate pH, providing a more suitable environment for yeast and bacteria to grow while also providing more possibilities for flavor diversity [14]. Ensuring the reproduction of dominant flora in JIUYAO is the key to the manufacture of high-quality JIUYAO.

The quality evaluation of JIUYAO has always used color, odor, and saccharification power as the main indicators, but these indicators cannot fully reflect the quality of JIUYAO. JIUYAO production is the result of microbial interaction. By analyzing the micro-ecosystem in JIUYAO, its structure can be understood fundamentally. High-throughput sequencing technology has high throughput, high sensitivity, and high precision and can detect a large number of unculturable micro-organisms, which is an important tool for microbial structure analysis in JIUYAO. The volatile flavor compounds (VFCs) in JIUYAO are produced by microbial metabolism, which is of great importance to their quality assessment [15]. The VFCs in JIUYAO are produced by microbial metabolism, which greatly influences their quality [1,15], but there is no report on the flavor of jiuqu in hubei. The commonly used flavor analysis method is headspace solid-phase microextraction combined with gas chromatography-mass spectrometry (SPME-GC/MS). Meanwhile, full two-dimensional gas chromatography-mass spectrometry (GC × GC-MS) has been generally used in the analysis of huangjiu and other alcoholic beverages [9]. The instrument has two chromatographic columns with different properties, which gives it a better separation effect. The instrument also can be used to detect the VFCs in JIUYAO so as to offer further detailed real data for the quality control of JIUYAO.

In the present work, amplicon sequencing was applied to perform a statistical analysis of the bacterial and fungal communities of the micro-organisms, identifying the dominant micro-organisms and predicting their metabolic mode in JIUYAO from Fangxian. Then, the VFCs in JIUYAO were determined by HS-SPME/GC × GC-MS for the first time. Finally, a correlation analysis was conducted to explore the relationship between VFCs and the dominant micro-organisms in JIUYAO and further reveal the mechanism of the microbial metabolism of flavor compounds. This study provided a theoretical basis for the production and quality evaluation of JIUYAO.

2. Materials and Methods

2.1. Samples Collection

The six JIUYAO samples were collected from Fangxian, Shiyan City, Hubei Province, China, and named FMQ, FZQ, HJQW, HJQF, HJQY, and TJQY. All the starter samples were stored at 4 °C until further analysis.

2.2. DNA Extraction, PCR Amplification, and Illumina MiSeq Sequencing

The steps for DNA extraction and amplification were as described in the previous study [16]: polymerase chain reaction (PCR) was performed using a GeneAmp 9700 (ABI, Carlsbad, CA, USA) instrument. Bacteria were amplified using primers 338 F and 806 R for the V3-V4 highly variable region of the 16s rRNA gene. The amplification conditions were as follows: 95 °C, incubated for 3 min, followed by 27 cycles performed at 95 °C for 30 s; then, 55 °C for 30 s and 72 °C for 45 s, and finally extended to 72 °C for 10 min. The PCR amplification conditions for the fungal ITS region were as follows: 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s with a final extension at 72 °C for 10 min, with primers ITS1F and ITS2. The PCR products were purified and qualified before being sent to the Majobio Institute (Shanghai, China). Sequencing was performed on the MiSeq platform of an Illumina high-throughput sequencer (Illumina, San Diego, CA, USA).

2.3. Determination of Volatile Flavor Compounds

The VFCs of the six JIUYAO samples were detected using HS-SPME-GC × GC/MS methods. Briefly, 4.00 g of each sample and 0.020 mL of 2-octanol (99.07 mg/L) were transferred into a 10.0 mL headspace bottle, and the bottles were balanced at 50 °C for 30 min. Then, SPME extraction fiber (50 μm DVB/CAR/PDMS, Supelco) was inserted into headspace bottles to extract at 50 °C for 30 min [17]. The VFCs were desorbed from the fiber in the GC inlet for 5 min at 250 °C [9].

The instrument method was slightly modified from the previous study [9]: the volatile compounds were separated and analyzed by GC × GC/MS (7890B-5977A, Agilent, Palo Alto, CA, USA) with two columns. The first column was a HP-INNOWAX column (30 m × 0.25 mm i.d. × 0.25 μm, Agilent, Palo Alto, CA, USA), and the second column was a DB-17 MS column (1.0 m × 0.15 mm i.d., 0.30 μm, Varian, Palo Alto, CA, USA). The oven temperature was first held at 50 °C for 3 min, then increased from 50 °C to 130 °C at the rate of 3 °C/min, and finally increased from 130 °C to 240 °C at a rate of 6 °C/min and held for 10 min. The carrier gas was helium (99.999%), and the flow rate was maintained at 1.2 mL/min.

The qualitative analysis of the VFCs was determined by NIST library and retention index (RI) [18]. The matching degree of all VFCs in the NIST library was greater than 700. The RI of the VFCs calculated by n-alkene (C_5–C_{25}, o2si, Charleston, SC, USA) was less than 50 compared with the RI in the NIST library [19]. The quantification of the VFCs was calculated using the internal standard method [20]. All results were the mean of triplicates, and the results were expressed as mean ± standard deviation.

2.4. Statistical and Bioinformatic Analysis

High quality sequencing readings were extracted using Usearch (version 7.0.1090, Robert Edgar, www.drive5.com) software. On the Qiime platform, a Bayesian algorithm was applied to conduct taxonomic analysis on OTU representative sequences with 97% similarity level, and the confidence threshold was 0.7 [21].

Beta diversity was represented by nonmetric multidimensional scale (NMDS), and the similarity was analyzed by Bray–Curtis dissimilarity distance matrix. NMDS analysis and visualization were performed using the vegan package of the R Studio (version 4.0.5) [22]. The corrplot package in R Studio software (version 4.0.5, Robert Gentleman and Ross Ihaka, Boston, MA, USA) was used to calculate the Spearman rank correlation coefficient of OTU of the dominant micro-organisms and draw the correlation heat map of microbial genera [1].

The R Studio software (version 4.0.5) was used to calculate the Spearman correlation coefficient between the microbial genera (relative abundance > 1%) and the VFCs [23] and the correlations networks between the selected volatile metabolites and the microbial community were visualized via the Cytoscape software (version 3.9.0, Shannon P, Bethesda, MD, USA) [17].

PICRUSt software package was used to predict the functional composition of the microbial bacterial community, and compare with the evolutionary genealogy of the genes: Nonsupervised Orthologous Groups database (egg NOG) and Kyoto Encyclopedia of Genes and Genomes database (KEGG) for functional annotation [15].

The data obtained were subjected to an analysis of variance (ANOVA), and the significance level in the analyses was considered at $p < 0.05$. SPSS (27.0, Norman H. Nie, C. Hadlai (Tex) Hull and Dale H. Bent, Chicago, IL, USA) software was used for significance analysis, and the difference was significant when p value < 0.05 [19].

3. Results

3.1. Sequencing the JIUYAO Samples

The fungal and bacterial community diversity of the six JIUYAO samples was analyzed. For the bacterial communities, 1,105,818 high-quality sequences were obtained after the exclusion of the low-quality reads and chimeras. The average sequence length was 424 bp. Based on a 97% similarity, 502 operational taxonomic units (OTUs) were clustered, which were annotated based on the Silva classification database. For the fungal communities, 1,383,802 high-quality sequences from all samples were acquired after quality control processing. The average sequence length was 231 bp. After fungi clustering, 157 OTUs were obtained, and the OTUs were classified and annotated based on the UNITE classification database. The number of fungal sequences was much higher than the bacterial sequences in all samples; however, the total number of fungal OTUs was much smaller than that of the bacterial OTUs.

3.2. α-Diversity Analysis of the JIUYAO Samples

The alpha diversity of six species of JIUYAO was compared; the rarefaction curves tended to be gentle (Figure S1), indicating that the amount of sequencing data was large enough and the sequencing depth could reflect the vast majority of the microbial diversity information in the samples. The parameters of species richness and diversity, including the Shannon, Chao1, Simpson, and ACE indices, are presented in Figure S2.

For the bacteria, the HJQW samples had greater Chao1 and ACE index values than those of the other five JIUYAO samples. These results implied that the HJQW samples had the highest bacterial richness and the highest total number of species. Subsequently, the HJQY and HJQF had a higher bacterial richness. TJQY has a low bacterial abundance. The Shannon and Simpson indices showed that HJQY had the highest bacterial community diversity. The diversity of TJQY and FZQ is relatively low.

For the fungi, the Chao1 and ACE indices showed that the HJQW had greater fungal richness than those of the other five samples. HJQY and HJQF had a higher total number of species. The Shannon and Simpson indices also showed that HJQW and HJQF had the highest fungal community diversity. FZQ and FMQ had the lowest richness, and the Simpson and Shannon index values showed that they also had the lowest community diversity. The above diversity index was consistent with the OTU results, indicating that the total fungal diversity was much lower than the total bacterial diversity.

3.3. Microbial Composition Analysis in JIUYAO

For the bacteria, using 16S rRNA gene sequence analysis, a total of 286 genera, 163 families, and 18 phyla were identified. The Circos samples and species relationship diagrams not only reflected the distribution proportion of the dominant species at different levels of the flora in different JIUYAO but also intuitively showed the composition proportion of the dominant species of micro-organisms in each JIUYAO sample. Among the bacterial communities, the dominant flo-

ras at the phylum level were *Firmicutes*, *unclassified_k_norank_d_Bacteria*, *Proteobacteria*, *Cyanobacteria*, and *Actinobacteriota* (Figure 1a). At the genus level, 15 core bacterial genera were detected (relative abundance > 1%), which included *Weissella*, *Pediococcus*, *unclasssified_k_norank_d_Bacteria*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, etc. (Figure 2a). There were seven dominant bacterial genera with a relative abundance of greater than 1.0% in FMQ, which included *Weissella* (37.90%), *Pediococcus* (21.17%), *Leuconostoc* (17.84%), *norank_f_norank_o_Chloroplast* (3.87%), *unclasssified_k_norank_d_Bacteria* (3.76%) *Lactobacillus* (2.59%), etc. Six of dominating genera proportions were above 1.00% in FZQ, including *Pediococcus* (41.03%), *Weissella* (37.16%), *Lactobacillus* (8.40%), *Lactococcus* (5.44%), *Staphylococcus* (2.23%), etc. *Unclasssified_k_norank_d_Bacteria*, *Weissella*, and *Leuconostoc* were the dominant genera for HJQF and HJQW. *Lactobacillus*, *Lactococcus*, and *Leuconostoc* were the dominant genera for HJQY. *Weissella*, *Pediococcus*, and *Kosakonia* were the dominant genera for TJQY. These results are consistent with previous studies, where *Weissella* and *Pediococcus* were the predominant group of bacteria and also the acid-producing bacteria in JIUYAO [1,15,23]. *Pediococcus*, *Lactobacillus*, *Leuconostoc*, and *Lactococcus* are important lactic acid bacteria in JIUYAO, both as acid-producing and as contributors to the flavor, such as ethyl lactate and ethyl acetate [12,22,24]. Lactic acid bacteria can also inhibit the growth of miscellaneous bacteria and reduce the production of Ethyl carbamate [25], and the proper amount of lactic acid bacteria reproduction has an important role in JIUYAO. The bacterial flora is characterized by high abundance and variety, which provides rich possibilities for the aroma and taste of huangjiu [15].

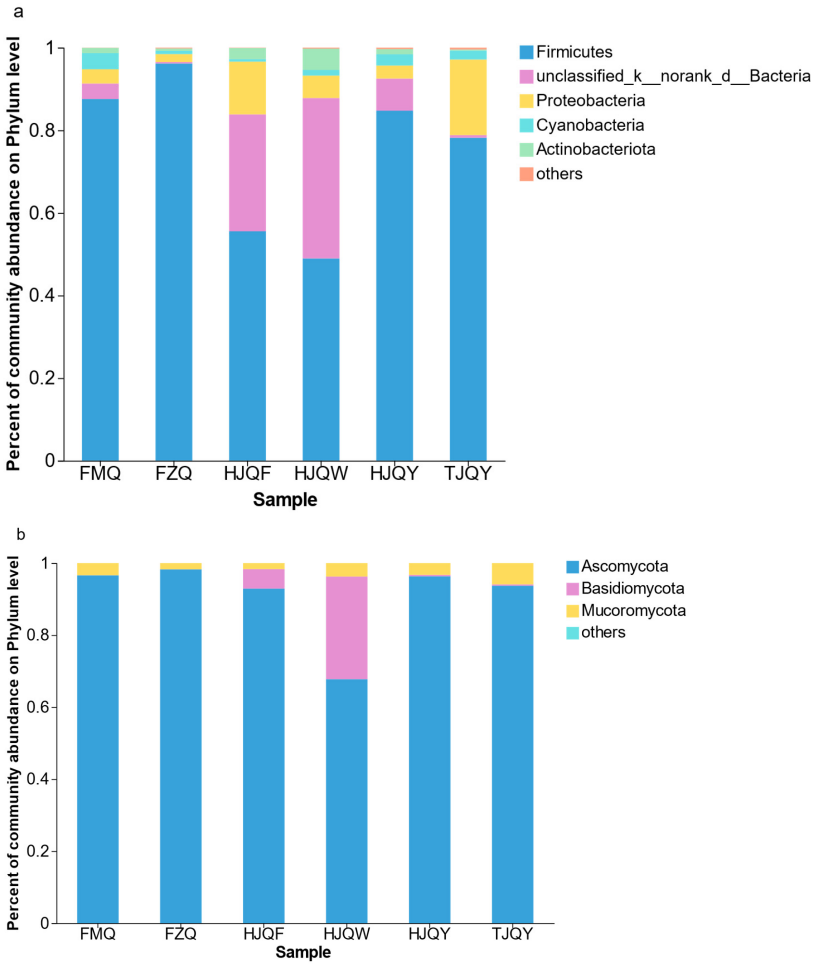

Figure 1. Microbial community composition at the phylum level of JIUYAO; (**a**): relative abundance of bacteria; (**b**) relative abundance of fungi.

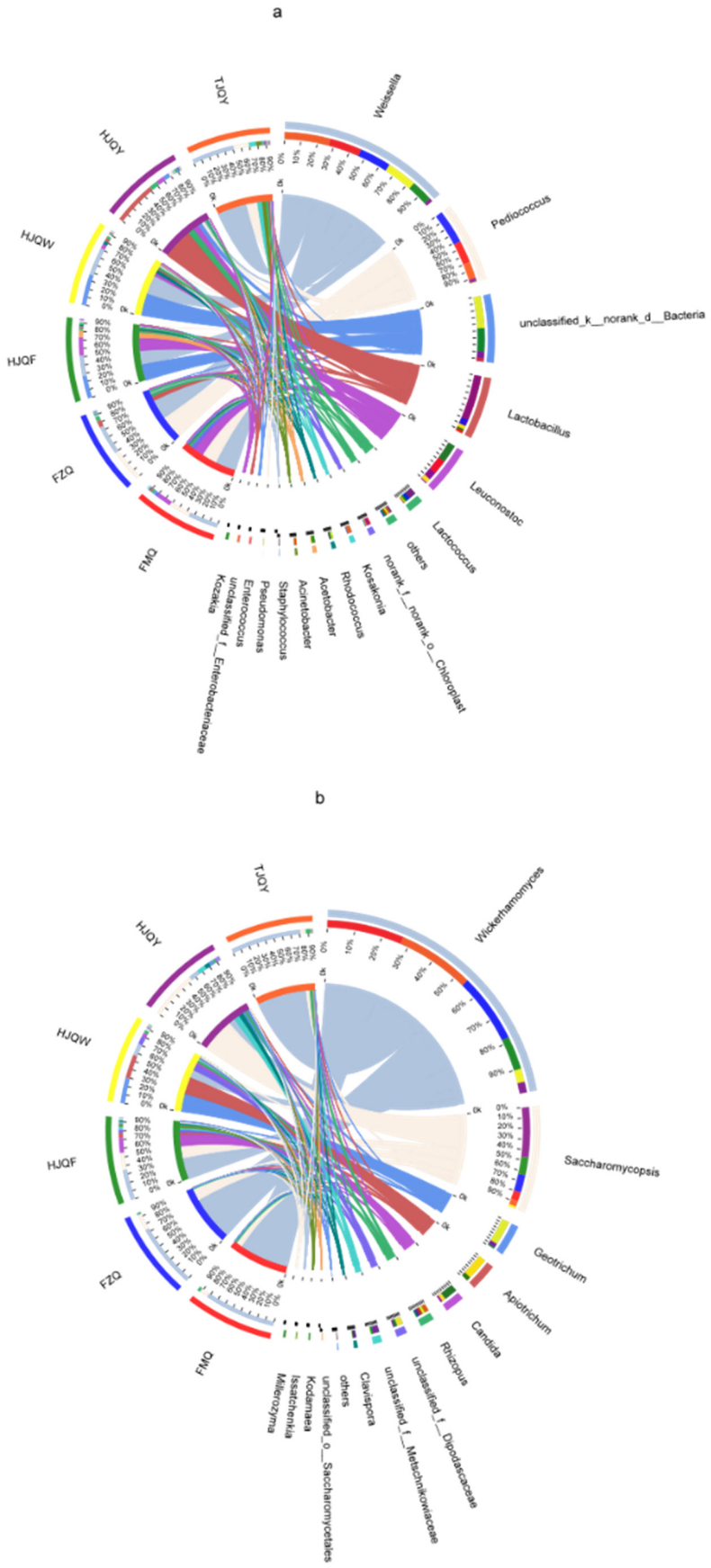

Figure 2. Microbial community composition at the genus level of JIUYAO; (**a**) relative abundance of bacteria; (**b**) relative abundance of fungi.

For the fungi, the ITS gene sequences were classified at the phylum, family, and genus levels. The results showed that five fungal phyla, 39 families, and 69 genera were identified. The dominant floras at the phylum level with relative abundance above 0.1% were *Ascomycota*, *Mucoromycota*, and *Basidiomycota* (Figure 1b). At the genus level, 13 core fungi genera were detected (relative abundance > 1%) (Figure 2b), which included *Wickerhamomyces*, *Saccharomycopsis*, *Rhizopus*, *Candida*, etc. *Wickerhamomyces* was the dominant fungi genus, followed by *Saccharomycopsis* and *Rhizopus*. In FZQ and FWQ, *Wickerhamomyces*, *Saccharomycopsis*, and *Rhizopus* were the dominant fungi genera, and these three dominant fungi genera accounted for 99.67% and 99.29% of FZQ and FMQ, respectively. The proportions of the four dominating genera were above 1.00% in TJQY, including *Wickerhamomyces* (84.96%), *Saccharomycopsis* (6.39%), *Rhizopus* (5.85%), and *unclassifies_o_Saccharomycetales* (1.51%). The 10 dominating genera proportions were above 1.00% in HJQF, including *Wickerhamomyces* (37.09%), *Saccharomycopsis* (20.43%), *Candida* (18.17%), *Apiotrichum* (5.36%), etc. The six dominating genera proportions were above 1.00% in HJQW, including *Geotrichum* (30.83%), *Apiotrichum* (28.14%), *Wickerhamomyces* (14.84%), *unclassifies_f_Dipodascaceae*, etc. The six dominating genera proportions were above 1.00% in HJQY, including *Wickerhamomyces* (56.90%), *Saccharomycopsis* (11.95%), *Clavispora* (6.32%), *unclassifies_f_Metschnikowiaceae*, etc. In the yeasts, *Wickerhamomyces* has a strong flavor production ability, especially ethyl acetate [26], which provides rich fruit aroma for JIUYAO and the brewing of huangjiu [27]. It also can produce a small amount of ethanol. *Saccharomyces* is also an important flavor-producing yeast in the starters of huangjiu, and it has a high proportion in rice starters [14]. This strain can not only play an obvious role in aroma enhancement during brewing [28] but also has the characteristics of secreting amylase, which can work with saccharifying bacteria to improve the hydrolysis efficiency of raw materials. In the molds, *Rhizopus* is the dominant fungus in JIUYAO and an important saccharifying bacteria, which has the ability to hydrolyze α-1,4 glycosidic bonds in starch and the ability to produce flavor compounds [29,30], and it can also secrete acidic protease [8]. Molds and some yeasts have the ability to secrete hydrolytic enzymes. Yeasts are primarily a producer of ethanol and flavor, and molds can also metabolize to produce flavor compounds combined with yeast metabolites. The two kinds of micro-organisms play a role in JIUYAO together to form a complex fungal community.

3.4. β-Diversity Analysis of JIUYAO

The production of JIUYAO occurs in an open environment, resulting in a certain diversity in the species and quantity of micro-organisms in different JIUYAO. The NMDS analysis based on the Bray–Curtis distance was used to analyze the differences in microbial communities among the different samples. The analysis of the similarities and adonis determined that the differences among the JIUYAO samples were significantly greater than the differences within the groups, enough to judge that the grouping was meaningful (Figure 3a,c). In the analysis of the bacteria (Figure 3b), the stress was <0.1, and the six kinds of samples were completely separated; the six kinds of the nine drugs are completely separated in the figure, so their bacterial compositions were quite different. In the analysis of the fungi (Figure 3d), the stress was <0.05, and the samples had good representativeness. The HJQY, HJQF, and HJQW samples could be completely distinguished, which indicated that the fungal communities of these three groups were more different among the groups. The FMQ, FZQ, and TJQY samples were close to each other, and the difference in fungal community composition was relatively small and stable, but the difference was significant from the other three samples, indicating that the four groups of the samples had greater discrepancies regarding fungal communities.

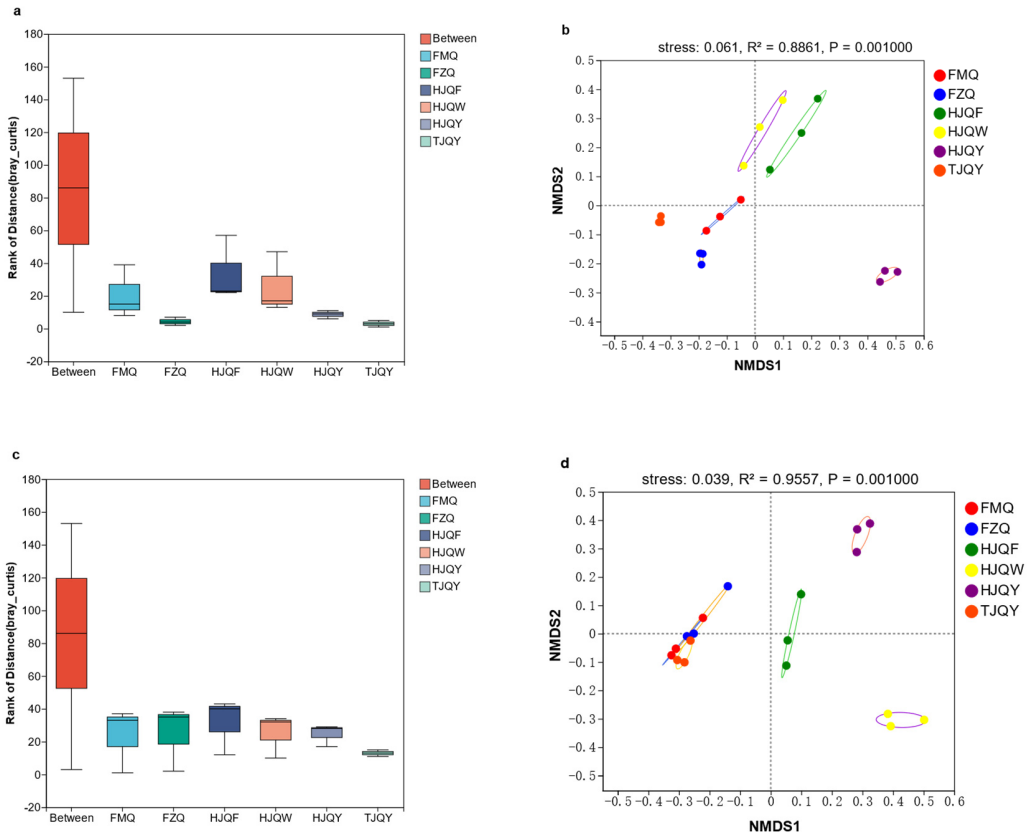

Figure 3. Comparison of the intergroup differences among microbial communities in JIUYAO; (**a**) box diagram of the similarity analysis between the bacterial groups based on Bray−Curtis distance algorithm; (**b**) NMDS analysis at the bacterial genus level; (**c**) box diagram of similarity analysis between the fungi groups based on Bray−Curtis distance algorithm; (**d**) NMDS analysis at the fungi genus level.

3.5. Analysis of Microbe Interactions in JIUYAO

A Spearman's rank correlation analysis was used to determine the correlations and dominant micro-organisms in the complex microbial communities (Figure 4). For the bacterial communities, *Weissella*, *Pediococcus*, *Kosakonia*, and *Staphylococcus* exhibited negative correlations with *unclasssified_k_norank_d_Bacteria*, *Lactobacillus*, *Leuconostoc*, and *Lactococcus*. A positive correlation was found between *Lactobacillus* and *Lactococcus*. For the fungi, *Wickerhamomyces*, *Saccharomycopsis*, and *Rhizopus* exhibited negative correlations with almost all of the abundant fungal genera. A positive correlation was found between *Rhizopus* and *Wickerhamomyces*, and a positive correlation was found among *Saccharomycopsis*, *Clavispora*, and *unclassifies_f_Metschnikowiaceae*. *Candida* appeared to have strong positive correlations with *Apiotrichum*, *Issatchenkia*, *Kodamaea*, *Millerozyma,* and *unclassifies_f_Dipodascaceae*. Interestingly, the Spearman correlation analysis between the bacteria and fungi indicated that *Weissella* correlated positively with *Wickerhamomyces*, *Rhizopus,* and *unclassifies_o_Saccharomycetales*. *Pediococcus* positively correlated with *Wickerhamomyces*. There were significant positive correlations between two bacteria (*Lactobacillus* and *Lactococcus*) and three fungi (*Saccharomycopsis*, *Clavispora,* and *unclassifies_f_Metschnikowiaceae*). Significant negative correlations between *Weissella* and *Saccharomycopsis*, *Clavispora*, and *unclassifies_f_Metschnikowiaceae*. *Wickerhamomyces* exhibited negative correlations with *unclasssified_k_norank_d_Bacteria*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Rhodococcus,* and *Pseudomonas*. *Pediococcus* exhibited negative correlations with almost all of the abundant fungal genera, except *Wickerhamomyces*.

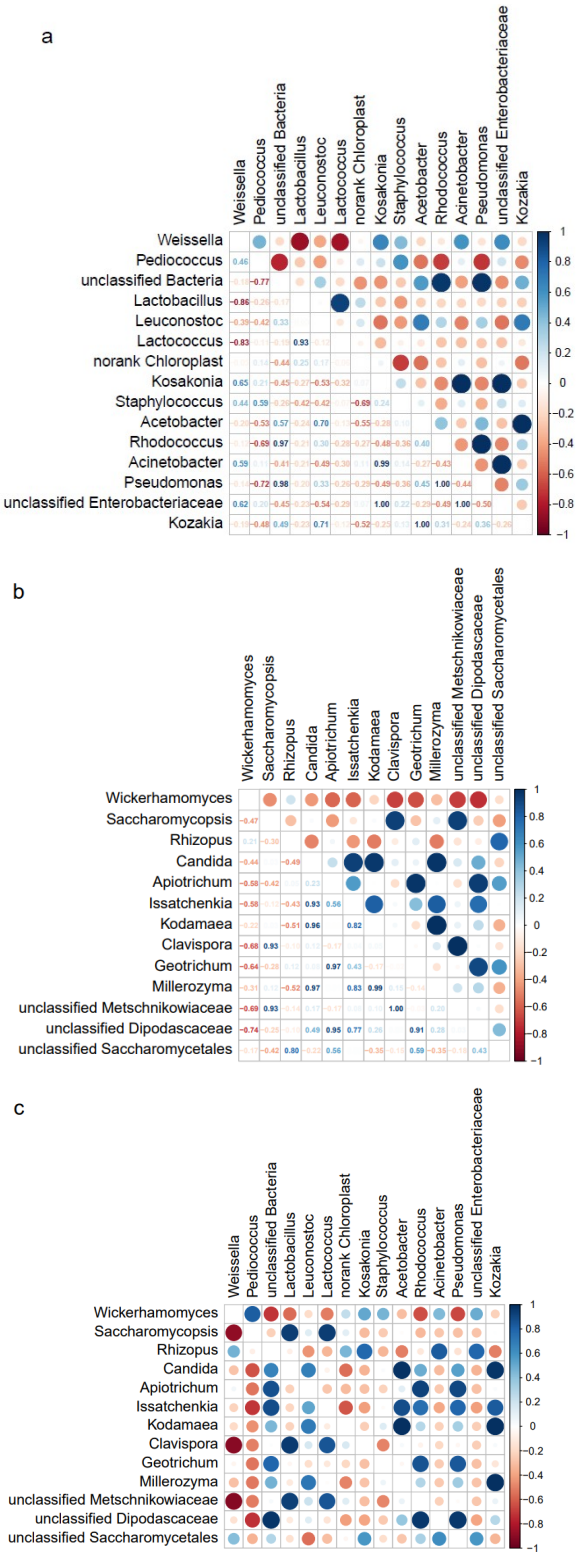

Figure 4. Correlation matrix of Spearman rank correlation between the dominant microbial genera. Spearman's rank correlation coefficient is between 1.0 and −1.0, indicating a range from strong positive to strong negative correlations. Circle size indicates the strength of correlation; the large circle is a strong correlation, and the small circle is a weak correlation. The color of the scale bar indicates the nature of the correlation, with blue indicating a positive correlation and red indicating a negative correlation. (**a**) Spearman rank correlation matrices are shown for bacteria with >1% abundance; (**b**) Spearman rank correlation matrices are shown for fungal species with >1% abundance; (**c**) Spearman rank correlation matrices are shown for bacterial/fungal species with >1% abundance.

3.6. Analysis of Volatile Flavor Compounds in JIUYAO

The volatile compounds in JIUYAO are directly related to the flavor of JIUYAO; therefore, flavor compounds play an important role in the sensory evaluation of JIUYAO. The VFCs in the six JIUYAO samples were identified by HS-SPME-GC×GC/MS (Table S1). A total of 191 volatile flavors compounds were identified and quantified and included 36 esters, 34 alcohols, 12 acids, 17 aldehydes, 26 ketones, 5 ethers, 4 furans, 7 lactones, 2 sulfur containing compounds, 17 nitrogen containing compounds, 7 phenols, and 24 hydrocarbons. The VFCs of the six JIUYAO samples were different in species and contents. The contents of the VFCs in JIUYAO were 4554.02–12834.53 μg/kg (Figure 5). Among them, ester and alcohol content was much higher than other compounds. The contents of the VFCs in wheat Qu were 1.70–3.80 mg/kg [31]. The most abundant volatile compounds in JIUYAO were hexanoic acid ethyl ester, 2,3-butanediol, phenylethyl alcohol, ethyl acetate, pentanoic acid ethyl ester, octanoic acid ethyl ester, 3-methyl-1-butanol acetate, heptanoic acid ethyl ester, nonanoic acid ethyl ester, and 1-hexanol, with a total content of 4940.79 μg/kg, accounting for 60.92% of the total volatile compounds detected.

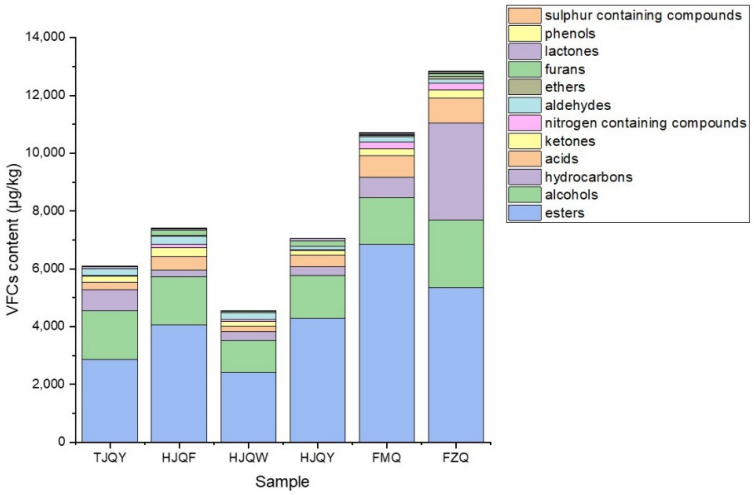

Figure 5. Contents of volatile flavor compounds in six JIUYAOs.

Esters accounted for the largest proportion in JIUYAO, with a total content of 2410.17–6854.59 μg/kg, accounting for 47.13–64.02% of the total volatile compounds detected. The high content of esters contributes to the fruity, floral, and sweet flavor of JIUYAO [7]. Hexanoic acid ethyl ester, with an aroma of sweet and banana [18], was the highest concentration among the ester compounds. Ethyl acetate, pentanoic acid ethyl ester, octanoic acid ethyl ester, 3-methyl-1-butanol acetate, heptanoic acid ethyl ester, and nonanoic acid ethyl ester were also important esters in JIUYAO. The ester content in FMQ was the highest, and the content of hexanoic acid ethyl ester (3187.42 μg/kg) was also the highest among the six JIUYAO, accounting for 47% of all ester compounds in FMQ. The contents of hexanoic acid ethyl ester in the other five samples of JIUYAO were 1147.49–2170.86 μg/kg. The contents of ethyl acetate and 3-methyl-1-butanol acetate in FZQ were the highest: 815.59 μg/kg and 677.81 μg/kg, respectively. Ethyl acetate has a fruity aroma [9], while *Wickerhamomyces* has a strong ability to produce ethyl acetate [7]. 3-Methyl-1-butanol acetate is an important compound in huangjiu with a fruity and sweet flavor [18]. Generally, yeast can metabolize to produce acetate [32], and acetyl coenzyme A or aliphatic coenzyme A and alcohols in yeast cells are catalyzed by alcohol acyltransferase to produce ethyl acetate and 3-methyl-1-butanol acetate, which are acetate or ethyl ester compounds [33,34]. The extracellular esterase produced by some molds is also one of the ways to synthesize esters. For example, pentanoic acid ethyl ester and butanoic acid ethyl ester are mainly produced in this way [35]. These esters are consistent with the research results of waxy maize huangjiu, the traditional fermentation of huangjiu, etc. [12,36]. Low-content compounds, such as benzeneacetic acid

ethyl ester, dodecanoic acid ethyl ester, ethyl (L)-ethyl lactate, and hexadecanoic acid ethyl ester, were also indispensable to the flavor composition of JIUYAO and huangjiu.

The alcohols had a total content of 1116.32–2336.66 μg/kg in the six samples, accounting for 15.01–27.56% of the total volatile compounds detected. 2,3-Butanediol, phenylethyl alcohol, 1-hexanol, and 3-methyl-1-butanol were the alcohols with a higher content in the six JIUYAOs. The content of the alcohols in FZQ was the highest, and the most kinds of alcohols were detected. The content of 1-hexanol, 3-methyl-1-butanol, and 1-octen-3-ol in FZQ was relatively high: 424.89 μg/kg, 357.99 μg/kg, and 101.11 μg/kg, respectively. 2-Borneol was only detected in FZQ. The content of 2,3-butanediol was the highest in TJQY at 1006.08 μg/kg. Its contents in FZQ, FMQ, HJQY, HJQW, and HJQF were 488.59 μg/kg, 398.818 μg/kg, 581.72 μg/kg, 453.94 μg/kg, and 440.93 μg/kg, respectively. Phenylethyl alcohol is aromatic with great contribution to the flavor of huangjiu and is also an important higher alcohol in huangjiu [7]. Phenylethyl alcohol has the highest content (729.64 μg/kg) in HJQF. Its contents in FZQ, FMQ, HJQY, HJQW, and TJYQ were 142.89 μg/kg, 242.11 μg/kg, 409.37 μg/kg, 30.71 μg/kg, and 263.00 μg/kg, respectively. As an important aroma-active compound of huangjiu, phenylethyl alcohol mainly contributed a rose and sweet aroma to huangjiu [20,37]. Phenylethyl alcohol is produced by *Saccharomyces cerevisiae* via the shikimic acid pathway or decarboxylation of phenylpyruvate [38]. Another route is the Ehrlich way, where L-phenylalanine produces phenylethyl alcohol via transamination, decarboxylation, and dehydrogenation [39]. The amount of L-phenylalanine in the raw material is important for phenylethyl alcohol synthesis [40]. As significant higher alcohols in huangjiu, 2,3-butanediol, 1-hexanol, and 3-methyl-1-butanol present a sweet, sweet and green, and an alcoholic and fruity aroma, respectively [41]. The production of higher alcohols is closely related to the metabolism of *Saccharomyces* and *bacteria* during fermentation, while 3-methyl-1-butanol may be most closely related to the interaction of various *Saccharomyces*, the metabolic pathways are mainly the amino acid catabolic metabolism and sugar anabolic metabolism [42,43].

The total contents of acids in JIUYAO were 182.89–862.80 μg/kg, accounting for 4.02–6.98% of the total volatile compounds detected. Formic acid, hexanoic acid, and acetic acid were the most abundant acids, and the contents of the acid compounds in FZQ were the highest, followed by FMQ. Formic acid was detected in FMQ (238.22 μg/kg) and FZQ (488.59 μg/kg) and was hardly detected in the other JIUYAOs. Acetic acid had higher contents in HJQY (189.77 μg/kg) and HJQF (187.31 μg/kg), while the contents of hexanoic acid were higher in FMQ (288.81 μg/kg) and FZQ (175.14 μg/kg). Butyric acid, 2-methyl-propanoic acid, pentanoic acid, and 3-methyl-butanoic acid were all detected in the six JIUYAO simples, which were the acid compounds with a high content in JIUYAO. These acids are closely related to the rich acid-producing micro-organisms, such as *Pediococcus*, *Weissella*, and *Lactobacillus* in JIUYAO [23]. In the process of alcohol fermentation, acetic acid and other compounds are also produced as byproducts [44]. Although the content of acid compounds is not high, it is essential for the formation of the unique flavor of huangjiu by regulating the sweetness, astringency, and bitterness of huangjiu so as to harmonize the aroma and taste of huangjiu [45].

The contents of the aldehydes and ketones were 119.55–277.27 μg/kg and 143.91–310.10 μg/kg, accounting for 1.18–3.84% and 2.04–4.19% of all volatile flavor compounds detected, respectively. Hexanal, benzaldehyde, nonanal, 2-octanone, 2-pentanone, 6-methyl-5-hepten-2-one, 2-undecanone, and 3-hydroxy-2-oxobutane had a high content for the aldehydes and ketones in JIUYAO. Aldehydes have green, fruity, and fatty aroma [9,18]. Benzaldehyde (almond aroma) can play a key role in the formation of the flavor in huangjiu aging [46]. Hexanal and nonanal were important sources of aroma in the cooked grain in huangjiu [47]. Aldehydes and ketones can be produced by mold, which has the ability to secrete lipase [7]. The fatty acids produced by lipase hydrolysis can be further decarboxylated, oxidized, and reduced to form aldehydes and ketones [48]. In huangjiu, they may also be brought in by jiu Qu [49], so the influence of aldehyde and ketone in JIUYAO on the flavor of huangjiu deserves attention.

A variety of nitrogenous compounds, such as pyrazines, were also detected in the six JIUYAO samples. Their contents in FMQ (238.30 μg/kg) were the highest, followed

by FZQ (233.83 μg/kg). Among them, nine pyrazines were detected. The pyrazines of FMQ and FZQ accounted for more than 95% of the total nitrogen compounds. Tetramethylpyrazine, 2,3,5-trimethyl-pyrazine, 2,6-dimethyl-pyrazine, and 2,3-dimethyl-pyrazine are the most abundant nitrogenous compounds. Pyrazine was an important flavor compound in Qu [15,50]. Pyrazines present a baking taste and nutty aroma [9] and were once detected in wheat Qu and huangjiu brewed with wheat Qu [15,49]. The formation pathways of the pyrazines are as follows: (1) nonenzymatic reactions: pyrazine is produced by a Maillard reaction during the drying and heating of the brewing or Qu-making materials [51]; (2) an enzymatic reaction: the microfermentation metabolism of Bacillus. Pyrazine compounds have been detected in Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis, etc. [52,53].

Only two sulfur compounds were detected. Dimethyl disulfide was detected in all six kinds of JIUYAO, and dimethyl trisulfide was detected only in FMQ and FZQ. 2-Pentylfuran had a high content in HJQF, HJQY, and FZQ. γ-Butyrolactone and γ-caprolactone were the lactones with a high content in the six JIUYAO samples, and lactone has a significant contribution to the caramel, coconut, and sweet aroma of rice wine [54,55].

The contents of phenol, p-methyl-phenol, and 4-ethyl-2-methoxy-phenol were higher. The contents of phenol and p-methyl-phenol were the highest in FZQ, which were 29.90 μg/kg and 7.19 μg/kg, respectively. 4-Ethyl-2-methoxy-phenol was detected in FZQ, FMQ, HJQY, HJQF, and TJQY, the contents of which were 3.03, 3.09, 2.81, 6.47, and 1.35 μg/kg, respectively. Phenolic compounds usually have the odor of "medicinal" and "smoky" [43]. The phenolic acid decarboxylase produced by yeast can convert hydroxycinnamic acid into volatile phenol [56]. For example, ferulic acid is converted into 4-ethenyl-2-methoxy-phenol and then is reduced to 4-ethyl-2-methoxy-Phenol [54]. During the production process of JIUYAO, herbal plants will be added to achieve the functions of loosening and bacteriostasis [4]. Chinese herbal medicines usually contain phenolic acids [57], which are the precursors of volatile phenolic compounds [43,54].

There were few reports on terpenoids in JIUYAO. In this study, 21 terpenoids (Table 1) were detected, including limonene, β-caryophyllene, copaene, 7-methyl-3-methylene-1,6-octadiene, γ-terpinene, etc. The content of terpenoids compounds in FZQ was the highest, followed by HJQF and TJQY. Camphene, α-pinene, and 2-borneol were the terpenoids with the highest content, which only existed in FZQ. It has been reported that the total amount of terpenoids in yam huangjiu was about 112.75 μg/kg [58], and the content of terpenoids in Chinese medicinal liquor was higher [59], indicating that the addition of Chinese herbal medicine had a great contribution to the content of terpenoids. Polygonum hydropiper is often added in the production of JIUYAO in Fangxian, which is one of the important sources of terpenoids in JIUYAO. Terpene compounds have certain health functions. It has been reported that β-caryophyllene, β-ionone, linalool, and other terpenes have antitumor, antimicrobia, antiviral, and anti-inflammation activity in vitro [60].

Table 1. Contents of terpene compounds in six JIUYAO.

Number	Terpenoids	TJQY (μg/kg)	HJQF (μg/kg)	HJQW (μg/kg)	HJQY (μg/kg)	FMQ (μg/kg)	FZQ (μg/kg)
1	α-Pinene	-	-	-	-	-	646.10 ± 32.50^{a}
2	Camphene	-	-	0.89 ± 0.09^{a}	-	-	1202.16 ± 143.23^{b}
3	4-Methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane	3.45 ± 0.47^{b}	-	0.96 ± 0.11^{ab}	2.20 ± 0.29^{ab}	-	40.22 ± 3.93^{c}
4	7-Methyl-3-methylene-1,6-octadiene	26.90 ± 1.70^{c}	-	0.67 ± 0.05^{a}	5.94 ± 0.55^{b}	-	66.83 ± 5.50^{d}
5	Limonene	61.80 ± 2.70^{b}	-	-	-	22.16 ± 0.64^{a}	185.87 ± 15.53^{c}
6	γ-Terpinene	1.87 ± 0.26^{ab}	1.65 ± 0.17^{ab}	2.29 ± 0.26^{ab}	2.78 ± 0.42^{b}	-	85.09 ± 3.38^{c}
7	3,7-Dimethyl-1,3,6-octatriene	0.27 ± 0.04^{a}	-	-	-	-	3.50 ± 0.55^{b}
8	1-Dethyl-4-(1-methylethylidene)-cyclohexene	0.73 ± 0.06^{ab}	0.93 ± 0.10^{abc}	1.36 ± 0.16^{bc}	1.87 ± 0.21^{c}	-	11.28 ± 1.36^{d}
9	(-)-α-Thujone	16.91 ± 0.90^{c}	-	-	-	2.13 ± 0.25^{a}	3.31 ± 0.06^{b}

Table 1. *Cont.*

Number	Terpenoids	TJQY (μg/kg)	HJQF (μg/kg)	HJQW (μg/kg)	HJQY (μg/kg)	FMQ (μg/kg)	FZQ (μg/kg)
10	Copaene	9.56 ± 0.34^{a}	-	-	-	-	34.06 ± 3.56^{b}
11	*δ*-Camphor	5.30 ± 0.15^{a}	14.01 ± 0.42^{cd}	14.76 ± 1.08^{d}	12.77 ± 1.04^{c}	7.61 ± 0.10^{b}	24.88 ± 1.09^{e}
12	3,7-Dimethyl-1,6-octadien-3-ol	6.53 ± 0.19^{e}	2.45 ± 0.09^{c}	1.08 ± 0.07^{a}	1.62 ± 0.18^{b}	1.59 ± 0.06^{b}	4.32 ± 0.46^{d}
13	Isophorone	3.95 ± 0.28^{c}	-	0.61 ± 0.02^{a}	-	-	1.06 ± 0.07^{b}
14	*β*-Caryophyllene	12.3 ± 0.93^{a}	143.04 ± 4.95^{d}	33.85 ± 3.42^{b}	56.91 ± 3.27^{c}	16.62 ± 0.33^{a}	39.22 ± 3.25^{b}
15	Terpinen-4-ol	3.22 ± 0.27^{bc}	1.85 ± 0.10^{ab}	4.77 ± 0.48^{cd}	5.56 ± 0.49^{d}	0.78 ± 0.15^{a}	25.93 ± 2.14^{e}
16	Isoborneol	-	0.17 ± 0.02^{a}	-	-	0.55 ± 0.05^{b}	2.94 ± 0.10^{c}
17	*α*-Caryophyllene	1.03 ± 0.12^{a}	5.73 ± 0.03^{e}	1.84 ± 0.27^{b}	3.14 ± 0.10^{c}	-	3.55 ± 0.23^{d}
18	2-Borneol	-	-	-	-	-	156.93 ± 11.33^{a}
19	Carvone	0.30 ± 0.02^{a}	-	-	-	-	0.78 ± 0.09^{b}
20	6,10-Dimethyl-5,9-undecadien-2-one	2.83 ± 0.21^{c}	1.28 ± 0.10^{a}	1.16 ± 0.17^{a}	1.40 ± 0.11^{a}	1.69 ± 0.13^{b}	1.21 ± 0.19^{a}
21	trans-*β*-Ionone	-	-	-	-	-	0.41 ± 0.04^{a}

NOTE: All values were means of triplicate determinations ± SD; "-", not detected; The letters ^{a}, ^{b}, ^{c}, ^{d}, ^{e} were used to indicate the significance; the same marked letter means no significant difference ($p \geq 0.05$), a different marked letter is a significant difference ($p < 0.05$).

3.7. Correlation between Micro-Organisms and Volatile Flavor Compounds

The network relationship between VFCs and the dominant micro-organisms was established by correlation analysis. A total of 538 correlation items were obtained (Table S2), including 166 positive and 372 negative correlation items (adjusted p value ≤ 0.05, $|r| > 0.7$). A total of 167 correlations were obtained between the flavor compounds with a VIP value of more than 1 and a dominant microbial genera (Table S3), including 84 positive correlations and 83 negative correlations (Figure 6). *Pediococcus* was positively correlated with 34 compounds, including formic acid, hexanoic acid, octanoic acid, acetic acid, ethyl benzoate, and 1-hexanol, to facilitate the release of most of the esters. It was negatively associated with benzeneacetaldehyde, phenylethyl alcohol, and 1-(1H-pyrrol-2-yl)-ethanone. *Leuconostoc* was negatively correlated with most compounds, such as 7-methyl-3-methylene-1,6-octadiene, carvone, and bornyl acetate. *Wickerhamomyces* was positively correlated with nonanoic acid ethyl ester, nonanoic acid, and (E)-2-octenal. There was a positive correlation between *Saccharomycopsis* and the esters, such as 3-methyl-butanoic acid ethyl ester, 2-methyl-propanoic acid ethyl ester, and propanoic acid ethyl ester. Yeast is a major contributor to ester compounds but also participates in the production of alcohols and acids [61]. *Rhizopus* was positively correlated with hexadecanoic acid ethyl ester and nonanal. It was negatively correlated with esters, such as butanoic acid ethyl ester, 3-methyl-butanoic acid ethyl ester, and acetic acid 2-phenylethyl ester. The correlation analysis showed that lactic acid bacteria were involved in the production of pyrazines, while most yeasts inhibited the production of pyrazines. Bacillus is the main species producing pyrazines [49], but no *Bacillus* was detected in this study. *Millerozyma*, *Clavispora,* and *Apiotrichum* had a positive correlation with phenylethanol. Yeast and *Pediococcus* were involved in the production of most ester compounds. Based on the correlation between the dominant micro-organisms and volatile compounds, it was concluded that *Pediococcus*, *Rhizopus*, *Saccharomycopsis,* and *Wickerhamomyces* were strongly associated with the flavor formation of huangjiu.

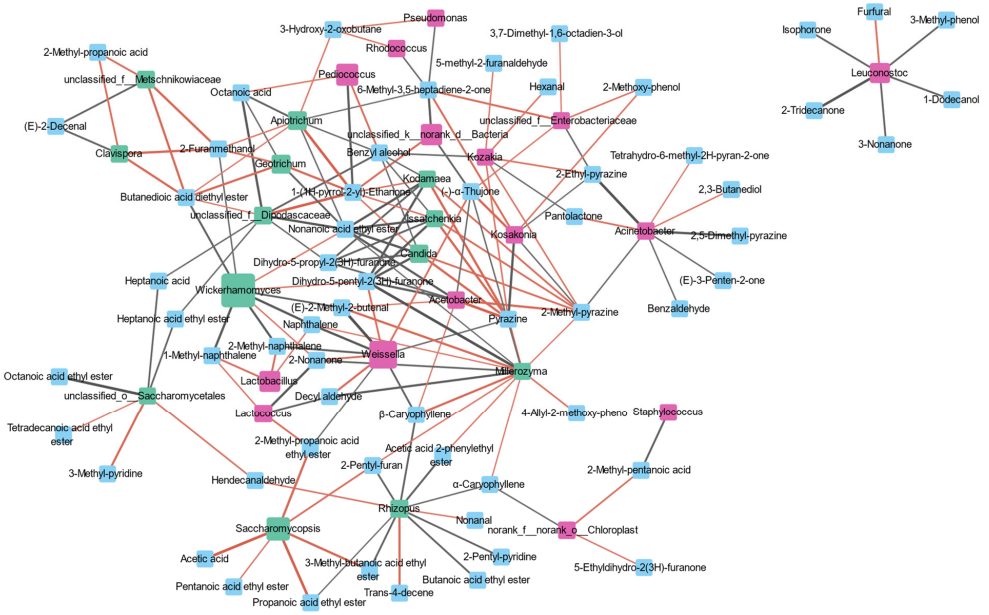

Figure 6. The relationship between flavor compounds (VIP > 1) and dominant microbial genera.

3.8. Functional Microbial Metabolic Pathways

In order to better explain the relationship between the micro-organisms and VFCs, the metabolic network of the dominant genus in JIUYAO was predicted based on the clusters of orthologous groups of proteins (COG) and KO information corresponding to PICRUSt, combining the eggNOG and Kyoto Encyclopedia of Genes and Genomes (KEGG) database corresponding to the metabolic pathways of the featured information and annotation of the related enzymes.

According to the eggNOG database, all these genes were clustered into 23 categories (Figure 7). The functional classification with the highest abundance was S (Function unknown), accounting for 19.29–20.89%, followed by J (Translation, ribosomal structure, and biogenesis), E (Amino acid transport and metabolism), and G (Carbohydrate transport and metabolism), which accounted for 9.35–10.77, 8.07–9.74, and 7.57–8.68%, respectively. The information storage and processing of J, K, L, and the cellular processes and signaling of M, O, and T were closely related to the growth and reproduction of micro-organisms. Metabolism was the highest among the four functional types of COG, accounting for about 39%. E and G, which were related to amino acid and carbohydrate metabolism, were the main functions of metabolism, accounting for 22.49% and 20.98% of metabolism. Nucleotide transport and metabolism, inorganic ion transport and metabolism, energy production and conversion, lipid transport and metabolism, and coenzyme transport and metabolism accounted for 13.31, 13.60, 12.03, 7.87, and 7.74% of metabolism, respectively.

According to the annotation level 1 of the KEGG database (Figure 8), all genes were classified into six major groups, where metabolism was the most abundant, accounting for 75.77% of all annotated genes in JIUYAO micro-organisms, followed by genetic information processing and environmental information processing, accounting for 9.62% and 6.68% of the annotated genes, respectively. A total of 46 level 2 pathways were annotated, and the most abundant pathway in JIYAO micro-organisms were global and overview maps (38.73%), carbohydrate metabolism (10.64%), and amino acid metabolism (5.95%). Energy metabolism (3.80%), nucleotide metabolism (3.78%), metabolism of cofactors and vitamins (3.46%), lipid metabolism (2.66%), metabolism of other amino acids (1.62%), xenobiotics biodegradation and metabolism (1.63%), glycan biosynthesis and metabolism (1.34%), and the Biosynthesis of other secondary metabolites (1.19%) were also important functions of JIUYAO microbiota. These metabolic pathways suggested that JIUYAO had a powerful gene to make use of carbohydrates, amino acids, and fats in brewing raw materials. A total of 369 level 3 pathways were annotated on the KEGG metabolic database. Among

them, 72 metabolic pathways were associated with human diseases, and 10 metabolic pathways had relative abundances greater than 0.1%. Previous studies, monitoring changes in functional micro-organisms during rice wine fermentation, found that the number of gene annotations associated with human disease gradually decreased as fermentation progressed [62]. Due to the production of JIUYAO in an open environment, this enriches a large number of unknown micro-organisms in the production environment, meaning that many metabolic pathways were unknown, or that JIUYAO could have been contaminated with human disease-related genes. There were 15 categories for carbohydrate metabolism associated with winemaking, including KO00520, KO00010, KO00620, KO00500, and KO00030. The relative abundance of five of the metabolic pathways was more than 1%, accounting for 60% of carbohydrate metabolism. Through the prediction of these metabolic pathways, a more comprehensive understanding of the role of micro-organisms in JIUYAO could be obtained.

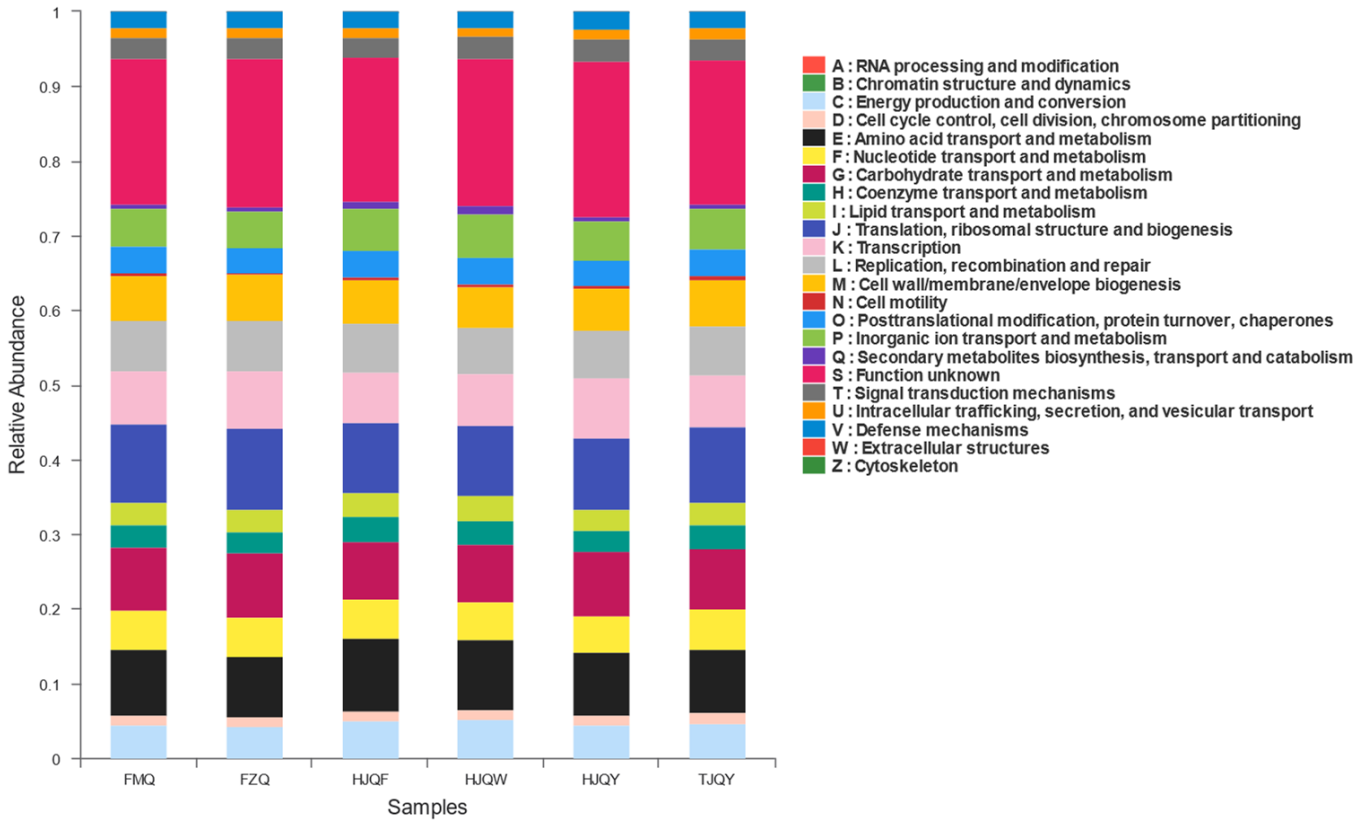

Figure 7. Statistical analysis of COG functional taxonomic abundance.

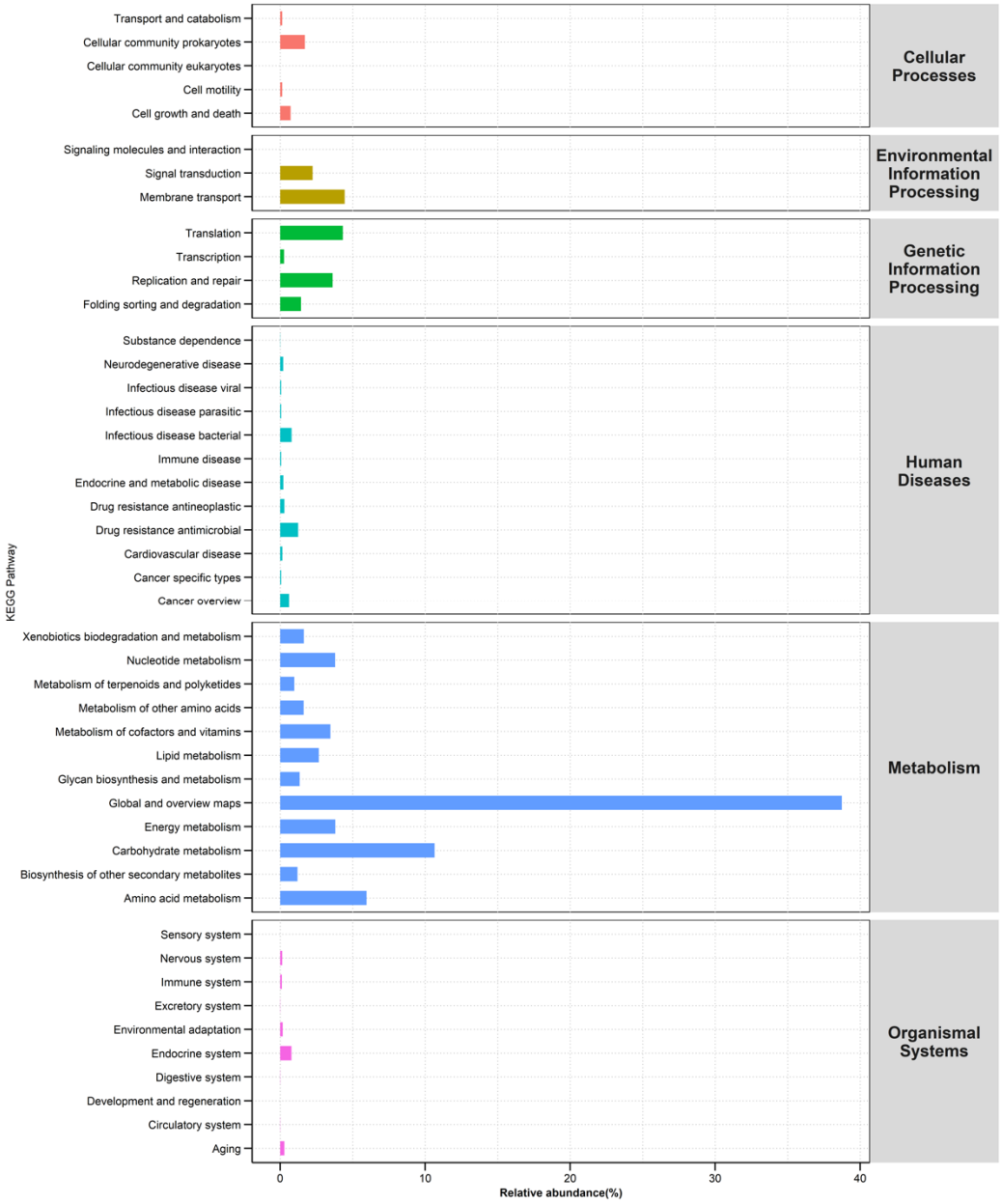

Figure 8. KEGG pathway taxonomic abundance map.

4. Conclusions

Through the analysis of microbial diversity and volatile metabolites in six kinds of JIUYAO from Fangxian, it was found that 15 of the dominant bacterial genera included *Weissella*, *Pediococcus*, *unclasssified_k_norank_d_Bacteria*, *Lactobacillus*, *Leuconostoc*, etc., and the 13 dominant fungal genera included *Wickerhamomyces*, *Saccharomycopsis*, *Rhizopus*, etc. The diversity of bacteria in JIUYAO was higher than that of fungi, and bacteria have a greater influence on the differences in JIUYAO. A total of 191 volatile flavor compounds were detected using HS-SPME and GC×GC-MS. Esters, alcohols, and acids accounted for a large proportion of JIUYAO, and 21 terpenoids were also detected for the first time. Ethyl caproate, ethyl acetate, 2,3-butanediol, and phenylethanol were important compounds that constituted the flavor of JIUYAO. In addition, the microbial function prediction in JIUYAO found that global and overview maps, amino acid metabolism, and carbohydrate metabolism were the main metabolic modes. By establishing the correlation between the dominant micro-organisms and volatile flavor compounds, 538 correlations were obtained, which provided more evidence for the correlation between micro-organisms

and the production of flavor compounds. These results provide theoretical guidance for the improvement of the microbial flora of JIUYAO in Fangxian and the improvement of huangjiu flavor.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8120754/s1, Figure S1 Dilution curves of α-diversity index (a1: sobs diversity index of bacterial, a2: sobs diversity index of fungi, b1: Shannon diversity index of bacterial, b2: Shannon diversity index of fungi); Figure S2 Distributions of alpha diversity indices(a1: sobs diversity index of bacterial, a2: sobs diversity index of fungi, b1: ace diversity index of bacterial, b2: ace diversity index of fungi, c1: Chao diversity index of bacterial, c2: Chao diversity index of fungi, d1: Shannon diversity index of bacterial, d2: Shannon diversity index of fungi, e1: Simpson diversity index of bacterial, e2: Simpson diversity index of fungi.); Table S1 Contents of volatile flavor compounds in six JIUYAO; Table S2 The relationship between flavor compounds and dominant microbial genera; Table S3 The relationship between flavor compounds (VIP > 1) and dominant microbial genera.

Author Contributions: W.Z. completed the experiment; W.Z., M.H., Q.R., J.W. and B.S. conceived and designed the experiment; W.Z., Q.R., Z.W. and H.L. analyzed the data of microbial; W.Z. and M.H. analyzed the data of volatile flavor compounds; W.Z., Q.R. and M.H. wrote the manuscript; W.Z., M.H. and Q.R. drew the illustration. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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