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Analysis of Bacterial Diversity in Fermented Grains of Baijiu Based on Culturomics and Amplicon Sequencing

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Abstract: Baijiu is a traditional distilled liquor in China. The unique flavor developed during the fermentation process of Baijiu is closely related to the microorganisms in the fermented grains of the Baijiu. Fermented grain is a solid material that has not been distilled after the fermentation of Baijiu. It is of great significance to study the bacterial diversity in fermented grains and to isolate and culture them to reveal the formation mechanism of the flavor substances in Baijiu. In this study, the diversity of bacteria in fermented grains was studied by the combination of amplicon sequencing and culturomics, and a pure culture of culturable strains was obtained. The results of amplicon sequencing showed that the bacteria detected in the fermented grains were classified into 5 phyla, 8 classes, 24 orders, 34 families, and 45 genera, of which *Lactobacillus* (66.95%) and *Acetobacter* (32.36%) were the dominant genera. By using the method of culturomics, five pH gradients (pH = 3, 4, 5, 6, and 7) of enrichment culture medium and solid medium with different components were designed to enrich and isolate the culturable bacteria in the fermented grains. A total of 323 strains of bacteria were obtained, belonging to 53 genera and 118 species, and 14 suspected new species were obtained. Among the bacteria isolated by the culturomics, 14 genera were detected by amplicon sequencing, and 39 genera were not detected, indicating that the culturomics method can isolate and culture bacteria with low abundance in fermented grains. Compared with the traditional culture method, culturomics expanded the culturable bacteria in the fermented grains to 188%. The diversity of the bacteria in the fermented grains of Baijiu was analyzed by amplicon sequencing and culturomics, and the microbial community composition and relative abundance of fermented grains were comprehensively revealed. At the same time, the acquisition of more pure cultures of culturable bacteria provides more choices for the liquor-making microbial resource library.

Keywords: fermented grains microorganism; bacteria; microbial diversity; amplicon sequencing; culturomics

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1. Introduction

Baijiu is a traditional grain distilled liquor in China, which has a unique flavor and a long history of brewing [1]. The flavor types of Baijiu can be divided into 12 kinds [2], among which the strong flavor, Maotai flavor, light flavor, and Laobaigan flavor are the most popular. Figure 1 shows the distribution of the four flavor types of Baijiu production areas. Fermented grain is a solid material that has not been distilled after fermentation of traditional Chinese Baijiu, and it is the material to be distilled after crushing, steaming, adding an appropriate amount of water, inoculating with Jiuqu (saccharifying starter culture of Baijiu) [3,4], and then fully fermenting. The microorganisms in the fermented grains are very rich, and some of them are closely related to the flavor quality of Baijiu [5,6]. Therefore, it is of great significance to study the diversity of bacteria in the fermented grains, isolate the strains to analyze the flavor formation mechanism of Baijiu, and improve the flavor quality.

Figure 1. Regional distribution of four types of Baijiu in China.

Bacteria are widely present in fermented grains and play important roles in the fermentation and flavor formation of Baijiu. In the early years, traditional culture methods were used to study microorganisms in fermented grains. Zhang et al., used a solid plate culture method to isolate and identify five lactic acid bacteria from fermented grains of Baiyunbian in Hubei Province [7]. Liu et al., used traditional culture technology to isolate and culture acid-producing bacteria from fermented grains of Jiangsu Yanghe, and isolated and cultured six genera, including *Pediococcus*, *Lactobacillus*, *Bacillus*, *Weissella*, *Lactococcus*, and *Acetobacter*. These six genera were positively correlated with the formation of acetic acid and lactic acid in the Baijiu fermentation process [8]. Liu et al., used a sodium carboxymethylcellulose (CMC-Na) liquid medium to isolate and screen three strains with strong cellulose degradation ability from fermented grains, which were classified as *Bacillus*, *Acinetobacter*, and *Gluconobacter* [9]. Dou et al., used different media to isolate and identify 258 strains of bacteria from fermented grains in different fermentation periods during the fermentation of Luzhou-flavor liquor [10].

Fermented grains contain a large number of microorganisms, which are difficult to culture and cannot be isolated successfully by traditional culture methods. In recent years, various non-culture methods have been applied to microbial diversity studies; examples include phospholipid fatty acid (PLFA) spectrogram analysis, Biolog MicroPlate analysis, denaturation gradient gel electrophoresis (DGGE), fluorescence quantitative PCR, single-chain conformation polymorphism (SSCP), amplificon sequencing, metagenomics sequencing, etc. [11–14]. Amplicon sequencing is used to design specific primers for PCR amplification of the target region, and then conduct second-generation sequencing (NGS) to obtain information, such as the community composition and relative abundance in the samples. Sun et al., used the Illumina MiSeq sequencing platform for the first time to study the bacterial succession during the fermentation of fermented grains of the strong-flavor liquor in winter and summer, and a total of 10 families and 13 families were detected [15], respectively. Du et al., used fluorescence quantitative PCR and amplicon sequencing techniques to conduct an absolute quantification of bacterial flora in fermented grains of sesame-flavor liquor by screening a natural internal standard [16]. Zhao used high-throughput sequencing to study the diversity of microorganisms in fermented grains of Maotai-flavor liquor. It was found that the *uncultured_bacterium_f_Bacillaceae* was the dominant genus in the fermented grains, and its relative abundance was between 5% and 12% [17].

Culturomics was proposed in 2012. In their study of human intestinal microflora, Lagier et al., designed 212 different culture conditions for the cultivation of "unculturable" bacteria in the intestine and identified the bacterial species by MALDI-TOF MS and 16S rRNA sequencing [18]. This led to the concept of culturomics, which uses multiple culture conditions to promote the growth of difficult-to-culture bacteria and combines techniques such as MALDI-TOF MS and 16S rRNA sequencing to identify the species information of microorganisms.

Due to the diverse culture conditions designed by culturomics, it has the potential to screen low-abundance microorganisms, which, to a certain extent, supplements the shortcomings of traditional culture techniques. Sun et al., studied the bacterial diversity in pit mud in Baijiu by using culturomics combined with amplicon sequencing, analyzed the bacterial community composition in the pit mud, and achieved pure cultures of many microorganisms [19]. In recent years, the combination of culturomics and the amplicon sequencing method has become a new strategy for analyzing the microbial composition of samples.

2. Materials and Methods

2.1. Sample Collection

Fermented grains of Baijiu were collected from Rongxiantang Distillery Co., Ltd. (Chengdu, China), Sichuan Province (104°07′ E, 30°67′ N). Some of the samples were used for amplification sequencing, and the others were used for microbial isolation by culturomics.

2.2. Amplificon Sequencing Sample Pretreatment

Three biological replicates were set for the samples of the fermented grains, and the sample size was set to 10 g. The samples were treated as follows: firstly, the 10 g samples were weighed and packed (5 g each) into two 50 mL straight-mouthed pressed-lid centrifuge tubes. Next, 30 mL of sterile water was added to the centrifuge tubes containing the samples on an ultra-clean bench. Then, a QL-901 scroll mixer was used to scroll for 3–5 min so that the samples were evenly mixed. After vortexing, they were filtered with two layers of sterile gauze. The filtrate was collected in a 250 mL blue cap bottle, and the gauze was washed several times with an appropriate amount of sterile water to recover any residual microorganisms. The filtrate was combined and equally divided into 50 mL straight-mouthed pressed lid centrifuge tubes. Then, the filtrate was centrifuged with a tabletop high-speed frozen centrifuge at 12,000 rpm for 20 min at 4 °C. Finally, the centrifuged precipitates were collected in a 10 mL centrifuge tube.

2.3. DNA Extraction and PCR Amplification

The genomic DNA of the microbial communities in the fermented grains was extracted by an E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). After the genomic DNA extraction, the quality of the DNA was measured using 1% agar gel, and the concentration and purity of the DNA extract were determined by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, NC, USA).

The genomic DNA was amplified using a PCR instrument (ABI, GeneAmp 9700 PCR thermocycler, Los Angeles, CA, USA). The PCR amplification reaction was set to three replicates.

The primers 33 (5′ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGG GTWTCTAAT-3′) were used to amplify the V3–V4 in the highly variable region of the 16S rRNA gene of the bacteria [20]. The PCR polymerase used for amplification was TransStart Fastpfu DNA Polymerase (Beijing TransGen Biotech Co., Ltd., Beijing, China). The PCR amplification procedure was set as follows: predegeneration: 95 °C, 3 min; 27 cycles of denaturation: 95 °C, 30 s; annealing: 55 °C, 30 s; extension: 72 °C, 45 s; and the final elongation: 72 °C, 5 min.

The amplified products were quality-tested using 2% agarose gel, purified, and recovered using a gel recovery kit (Axygen Biosciences, Union City, CA, USA), and quantified using a Quantus fluorometer (Promega, Madison, WI, USA).

2.4. Illumina MiSeq Sequencing and Data Processing

The amplicon sequencing of the pretreated samples was completed by Shanghai Meiji Biomedical Technology Co., Ltd. The purified PCR products were subjected to high-throughput sequencing on the MiSeq platform (Illumina, San Diego, CA, USA). The MiSeq sequencing obtained paired-end sequence data, using fastp and FLASH [21] software for sequence decontamination to obtain optimized data. The sequence decontamination parameters were set as follows: 1. If the filter reads the tail quality value at <20 bases, set

the 50 bp window; if the average quality value in the window is <20, cut the back-end base from the window, filter out the reads below 50 bp after quality control, and remove the reads containing N bases. 2. According to the overlapping relationship between the PE reads, the paired reads were merged into a sequence, the minimum overlap length was 10 bp, and the maximum mismatch ratio allowed by the overlap region of the splice sequence was 0.2. 3. The samples were distinguished according to the barcode and primers at both ends of the sequence, and the sequence direction was adjusted. The barcode allowed a mismatch number of 0, and the primer allowed a mismatch number of 2.

After obtaining pure amplicon data, representative sequences need to be selected as species substitutes. UPARSE (version 7.1, http://drive5.com/uparse/, accessed on 10 September 2020) was used for the operation on the truncated strings at a rate of 97% similarity classification unit (OTUs) clustering, and for identifying and deleting chimeric sequences. The RDP classifier (http://rdp.cme.msu.edu/, accessed on 10 September 2020) was used to classify and analyze the representative sequences of each OTU into seven main levels, kingdom, phylum, class, order, family, genus, and species, providing information on the species composition of the microorganisms.

2.5. Bioinformation Analysis

A bioinformatic analysis of the amplicon data was performed on the Majorbio Cloud Platform (https://cloud.majorbio.com/, accessed on 25 October 2020), including species annotation and assessment, a species composition analysis, comparative sample analysis, evolutionary analysis, and functional prediction analysis.

To analyze the evolutionary relationships among the species, a phylogenetic evolutionary tree was constructed for 45 bacterial genera using Mega software (version 10.0), based on neighbor-joining (NJ) plotting with a bootstrap of 500.

PICRUSt software was used to predict the function of the bacterial OTU. Firstly, the OTU abundance table was standardized by PICRUSt (PICRUSt process stored COG information and KO information corresponding to its Greengenes ID); that is, the effect of the copy number of the 16S marker gene in the species genome was removed. Then, through the Greengenes ID corresponding to each OTU, the COG family information and KEGG Orthology (KO) information corresponding to each OTU were obtained. The COG abundance and KO abundance were calculated.

2.6. Enrichment Culture of Samples

Five media with different pH gradients were designed; the pH values were 3, 4, 5, 6, and 7, and the medium volume was 200 mL. The medium was composed of 0.24% NH_4Cl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% yeast extract, 2% peptone, 0.1% EDTA-Na_2, 0.11% sodium pyruvate, 3.30% glucose, 2.2 mg sodium acetate, 4.5% ethanol, and 3.5% soluble starch. The pH of the medium was adjusted to the corresponding gradient. The media were sterilized by autoclaving. A 10% (*w*/*v*) $NaHCO_3$ solution was filtered and sterilized by autoclave separately from 4.91–9.15 mg (*w*/*v*) of KH_2PO_4 solution. The above two solutions were added to the autoclaved medium (every 200 mL/2 mL).

The five enriched liquid culture media were placed in five 500 mL sealed glass bottles (200 mL of liquid culture medium and 10 g of sediment samples) in a full-temperature oscillating incubator at 25 °C for 0, 5, 12, 21, and 33 days.

2.7. Pure Cultures Isolation

Bacterial growth requires nutrition and an appropriate environment, including a carbon source, nitrogen source, inorganic salts, growth factors, and water, and an environment including the temperature, pH, aerobic condition, and interface. According to the nutrient requirements and growth conditions of the bacteria reported in the literature on fermented grains, different solid plates were selected and designed for the bacterial isolation and culture. The media used for the bacterial culturing are shown in Table 1. A total of nine media were designed for the isolation and culture of the bacteria in the fermented grains.

Table 1. Solid plate medium for bacterial culturomics in fermented grains.

Number	Medium Name (Abbreviation)	Complementary Ingredient	Type of Medium
1	MRS Agar (MRS)	200U/plate Catalase solution	Selective culture medium
2	Actinomycetes Culture Medium (ACM)	–	Selective culture medium
3	Tryptone Soy Agar (TSA)	Vitamin K	Non-selective culture medium
4	R2A Agar (R2A)	5% sterile defibrillated sheep blood	Non-selective culture medium
5	ISP Medium 2 (ISP2)	–	Selective culture medium
6	ISP Medium 3 (ISP3)	–	Selective culture medium
7	In-situ Simulation Medium (ISM)	Meat extracts, Peptone, NaCl, Glucose	In situ simulated culture medium
8	Lactobacillus Selective Agar (LSA)	200U/plate Catalase solution	Selective culture medium
9	Nutrient Agar (NA)	–	Non-selective culture medium

Note: "–" indicates no supplementary ingredient.

One mL enrichment culture suspensions of the different pH gradients were absorbed, respectively, during the specified enrichment days as the mother liquor (the concentration was specified as one). According to the 10-fold gradient dilution method, aseptic water was added successively under aseptic conditions for full shock dispersion and diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} in aseptic tubes. Then, 200 µL of diluent of various concentrations was absorbed and coated on the solid plate medium with a coating rod. The diluent was incubated continuously at 30 °C for 40 days. Single colonies were selected every 2–15 days during the culture. The colony-picking interval depended on the colony growth conditions. The single colonies were purified on the nutrient agar (NA) more than three times by the three-line method to obtain a pure culture of the strain.

2.8. Preliminary Identification of Bacteria

The species of bacteria in the fermented grains were identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and bacterial 16S rRNA gene sequencing.

The main principle of MALDI-TOF MS for identifying microorganisms is to establish a database by using the fingerprint of the ribosomal protein of known bacterial species, obtain the ribosomal protein fingerprint of the bacteria to be identified by mass spectrometry, and compare it with the reference fingerprint in the database to obtain the identification result [22]. A fresh sample of pure colonies was selected; the gun head was used to pick out an appropriate number of single colonies on the target plate and smear them evenly; 1 µL of lysate 1 (formic acid) was added by drops and dried; 1 µL of the matrix solution (lysate 2, buffer, by the matrix) was added by dropsand dried; then, the sample was put into the automatic microbial mass spectrometer for target shooting. The mass fingerprint of the ribosome protein in the bacteria to be identified was compared with the mass spectrum of the bacteria in the database. If the score interval matched the mass spectrum result (9,10], the result was reliable. For strains that had not been successfully identified after three repeated operations, the species information was determined by 16S rRNA gene sequencing.

A TIANGEN Bacterial DNA kit (TIANGEN Biotech Beijing Co., Ltd., Beijing, China) was used to extract the bacterial genome, according to the instructions. After the genome extraction was completed, it was used as a template for the PCR amplification. Premix Taq (Ex Taq™ Version 2.0 plus dye) (Takara Biomedical Technology (Beijing, China) Co., Ltd.), a 25 µL PCR reaction system, and bacterial universal primers 27F (5′-AGAGTTTGATCCTGGC TCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) were selected to amplify the full length of bacterial 16S rDNA. The PCR procedure was 94 °C for 3 min and 30 cycles; 94 °C for 30 s; 55 °C for 30 s; 72 °C for 2 min; and 72 °C for 5 min.

The quality of the PCR-amplified products was tested by 1% agarose gel electrophoresis. If clear bands appeared at about 1500 bp, they were considered qualified. The PCR products with the qualified bands were sequenced bidirectionally and spliced by Sangon Biotech Co., Ltd. (Shanghai, China). The sequencing results were identified using the NCBI BLAST [23]

(https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 20 December 2020) and EzBioCloud (https://www.ezbiocloud.net/, accessed on 20 December 2020) [24] sequence comparison.

Lagier et al., found from a study and summary of a large number of human gut microorganisms that 16S rRNA gene sequencing of an isolated pure culture could be classified as a potential new species if its sequencing result was <98.65% similar to the most similar model strain [25]. However, if it is to be determined as a new species, it cannot be judged from the 16S rRNA gene similarity alone. The whole genome of the potential new species should be determined and analyzed for DNA–DNA hybridization (DDH) and average nucleotide identity (ANI) values with the whole genome of the most similar model strain. If the isolated suspected new species has a DDH < 70% and an ANI < 95% of the closest model species, the isolated strain can be recognized as a new bacterial species [26].

2.9. Preservation of Bacteria

The isolated and identified pure cultures of bacteria were scraped from the plate medium, mixed with the bacteria-preserving solution containing 30% glycerol and 70% liquid LB medium, and evenly added into the freezing tube. The freezing tube containing the bacteria suspension was numbered and put into the plastic box for storage and storage at a −80 °C ultra-low temperature.

3. Results and Discussion

3.1. Amplicon Sequencing of Bacteria

Three bacterial amplicons were sequenced from the samples of fermented grains, and 143,056 optimized sequences and 60,233,065 effective bases were obtained. The average sequence length was 421 bp. The sample sequencing information is shown in Table 2.

Table 2. Bacterial amplicon sequencing sample information statistics.

Sample Name	Sequence Number	Base Number	Mean Length	Minimum Sequence Length	Longest Sequence Length
Fermented grains 1	48430	20354273	420.282325	277	450
Fermented grains 2	46219	19488183	421.648738	287	478
Fermented grains 3	48407	20390609	421.232652	277	452

The samples of fermented grains were analyzed at the OTU level. The species with sequence numbers greater than or equal to 5, and the sum of sequence numbers greater than or equal to 20, were kept, and the samples were drawn-out based on the minimum sequence number of the samples (46,219). In total, 64 OTUs were obtained by 97% OTUs clustering, which were classified into 1 kingdom, 5 phyla, 8 classes, 24 orders, 34 families, and 45 genera.

The Shannon dilution curve of bacterial OTU levels in the fermented grain samples showed that the curve tended to be stable with the increase in sequencing depth, indicating that the data quantity of the fermented grain samples was reasonable, the species richness of fermented grains samples was high, and the sequencing depth was sufficient (Supplementary Figure S1).

3.2. Analysis of Bacterial Community Structure

The species abundance of the samples was counted at the taxonomic level of class, order, family, and genus. Community pie charts were used to visualize the bacterial species community composition of the samples of fermented grains, and the species with an abundance of less than 0.01% in all the samples were assigned to others. At the class level, Bacilli was the most abundant (67.26%), followed by Alphaproteobacteria (32.47%) and Gammaproteobacteria, Actinobacteria, and Clostridia with lower abundances (Supplementary Figure S2A). At the order level, Lactobacillales was the most abundant bacterial order (67.04%), which was the dominant order under the Bacilli, followed by Acetobacterales (32.46%) (Supplementary

Figure S2B). At the family level, Lactobacillaceae was the most abundant (66.99%) and was the dominant family under the Lactobacillales, and Acetobacteraceae, which is the dominant family under Rhodospirillales (Supplementary Figure S2C) was also abundant (32.46%). At the genus level, as shown in Figure 2, *Lactobacillus* was the most abundant bacterial genus in the mash sample (66.95%), followed by *Acetobacter* with 32.36%, *Kroppenstedtia,* accounting for about 0.13% in the mash sample, and the unclassified representatives of Acetobacteraceae family (unclassified_f__Acetobacteraceae), *Acinetobacter*, *Bacillus*, *Enterobacter*, *Weissella*, *Pediococcus*, *Ralstonia*, *Brevibacterium*, *Staphylococcus,* and *Brachybacterium* were less abundant in the fermented grain samples (approximately 0.01–0.1%). The relative abundances given in the section were based on the mean of three replicates.

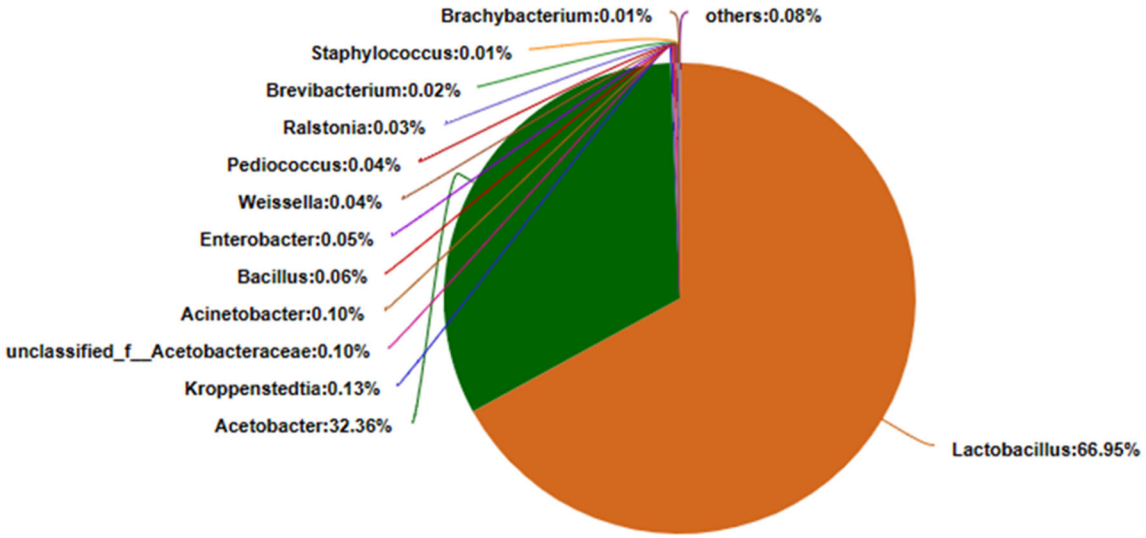

Figure 2. Bacterial community composition of fermented grain samples at genus level. Note: Relative abundances given in the figure were based on the mean of three replicates.

By changing the others' merge values, it was found that the middle rank of the bacterial species in most taxonomic lineages did not have scientific names and were marked with "no rank". After a taxonomic comparison, according to the screening of the confidence threshold, there will be some taxonomic lineages below the confidence threshold, do not receive classification information, and use "Unclassified" as a mark without classification information in the statistics. It shows that there are many examples of "dark matter" in the wine mash [27], that is, unknown bacterial microbial species resources to be elucidated, which suggests that the isolation and culture of microorganisms in the wine paste need to be further studied.

A community heat map analysis was performed on 45 bacterial genera obtained by the OTU clustering analysis (Figure 3), and the similarity in abundance between the species was clustered, so that the high-abundance and low-abundance bacterial genera were clustered in blocks, and it could be seen that *Lactobacillus* and *Acetobacter* were the dominant bacterial genera in the three wine brew samples.

A single-factor correlation network analysis was performed on the top 20 bacterial genera's relative abundance in the fermented grain samples, as shown in Figure 4. Two major clusters were identified in the network diagram, each of which contained a large number of nodes, and *Ralstonia* had little correlation with the other genera. The first 20 bacterial genera are distributed in four phyla, namely Actinobacteriota, Firmicutes, Proteobacteria, and Cyanobacteria. *Lactobacillus* and *Acetobacter*, as the two genera with the highest abundance, were highly correlated with the other bacterial genera. *Lactobacillus* was associated with eight genera; *Ralstonia* and *Lactococcus* were positively correlated; and the unclassified_f__Acetobacteraceae, *Enterobacter*, *Ruminococcus*, *Acinetobacter*, *Pseudomonas*, and *Sanguibacter* were negatively correlated. *Acetobacter* was associated with five genera,

respectively, with the unclassified_f__Intrasporangiaceae, *Brevibacterium*, *Brachybacterium*, and *Bacillus* positively correlated, and negatively correlated with *Rhodococcus*.

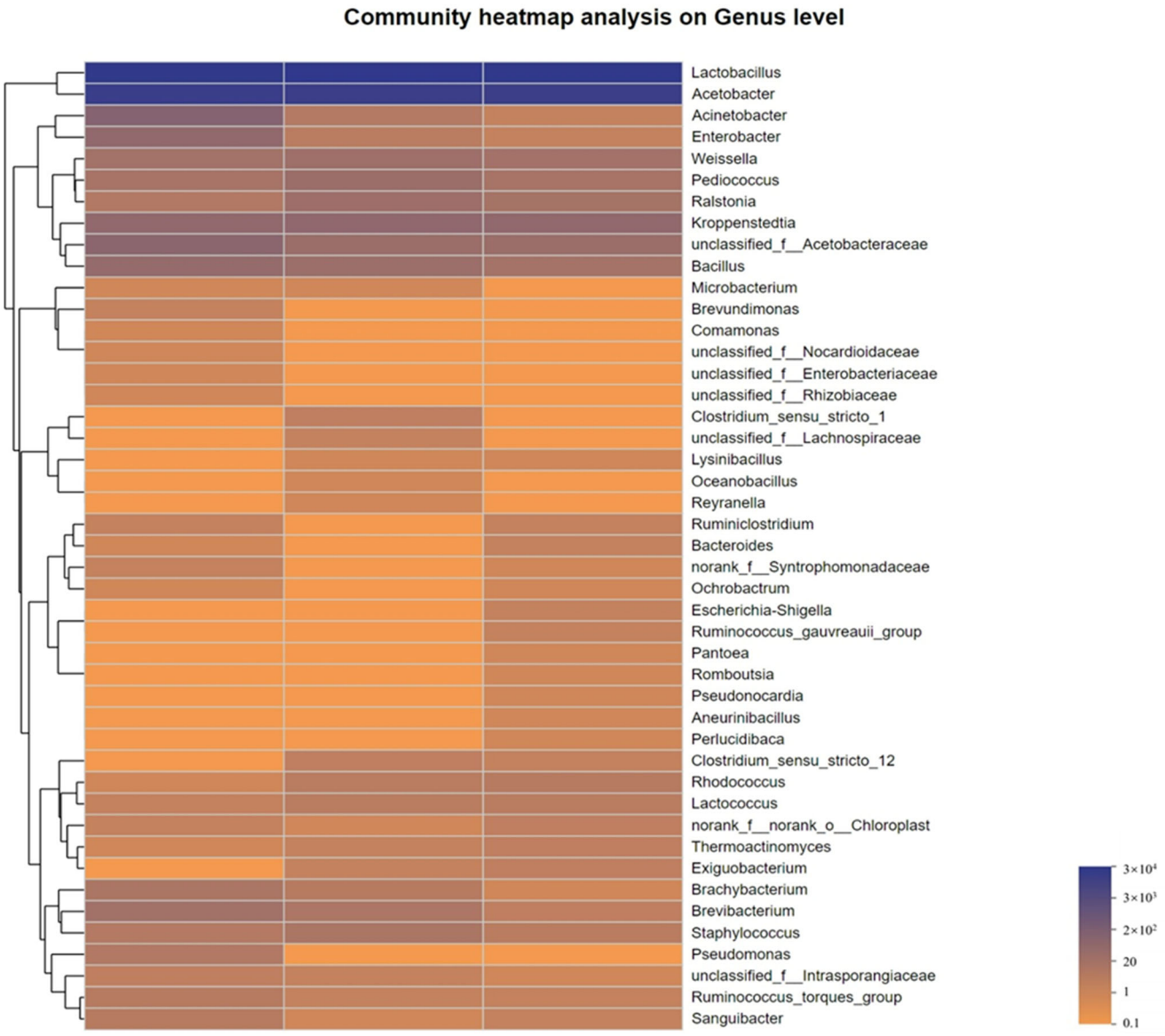

Figure 3. Heat map of horizontal communities of bacteria in fermented grain samples. Note: the color gradient of the color patch represents the change in abundance of different species in the sample, with the species cluster tree on the left and the value represented by the color gradient on the right.

3.3. Construction of Phylogenetic Evolutionary Trees

In order to analyze the evolutionary relationships between the species, a phylogenetic evolutionary tree of 45 bacterial genera was constructed by the NJ (neighbor-joining) method, as shown in Figure 5. The results showed that, in the five phyla, the evolutionary distance between the different bacterial genera was significantly different, and some bacterial genera in the same phyla were also far from each other, such as *Lactobacillus* and *Clostridium*.

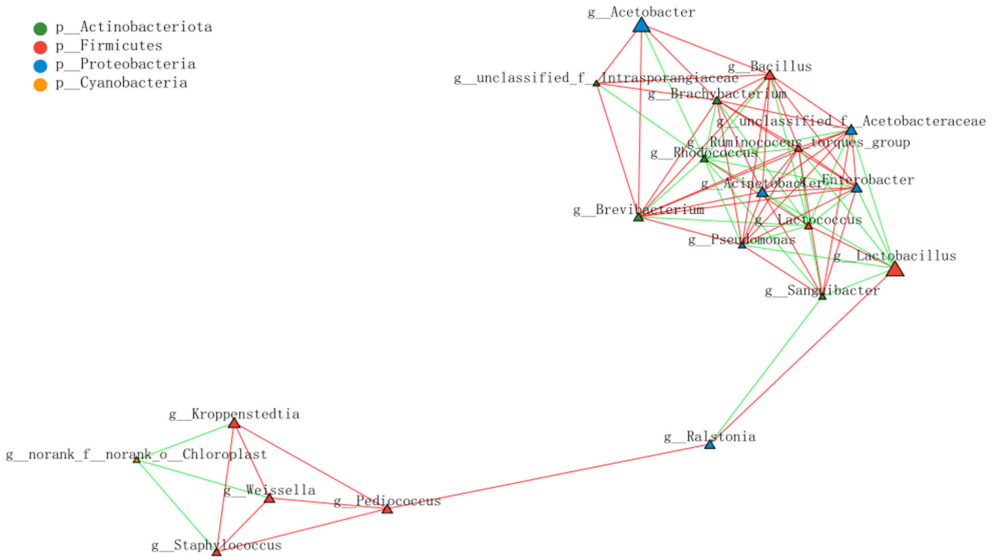

Figure 4. Horizontal correlation network analysis of bacteria genera in fermented grain samples. Note: The size of the regular triangle represents the abundance of species, and the different colors represent different phyla. The colors of the line represent positive and negative correlations: red represents positive correlations and green represents negative correlations. The more lines there are, the more closely related the species is to other species.

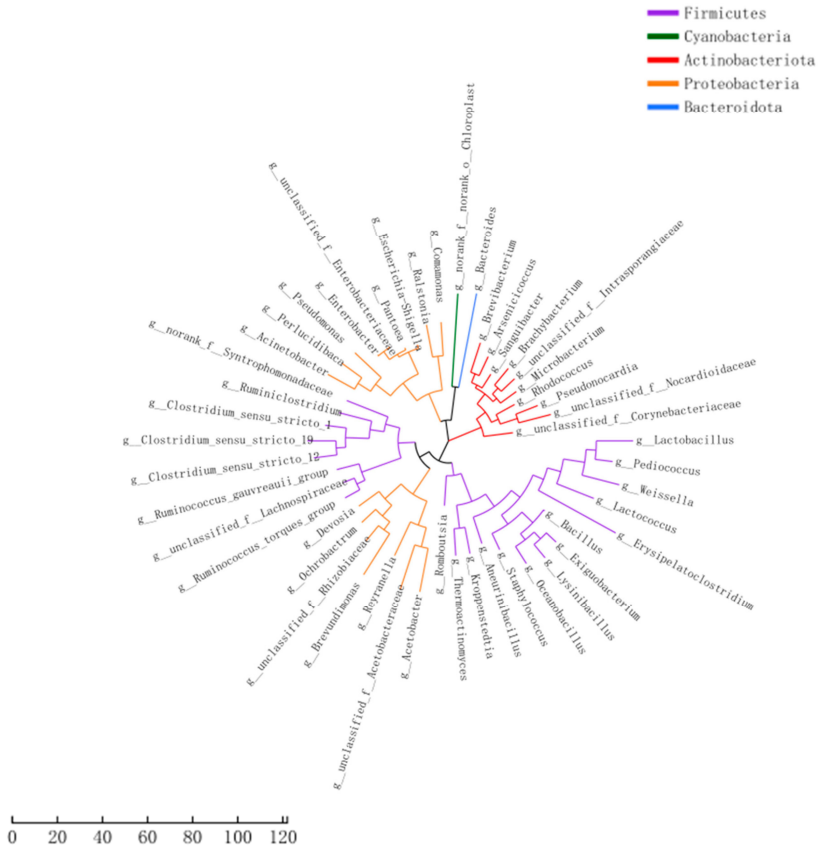

Figure 5. Phylogenetic tree of bacteria in fermented grain samples. Note: each branch corresponds to a genus detected by amplicon sequencing, and the length of the branch is the evolutionary distance between the two genera, i.e., the degree of difference between the genera.

3.4. Functional Predictive Analysis

A COG function prediction was carried out for the bacteria OTUs in the fermented grain samples, as shown in Figure 6. The number of genes in the OTUs corresponding to the unknown function (S) in the fermented grain samples was the highest; that is, the functional abundance was the highest, followed by the replication, reconstitution, and repair functions (L), amino acid transport and metabolism functions (E), cell wall/membrane/biogenesis functions (M), carbohydrate transport and metabolism (G), translation and ribosome structural functions (J), and inorganic ion transport and metabolism (P). The transcription function (K) and energy production and conversion function (C) were abundant, corresponding to the Greengenes ID of more than 600,000 OTUs. However, the RNA processing and modification functions (A), chromatin structure and kinetic functions (B), and cytoplasmic skeleton function (Z) had very low functional abundance, all corresponding to Greengenes IDs of OTUs below 7000. According to the COG functional pathway annotation, the Greengenes ID'd functional abundance of the bacteria OTUs in the fermented grain samples had significant differences in different functions.

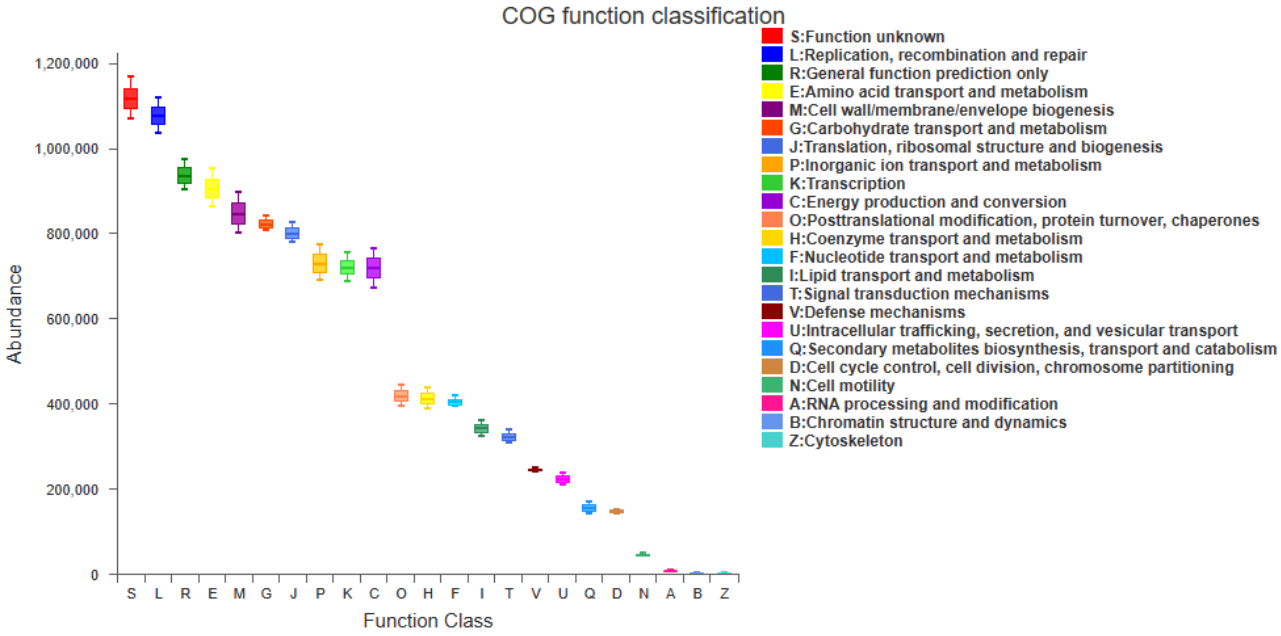

Figure 6. COG function annotation of bacterial OTU in fermented grains sample. Note: The abundance here refers to the functional abundance, that is, the number of genes in the OTUs corresponding to various predictive functions.

The KEGG pathways of the bacterial OTUs in the fermented grains samples were functionally annotated at the level of Pathway level1, Pathway level2 and Pathway level3, respectively. The results are shown in Figure 7. At Pathway level1, four functions were annotated, including metabolism, gene information processing, environmental information processing, and cellular processes, among which metabolism had the highest abundance. At Pathway level2, a total of 16 functions were annotated. The functional abundance from high to low was carbohydrate metabolism, amino acid metabolism, lipid metabolism, energy metabolism, cofactor and vitamin metabolism, terpenoid and polyketide metabolism, nucleotide metabolism, biodegradation and metabolism, other amino acid metabolism, polysaccharide biosynthesis and metabolism, replication and repair, folding, classification and degradation, translation, membrane transport and signal transduction, and cell growth and death. At Pathway level3, the functions with higher abundance were ABC transporters, purine metabolism, ribosome, pyrimidine metabolism, oxidative phosphorylation, glycolysis/gluconeogenesis, aminoacyl-tRNA biosynthesis, the two-component system, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, fructose and mannose

metabolism, cysteine and methionine metabolism, porphyrin and chlorophyll metabolism, the bacterial secretion system, butanoate metabolism, arginine and proline metabolism, pentose phosphate pathway, alanine, aspartate and glutamate metabolism, glycine, serine and threonine metabolism, and methane metabolism.

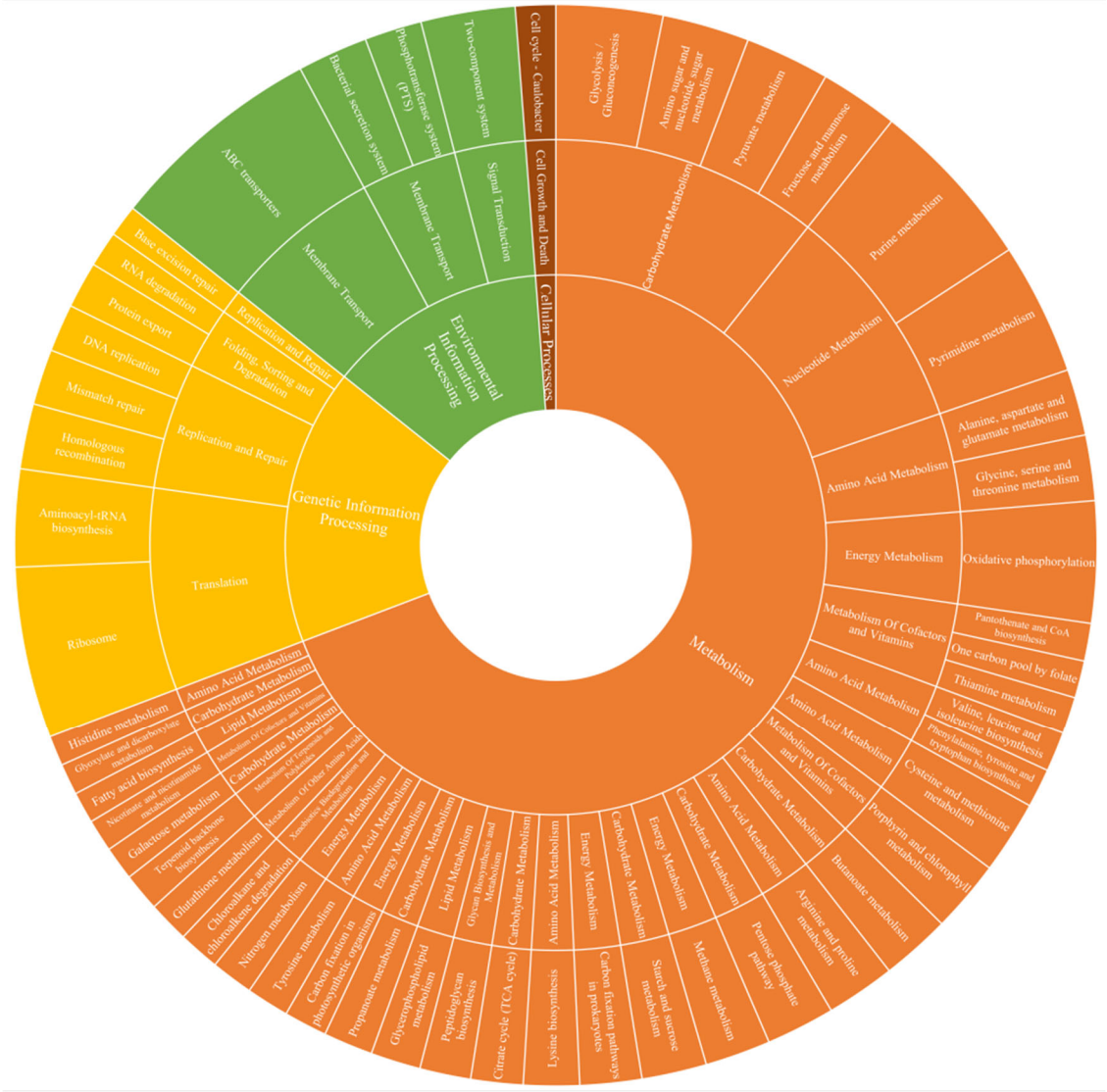

Figure 7. Annotation of KEGG functions of bacterial OTUs in fermented grain samples. Note: The innermost ring, Pathway level1; intermediate ring, Pathway level2; outermost ring, Pathway level3; sector area, OTU abundance size.

3.5. Analysis of Culturomics Results of Bacteria

Bacterial culturomics was used on fermented grain samples, which were enriched, isolated, and cultured, and the pure cultures were identified by MALDI-TOF MS and 16S rRNA sequencing. A total of 53 bacterial genera were isolated and cultured, among which 8 genera were found to be suspected new species, and 118 bacterial species were isolated and cultured. Among them, 11 species were listed as suspected new species, and a total of 323 isolates were isolated and cultured, among which 14 were suspected new species isolates. The results of the bacterial isolation and identification by the culturomics method are shown in Table 3, below, and the results of the suspected new species isolation and identification are shown in Table 4 below.

Table 3. The results of isolation and identification of fermented grains by culturomics.

Number	Genus Name	Specific Epithet
1	*Bacillus*	*B. aerius* *B. aerophilus* *B. altitudinis* *B. anthracis* *B. subtilis* *B. pumilus* *B. oleronius* *B. megaterium* *B. licheniformis* *B. safensis* *B. cereus* *B. kochii* *B. aryabhattai* *B. simplex* *B. toyonensis* *B. methylotrophicus* *B. idriensis* *B. siamensis* *B. amyloliquefaciens* *B. velezensis* *B. australimaris* *B. infantis* *B. marisflavi* *B. zhangzhouensis* *B. invictae* *B. thuringiensis* *B. stratosphericus*
2	*Microbacterium*	*M. paraoxydans* *M. paraoxydans* *M. testaceum* *M.trichothecenolyticum* *M. resistens* *M. esteraromaticum* *M. ginsengisoli*
3	*Acetobacter*	*A. oryzoeni* *A. pasteurianus*
4	*Brevundimonas*	*B. diminuta* *B. aurantiaca* *B. faecalis* *B. albigilva* *B. vancanneytii*
5	*Pseudomonas*	*P. fluorescens* *P. oryzihabitans* *P. hibiscicola*
6	*Ralstonia*	*R. pickettii*
7	*Enterobacter*	*E. hormaechei* *E. cloacae*
8	*Rhizobium*	*R. radiobacter*
9	*Variovorax*	*V. paradoxus*
10	*Stenotrophomonas*	*S. maltophilia*

Table 3. *Cont*.

Number	Genus Name	Specific Epithet
11	*Paracoccus*	*P. yeei* *P. chinensis* *P. salipaludis*
12	*Providencia*	*P. rettgeri*
13	*Micrococcus*	*M. flavus* *M. luteus*
14	*Brevibacterium*	*B. frigoritolerans* *B. casei* *B. aureum*
15	*Moraxella*	*M. osloensis*
16	*Sphingomonas*	*S. aquatilis* *S. melonis* *S. carotinifaciens*
17	*Cupriavidus*	*C. metallidurans*
18	*Ochrobactrum*	*O. anthropi*
19	*Staphylococcus*	*S. hominis* *S. gallinarum* *S. haemolyticus* *S. capitis*
20	*Nocardioides*	*N. exalbidus*
21	*Lysinibacillus*	*L. sphaericus* *L. massiliensis* *L. boronitolerans*
22	*Gordonia*	*G. rubripertincta*
23	*Streptomyces*	*S. griseoruber* *S. scabrisporus*
24	*Acinetobacter*	*A. lwoffii*
25	*Kocuria*	*K. rosea*
26	*Brachybacterium*	*B. paraconglomeratum*
27	*Micromonospora*	*M. aurantiaca* *M. echinospora*
28	*Klebsiella*	*K. aerogenes*
29	*Methylobacterium*	*M. extorquens* *M. hispanicum* *M. aquaticum*
30	*Rhodococcus*	*R. yunnanensis*
31	*Tsukamurella*	*T. tyrosinosolvens*
32	*Alcaligenes*	*A. faecalis*
33	*Enterococcus*	*E. gallinarum*
34	*Paenibacillus*	*P. barengoltzii* *P. alvei*
35	*Neobacillus*	*N. mesonae*
36	*Serratia*	*S. marcescens*

Table 3. *Cont.*

Number	Genus Name	Specific Epithet
37	*Rummeliibacillus*	*R. suwonensis* *R. stabekisii* *R. pycnus*
38	*Novosphingobium*	*N. panipatense*
39	*Pseudochrobactrum*	*P. asaccharolyticum*
40	*Sphingobacterium*	*S. daejeonense*
41	*Bosea*	*B. eneae*
42	*Chryseobacterium*	*C. taklimakanense* *C. hominis*
43	*Paraburkholderia*	*P. fungorum*
44	*Agrobacterium*	*A. deltaense*
45	*Pigmentiphaga*	*P. kullae*
46	*Brevibacillus*	*B. choshinensis* *B. nitrificans*

Table 4. Suspected bacteria new species isolated by culturomics.

Strain Number	Genus Name	Closest Type Species	BLAST Similarity	EZ Similarity
H9	*Sphingomonas*	*S. aquatilis*	100	98.34
H18	*Microbacterium*	*M. proteolyticum*	99.93	98.86
H14	*Microbacterium*	*M. proteolyticum*	100	98.93
H56	*Curvibacter*	*C.lanceolatus*	98.39	98.19
HC15	*Microvirga*	*M. indica*	99.41	98.38
HD21	*Xanthobacter*	*X. flavus*	96.95	96.89
HC47	*Xanthobacter*	*X. flavus*	96.96	96.85
HD30	*Alcanivorax*	*A. pacificus*	96.45	96.10
HC49	*Aneurinibacillus*	*A. aneurinilyticus*	98.74	98.34
HD31	*Cohnella*	*C. nanjingensis*	98.04	97.99
HD23B	*Alcanivorax*	*A. pacificus*	96.73	96.58
H9-1	*Lysinibacillus*	*L. macroides*	98.33	97.39
HD59	*Altererythrobacter*	*A. terrae*	98.9	99.49
HS3	*Microbacterium*	*M. pseudoresistens*	98.24	98.17

Fourteen suspected new species were identified from the samples of fermented grains (Table 4). The 14 strains were classified into eight genera, among which two isolates of *Microbacterium* with close to 100% similarity were identified as H14 and H18, and two isolates of *Xanthobacter* with close to 100% similarity were identified as HD21 and HC47. Two strains with close to 100% similarity were isolated from the genus *Alcanivorax*, whose numbers were HD30 and HD23B, respectively. The other suspected new species were all one strain, and the specific numbers are shown in Table 4.

3.6. Correlation Analysis between Dominant Bacteria and Culture Conditions

The results of the isolation and culture of the bacteria by culturomics showed that the dominant bacteria in the culturable microorganisms were *Bacillus*, *Microbacterium*, *Acetobacter*, and *Rummeliibacillus*. In the enrichment culture conditions with pH values of 3, 4, 5, 6, and 7, the different bacterial genera showed different pH preferences. In total, 86 strains of *Bacillus*, 22 strains of *Acetobacter*, 20 strains of *Microbacterium*, and 18 strains of *Rummeliibacillus* were isolated. The pH gradient correlation analysis was conducted for the above four bacterial genera, and the pH preference of the different bacterial genera

was discussed. The correlation analysis of *Bacillus* was isolated and identified after the enrichment culture was conducted, as shown in Figure 8A. It was found that, under the condition of pH = 5 in the enrichment culture medium, the number of *Bacillus* bacteria isolated was the largest, and the culture condition of pH = 5 may be more conducive to the isolation and culture of *Bacillus* bacteria. The association analysis of the *Acetobacter* isolated and identified after the enrichment culture is shown in Figure 8B. It was found that, under the condition of pH = 4 in the enriched culture medium, the number of *Acetobacter* bacteria isolated was the largest, accounting for 45% of all the *Acetobacter* bacteria, indicating that the abundance of *Acetobacter* may be higher when pH = 4, which is more conducive to the growth of *Acetobacter*. The correlation analysis of the *Microbacterium*, isolated and identified after the enrichment culture, was conducted, as shown in Figure 8C. It was found that, under the condition of pH = 6 in the enriched culture medium, the number of *Microbacterium* isolated was the largest, accounting for 35% of all the *Microbacterium* bacteria, indicating that pH = 6 may be more conducive to maintaining the bacterial diversity of *Microbacterium*. The correlation analysis of the *Rummeliibacillus*, isolated and identified after the enrichment culture, was conducted, as shown in Figure 8D. It was found that, under the condition of pH = 7 in the enrichment medium, almost all the *Rummeliibacillus* bacteria could be isolated, accounting for 94% of all the *Rummeliibacillus* bacteria, indicating that the enrichment condition of pH = 7 was conducive to the enrichment of *Rummeliibacillus*, and this enrichment condition could increase their abundance and diversity.

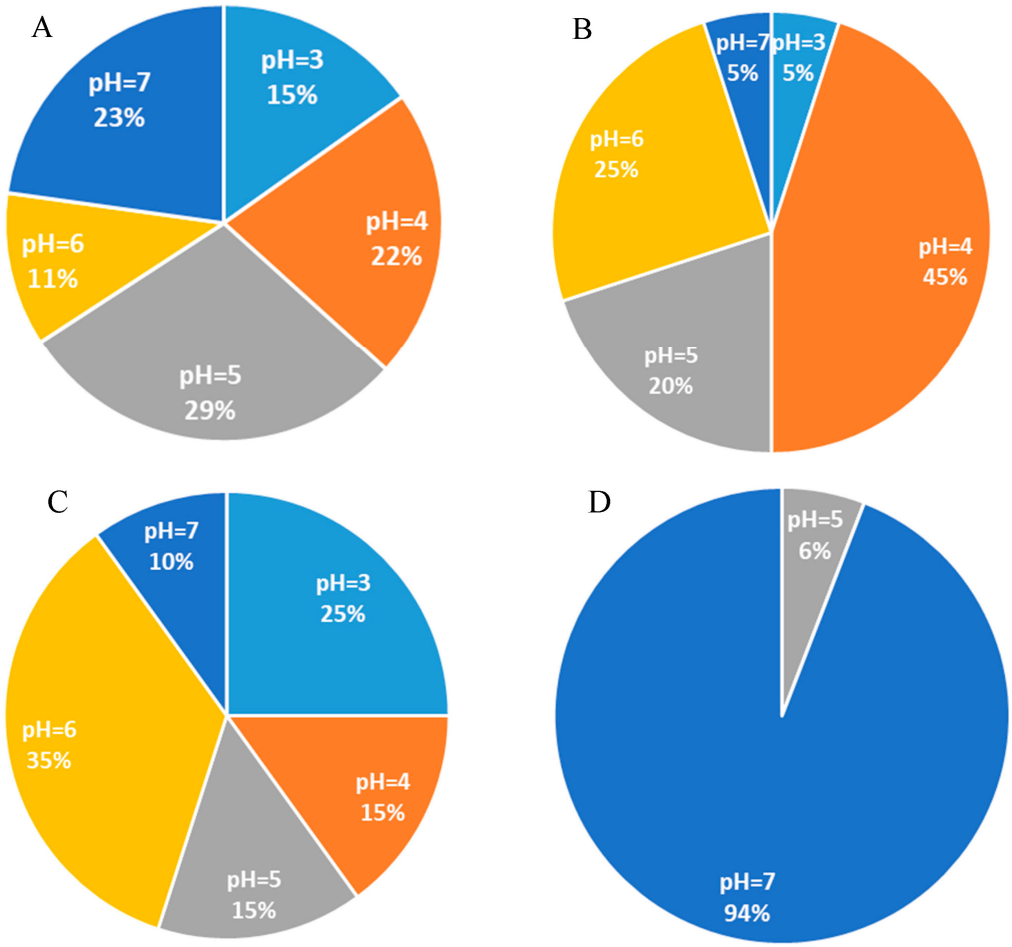

Figure 8. Relationship between dominant bacterial genera and pH of enriched culture conditions. (**A**) Bacillus, (**B**) Acetobacter, (**C**) Microbacterium, and (**D**) Rummeliibacillus.

3.7. Comparison of Bacterial Differences in Fermented Grains Cultured by Culturomic, Amplicon Sequencing and Traditional Culture Method at the Genus Level

A Venn diagram was drawn using online software (https://bioinformatics.psb.ugent.be/webtools/Venn/, accessed on 17 February 2023). The differences at the genus level between the bacteria in the fermented grains obtained by culturomics, amplicon sequencing, and traditional culture technology reported in the literature were compared, as shown in Figure 9. A total of 45 genera of bacteria were detected by amplicon sequencing. The bacteria of 14 genera (*Ochrobactrum*, *Aneurinibacillus*, *Ralstonia*, *Brachybacterium*, *Rhodococcus*, *Lysinibacillus*, *Brevibacterium*, *Acetobacter*, *Pseudomonas*, *Acinetobacter*, *Microbacterium*, *Enterobacter*, *Bacillus,* and *Staphylococcus*) were also cultured by culturomics. The other 31 genera of bacteria have not been isolated and cultured. The reason may be that the designed culture conditions are not suitable for the growth of some bacterial genera, or the bacteria have died or are in a viable but non-culturable (VBNC) state [28]. However, 39 genera of bacteria were only obtained by the culturomics method and not detected by the amplicon sequencing method. It may be, because of their low abundance, they could not be detected by amplicon sequencing, and the enrichment solution of different pH and various solid media designed by culturomics enable some bacterial genera with extremely low abundance to be isolated and cultured. A total of 43 genera of bacteria in the fermented grains were cultured by traditional culture techniques reported in the literature. Among them, 15 genera (*Novosphingobium*, *Paenibacillus*, *Micrococcus*, *Streptomyces*, *Enterococcus*, *Moraxella*, *Klebsiella*, *Acetobacter*, *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, *Microbacterium*, *Enterobacter*, *Bacillus,* and *Brevibacillus*) were also isolated by culturomics. In this experiment, 38 genera of bacteria were isolated from the fermented grains for the first time by culturomics. The culturomics method expanded the number of culturable bacteria in the fermented grains by 188%. This may be due to the differences between the samples and the diversity of the culture conditions.

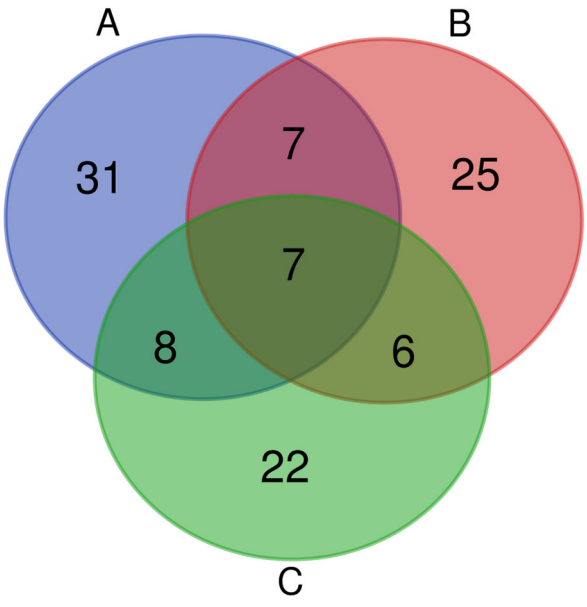

Figure 9. Comparison of genus-level bacteria detected by different methods in fermented grains. (**A**) Culturomics, (**B**) amplicon sequencing, (**C**) traditional culture technology.

4. Conclusions

In this study, the bacterial diversity in fermented grains of Baijiu was studied by the combination of culturomics and amplicon sequencing, and the bacteria in the fermented grains were isolated and cultured. The results of the amplicon sequencing showed that a total of 64 OTUs were obtained after the clustering of the bacterial OTUs in the fermented grains, which were classified into 1 kingdom, 5 phyla, 8 classes, 24 orders, 34 families and

45 genera. *Lactobacillus* was the most abundant genus (66.95%), followed by *Acetobacter* (32.36%), *Kroppenstedtia*, and the unclassified_f__Acetobacteraceae, *Acinetobacter*, *Bacillus*, *Enterobacter*, *Weissella*, *Pediococcus*, *Ralstonia*, *Brevibacterium*, *Staphylococcus*, and *Brachybacterium*. The bacterial community of the fermented grain samples was predicted by a COG functional classification. The results showed that the highest functional abundance in the fermented grain samples was an unknown function (S). At the same time, through the KEGG metabolic pathway prediction of the OTU sequences, it was found that the microbial metabolic function accounted for the main abundance. Through the method of culturomics, five pH gradient (pH = 3, 4, 5, 6, and 7) enrichment solutions, and various solid media were designed to isolate and culture bacteria in the fermented grains. A total of 323 strains of bacteria were isolated, belonging to 53 genera and 118 species. Bacteria belonging to 14 genera (*Ochrobactrum*, *Aneurinibacillus*, *Ralstonia*, *Brachybacterium*, *Rhodococcus*, *Lysinibacillus*, *Brevibacterium*, *Acetobacter*, *Pseudomonas*, *Acinetobacter*, *Microbacterium*, *Enterobacter*, *Bacillus*, and *Staphylococcus*) were detected by both amplicon sequencing and culturomics. No pure cultures of the remaining 31 genera were obtained. Among them, *Lactobacillus*, the absolute dominant genus detected by the amplicon sequencing method, was not obtained by culturomics. It may be because the designed culture conditions still could not meet their growth needs. *Lactobacillus* strains are facultative anaerobic bacteria, which grow faster under anaerobic conditions, and their growth state is poor under aerobic conditions. The limited nutrients in the enrichment solution may have been utilized by the rapidly growing bacteria, resulting in the *Lactobacillus* not being cultured [29]. Compared with traditional culture methods, 38 genera of bacteria were isolated from the fermented grains for the first time in this experiment using culturomics, which expanded the culturable bacteria in the fermented grains by 188%. This experiment also isolated 14 suspected new species, and they will be further studied in the future. At present, many new bacterial species have been isolated from the fermentation system of Baijiu [30–32]. Researchers have developed and utilized some strains to study their growth characteristics and functions [33,34], which is conducive to expanding the microbial resource pool of Baijiu-making and contributing to the development of the quality of Baijiu.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030260/s1, Figure S1: Shannon dilution curve of bacterial OTU levels in fermented grains samples; Figure S2: Bacterial community composition of fermented grains samples.

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