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Correlation between microbial communities changes and physicochemical indexes of Dazu Dongcai during different fermentation periods

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Abstract

Bacteria play the most important role in the fermentation process of Dongcai. At present, the flora in the fermentation process of Dazu Dongcai have not been detected. The physicochemical indexes association with microbiota changes during fermentation have not yet been evaluated. Therefore, it is necessary to explored the correlations between microbial communities and physicochemical indexes during Dazu Dongcai fermentation. Dazu Dongcai samples including five time points were prepared for four year during the spontaneous fermentation process. Microbial community dynamics and associated biochemical changes were characterized using metagenomics sequencing technology and physiochemical properties determination to identify associations between microorganisms and fermentation characteristics. These results indicated that *Staphylococcus, Lactobacillus*, *Terrisporobacter*, *Blautia*, *Romboutsia*, *Bifidobacterium* and *Streptococcus* dominated the whole fermentation process, which were strongly correlated with the physical and chemical indexes. These flora have promoting function during fermentation and may affect the quality of Dongcai. This study provides new detailed insights into the succession of microbial communities and their potential roles in Dazu Dongcai fermentation, so as to establish a correlation analysis model of key factors in the pickling process of Dazu Dongcai, which can lay a foundation for the standardized production of Dazu Dongcai.

Keywords: Dazu Dongcai; metagenomics; physicochemical indexes; microbial community; correlation analysis.

Practical Application: The correlation analysis model of key factors in the pickling process of Dazu Dongcai was established to provide the basis for the influence of microbial community on physicochemical indexes in the fermentation process, lay the foundation for the standardized production of Dazu winter vegetables, and also provide a theoretical reference for the production control of fermented vegetables.

1 Introduction

Dongcai are a kind of semi-dry pickled vegetables, which are divided into Sichuan Dongcai, Beijing Dongcai, Tianjin Dongcai and Shanghai Wuxiang Dongcai. Dazu Dongcai belongs to Sichuan Dongcai. its material is leaf mustard (Zhu, 2019; Song, 2019). The whole brewed process of Dazu Dongcai were from ancient method and have many complex process. The main process is raw material collection, fine selection, upper shelf air dehydration, lower shelf, salt pickling, packing seal, the next day repeated pickling open altar, before and after repeated 14 times more, then out of the altar around hoarding spit astringent water, then into the altar pressure seal, at least 3 years of fermentation, packaging products (Yang et al., 2016). Dongcai has the characteristics of dark and shiny color, rich aroma, delicious flavor, tissue tender crisp, which can improve appetite for food, and is loved by the majority of consumers (Zhang et al., 2021c).

Dongcai contains rich amino acids, lactic acid, protein, vitamins and a variety of trace elements needed by the human body. Through the fermentation process, microorganisms turned protein, sugar, fat and other substances in mustard raw materials into amino acids, alcohols, aldehydes, ketones, esters (Zhang et al., 2021d). Through the interaction of substances produced by microbial fermentation makes Dongcai have special color, fragrance and taste. However, due to the fermentation process is carried out in the open field, the fermentation conditions are difficult to control. The quality of Dongcai is not stable, as the diversity and abundance of microorganisms involved was influenced by environment. Therefore, the change of microbial community structure during fermentation period will make the product quality unstable, resulting in lower production (Wang & Ma, 2016).

In previous studies, the composition and diversity of bacterial communities in Dongcai from Nanchong, Sichuan province were studied. Fermented vegetables are mainly moderately halophilic bacteria, including *Virgibacillus*, *Bacillus megaterium* and *Gracilibacillus saliphilus* (Dong et al., 2012). Sequencing of amplicons targeting bacterial 16S rRNA gene in mustard showed that the bacterial community was dominated by *Halophilic* and *Lactobacillus* (Zhang et al., 2021a). Besides, a bacterial strain with high yield of phenyllactic acid was found in Sichuan Dongcai, and its content was more than 140 mg/L (Deng, 2014). Although some studies have been carried out on the fermentation dynamics and flavor formation of Dongcai, it is still unclear about the changes of bacterial phase and product phase and the correlation between them in the fermentation process of Dongcai, especially for Dazu Dongcai.

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Therefore, this study mainly uses metagenomics technology to study the correlation between the changes of microbial flora and product phase in the pickling process of Dazu Dongcai in different pickling periods, and establishes the correlation analysis model of key factors in the pickling process of Dongcai, which can provides the effect of microbial community on the physicochemical indexes during fermentation and lay a foundation for the standardized production of Dazu Dongcai vegetables.

2 Materials and methods

2.1 Materials acquisition and preprocessing

Dazu Dongcai samples were collected from local enterprises in Dazu District Chongqing, China. Briefly, Dazu Dongcai with four years of fermentation for 0-4 years were taken, and samples were collected from the top, middle and bottom of the fermentation tank, respectively. After taking, the samples were sealed, liquefrozen and transported to the laboratory immediately. Then, the samples were washed with deionized water, dried, sealed and placed in a freezer for further use.

After thawing before detection, fermentation samples were collected at one fermentation time points every one year from fermentation onset through the fourth year. Then, the 15 samples were cut into fragments of 2 mm thickness with cutting machine. which were placed in the crusher, then screen. After the final screening, which was dried at 105 °C for 6 h in an electrothermal constant temperature blast dryer and sealed according to the test year.

2.2 High-throughput sequencing of bacterial 16S rRNA gene

To analyze the composition of bacterial community in the whole fermentation process of Dongcai. Firstly, Microbial DNA was extracted from the Dongcai samples using CTAB method. To analyze the taxonomic composition of bacterial communities during the entire Dongcai fermentation period, two universal primer pairs, including (5'-TACGGRAGGCAGCAG-3') and (5'-GGACTACHVGGGTWTCTAAT-3'), were used to amplify the V3–V4 hypervariable regions of bacterial 16S ribosomal RNA (rRNA) genes. The PCR reactions were performed as described previously (Yang et al., 2020), and amplicons were sequenced on the Illumina NovaSeq platform.

According to the characteristics of the amplified 16S region, the library was subjected to double-terminal sequencing based on the Illumina NovaSeq sequencing platform. Quality filtering of the raw reads was then performed to obtain clean, high-quality Clean Data using the Cutadapt (V1.9.1) program (Itoh et al., 2014). The quality filtered sequences were then assigned to operational taxonomic units (OTUs) using a nucleotide similarity cutoff of 97% in the UPARSE (v10.0) program (Edgar, 2013). UCHIME was used to dected and remove chimeras by PCR amplification (Edgar et al., 2011). Then the OTU abundances were analyzed by the Usearch_global method. Bacterial sequences was taxonomically classified using the RDP classifier by referencing the SLIVA databases, respectively, at the phylum, genus, and species levels. Alpha diversity metrics (including the Chao1, observed species, and Shannon indices) and beta diversity metrics were calculated using the QIIME software program. Principal coordinates analysis (PCoA) based on Bray-Curtis community distances was conducted using R (v.3.5.2)

The results were expressed as mean ± standard deviation. Origin 2018 was used to plot. SPSS 22.0 was used for single factor analysis of variance, spearman correlation analysis and principal component analysis.

2.3 Physiochemical properties determination

Determination of nitrite content

Nitrite in the Dongcai samples was analyzd with spectrophotometry. The nitrite content was determined according to the national standard (Kang et al., 2019). The crushed sample (5 g) was mixed with water and saturated borax solution in a conical bottle (250 mL) in a water bath for 15 min and cooled, and then the extract was precipitated with potassium ferrocyanide solution and zinc acetate solution in a 200 mL volumetric bottle and the supernatant was extracted, finally measured with a spectrophotometer (538 nm) and blank experiment was done. The formula of nitrite content formula is as follows: The nitrite content $X_1 = (m_2 \times 1000) / (m_3 \times V_1 \times 1000 \div V_0)$.

X_1, the content of sodium nitrite in the sample, the unit is mg/kg; m_2, the mass of sodium nitrite in sample solution was determined in micrograms (μg); 1000, conversion coefficient; m_3, sample mass in g (g); V_1, liquid volume of the sample for determination, in milliliter (mL); V_0, sample treatment liquid volume in mL (mL).

Determination of total sugar content

To investigate total sugar contents, 3 g of dried Dazu Dongcai was accurately weighed. The total soluble sugar content was determined according to the method described previously. Put the crushed sample (3 g) and boiling water into a conical flask with a plug for 10 min after cooling and filtration, place the filtrate in a 50 mL volumetric flask, and finally dilute 2 mL of the extract in another 50 mL volumetric bottle for testing. Finally, measured with a spectrophotometer (620 nm) and blank experiment was done. The total soluble sugar formula is as follows: the mass percentage of total sugar=$\left(c \times f \times V_{\text{Sample total}} / 10^6 \times m\right) \times 10$ (c is sugar concentration of standard curve (ug/mL); f is dilution ratio; $V_{\text{Sample total}}$ is total volume of sample (mL); m is quality of sample (g)).

Determination of crude fiber content

Crude fiber contents were determined using national standard method (Zhou et al., 2022). Put the crushed sample (5 g) into 500 mL conical flask, adding 200 mL 1.25% concentrated sulfuric acid and potassium hydroxide and boiling 30 min before and after, then filtered, the washed samples placed in the oven drying and put into the muffle furnace ash. The crude fiber formula is as follows: $X = \left(\frac{G}{m}\right) \times 100\% X$, the content of crude fiber in the sample; g, the mass (or the mass lost by high temperature furnace) in gram (g); m, mass of the sample, in gram (g).

Determination of protein content

Protein content was determined by Kjeldahl nitrogen analyzer (Liu, 2021). 0.5 g of sample was placed in the digestion tube, and 0.4 g of copper sulfate, 6 g of potassium sulfate and 20 mL of sulfuric acid were added to the digestion furnace for digestion. When the temperature of the digestion furnace reached 420 °C, the digestion was continued for 1 h, and the liquid in the digestion tube was green and transparent. After cooling, 50 mL water was added. At the same time, the sodium hydroxide solution and the receiving bottle (10 mL boric acid and 1-2 d mixed indicator) were configured, and they were placed in an automatic Kjeldahl nitrogen analyzer for 6 min of distillation. After distillation, 200 mL of the receiving bottle was set for titration, and the consumption of sulfuric acid standard solution was recorded. At the same time do reagent blank. Protein content Formula 1 is as follows:

$$X = \frac{(V_1 - V_2) \times c \times 0.0140}{m \times V_3 / 100} \times F \times 100 \quad (1)$$

X, protein content in the sample, in gram per 100 g (g/100 g); V_1, the test solution consumed the volume of sulfuric acid or hydrochloric acid standard titration solution, in milliliter (mL); V_2, reagent blank consumption of sulfuric acid or hydrochloric acid standard titration solution volume, unit for mL (mL); c, sulfuric acid standard titration solution concentration, unit for mol per liter (moL/L); m. Sample quality; V_3, absorb the volume of digestive juice, unit for mL (mL); F, nitrogen conversion coefficient to protein, nitrogen conversion coefficient in various foods; 100, conversion factor.

Determination of amino acids content

The contents of 16 free amino acids(FAAs) (i.e., aspartic acid (Asp), gluta-mic acid (Glu), serine (Ser), glycine (Gly), threonine (Thr), alanine (Ala), lysine (Lys), histidine (His), arginine (Arg), tyrosine (Tyr), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), phenyl-alanine (Phe), and cystine (Cys)) in the Dongcai samples were analyzed with Hitachi L-8900 Automatic Amino Acid Analyzer (Zhang et al., 2021b).

2.4 Statistical analyses

All analyses were conducted with three biological replicates, and experimental results are presented as means ± SD. Statistical analysis methods such as T-test, Simper and LEfSe were used to test the significant difference in species composition and community structure of grouped samples (Ma et al., 2018; Kioroglou et al., 2020). Using SPSS statistical software program to analyze the data differences between samples. Significant differences between two groups are denoted by different letters and were identified at the $p < 0.05$ alpha level.

The correlation between microbial abundance, physicochemical characteristics and biochemical parameters (i.e. nitrite, total sugar, crude fiber, protein and amino acid) was analyzed by sequencing species distribution, inter-group differences and physicochemical properties charts.

3 Results

3.1 Changes of physicochemical properties during Dazu Dongcai fermentation

Nitrite, total sugars, crude fiber, protein profiles

Fresh Dazu Dongcai were fermented using traditional methods. The nitrite content of initial fermentation period was 2.11 mg/kg, and its variation exhibited similar patterns with other traditional Chinese fermented vegetables, such as Sichuan Dongcai (Yao et al., 2016). A slight decrease in nitrite occurred in the first 3 years (2.11-1.27), followed by sharp decrease to 0.26 in the fourth year (Figure 1A). Additionally, crude fiber and protein content showed similar changes. There were apparent decreases in crude fiber content and protein content during fermentation (Figure 1C and D), while the total sugars gradually increased (Figure 1B).

A total of 16 FAAs were detected in fermented Dazu Dongcai (Table 1). FAA profiles varied with fermentation process. Compared with profiles in fresh vegetables, total FAAs concentrations decreased with the increase of fermentation time. Besides, due to the decrease in available sugars of microorganisms, EAAs also showed a slight downward trend with the extension of curing time. EAAs account for about 28%~30% at the beginning of fermentation, but the proportion of EAAs increased to 33%~36% and remained stable as fermentation proceeded. FAAs also have taste function, Glu and Asp are common delicious amino acids, and Leu, Tyr and His are bitter amino acids, then combination of different flavor amino acids would produce good taste. The concentrations of umami-tasting FAAs (Glu and Asp) decreased from 1.06 to 0.24 g/100 g in four years. The concentration of sweet-tasting FAAs including Ser, Thr, Ala, Gly, and Lys, was decreased from 0.56 to 0.35 g/100 g with fermentation development. The concentration of bitter amino acid FAAs including His, Tyr, Val, Met, Ile, Leu, Arg, and Phe, was decreased from 1.15 to 0.85 g/100 g.

3.2 Microbial community profiles of Dongcai

Sequencing data summary

After quality filtering, a total of 2,902,721 high quality reads were obtained by 16S rRNA sequencing from the 45 samples. The number of bacterial OTUs from each sample ranged from a minimum of 194 (Z2021.9) to a maximum of 1798 (T2019.9). The rarefaction curves tended to reach plateaus, indicated that the sequencing depth was relatively sufficient in covering the bacterial diversity (Figure 2A). Venn analysis showed that the maximum number of OTUs was 2829 in F2017, the number of unique OTUs per year was 302,151,199,136 and 321, the number of common OTUs was 502, which accounts for 8.2% (Figure 2B). Detailed information regarding sequence quality control and community diversity indices is as follows: samples collected from the last stages of fermentation (i.e., at 3-4 years of fermentation) exhibited the highest bacterial alpha-diversity based on OTUs metrics, Chao1, and Shannon index (Figure 2C-E). As the fermentation proceeded, the Shannon index increased showed the higher of microbial flora diversity, bacterial richness and diversity subsequently increased gradually.

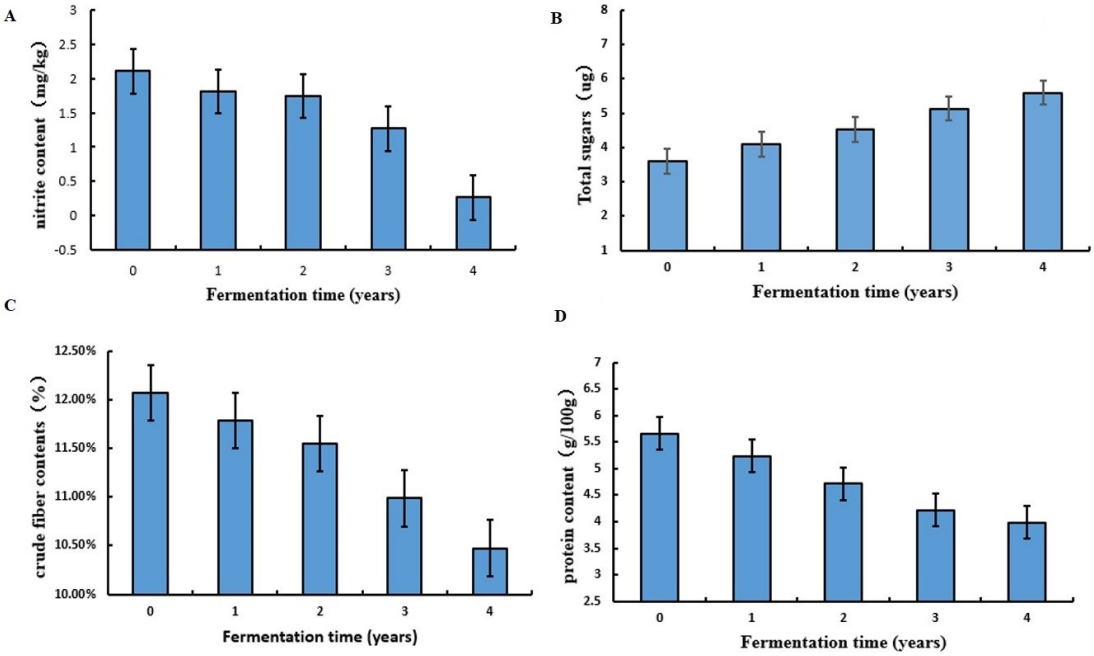

Figure 1. Content changes in nitrite (A), total sugars (B), crude fiber (C) and protein (D) during Dazu Dongcai fermentation. Data are shown as means ± standard deviation (n = 9). 3.1.2. Free amino acids profiles.

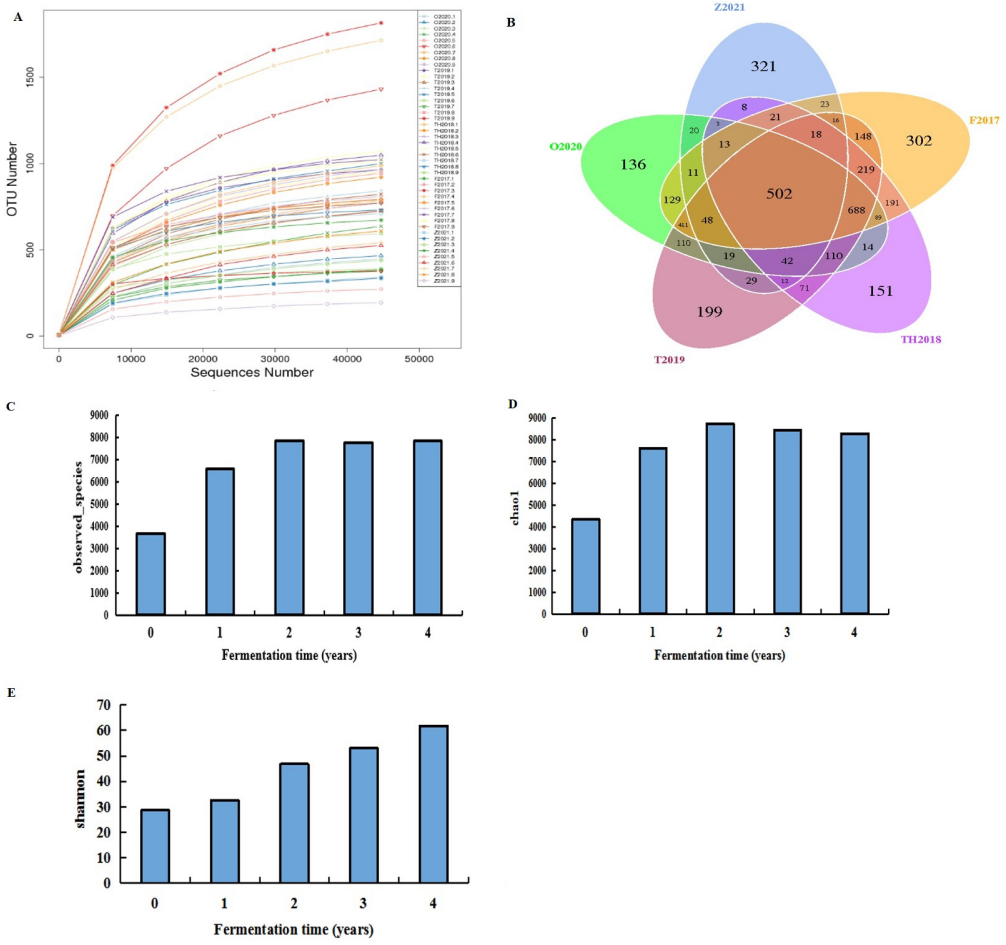

Figure 2. OTU Number (A), Venn (B), observed OTUs metrics (C), chao 1 (D) and Shannon (E) during Dazu Dongcai fermentation. Data are shown as means ± standard deviation (n = 9).

Table 1. Concentrations of free amino acids (FAA) in Dazu Dongcai during fermentation.

	Concentrations in Dazu Dongcai (g/100 g)				
FAA	Year 0	Year 1	Year 2	Year 3	Year 4
Asp	0.29 ± 0.03	0.24 ± 0.03	0.18 ± 0.01	0.20 ± 0.05	0.11 ± 0.01
Thr	0.10 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.11 ± 0.04	0.06 ± 0.01
Ser	0.14 ± 0.01	0.10 ± 0.01	0.12 ± 0.02	0.15 ± 0.04	0.08 ± 0.01
Glu	0.77 ± 0.05	0.34 ± 0.07	0.46 ± 0.06	0.37 ± 0.13	0.13 ± 0.02
Gly	0.05 ± 0.00	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.03 ± 0.01
Ala	0.19 ± 0.02	0.18 ± 0.03	0.15 ± 0.02	0.17 ± 0.05	0.15 ± 0.03
Cys	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Val	0.21 ± 0.02	0.18 ± 0.02	0.19 ± 0.03	0.22 ± 0.04	0.16 ± 0.02
Ile	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Leu	0.11 ± 0.01	0.12 ± 0.02	0.11 ± 0.03	0.15 ± 0.02	0.10 ± 0.03
Met	0.17 ± 0.03	0.12 ± 0.04	0.10 ± 0.04	0.19 ± 0.07	0.09 ± 0.02
Tyr	0.15 ± 0.04	0.24 ± 0.06	0.19 ± 0.06	0.24 ± 0.06	0.23 ± 0.12
Phe	0.17 ± 0.02	0.14 ± 0.02	0.16 ± 0.03	0.20 ± 0.04	0.11 ± 0.02
Lys	0.13 ± 0.01	0.08 ± 0.02	0.09 ± 0.03	0.12 ± 0.05	0.06 ± 0.01
His	0.07 ± 0.00	0.04 ± 0.01	0.08 ± 0.02	0.07 ± 0.02	0.03 ± 0.01
Arg	0.26 ± 0.02	0.19 ± 0.03	0.22 ± 0.03	0.23 ± 0.07	0.12 ± 0.02
EAA	0.81	0.65	0.74	0.88	0.53
TAA	2.84	2.12	2.19	2.48	1.48
EAA/TAA	28.52%	30.66%	33.79%	35.48%	35.81%

Data are shown as means ± standard deviation (n = 9).

Samples bacterial community structure

The bacterial community structures of samples were presented at the phylum level to evaluate taxonomic compositional dynamics of bacterial communities associated with Dazu Dongcai fermentation (Figure 3A). *Proteobacteria* and *Firmicutes* were the dominant phyla in communities across the entire fermentation period. During the four years of fermentation, the abundance of *Cyanobacteria* decreased from the initial ratio value of 58% to the final 2%, while the abundance of *Firmicutes* bacteria increased from the initial 2% to the final 52%. In addition, there was no significant change in the abundance of *Proteobacteria*, which always occupied about 30%. The abundances of *Halo bacterota* increased significantly from an initial level of 1% to the final level of 30% in the third year, and then decreased to the initial state. The relative abundances of *Bacteroides* and *Actino bacillus* increased from 1% and 4% of the initial values to 6% and 10%, respectively.

The bacterial community structures of samples were presented at the genus level to evaluate taxonomic compositional dynamics of bacterial communities associated with Dazu Dongcai fermentation. The dominant bacterial genera were *Staphyllococcus*, *Lactobacilus*, *Sphingomonas*, *Clostridium_sensu_stricto_1*, *Terrisporobacter* and *Pseudomonas* (Figure 3B). The results revealed *unidentified_ Chloroplast*, *Sphingomonas*, *unidentified_Mitochondria* and *Pseudomonas* were predominated bacterial genera in the periods of Z2021 and O2020. *unidentified_Chloroplast*, *Staphyllococcus*, and *Sphingomonas* were predominated bacterial genera in the second year of fermentation (T2019). Furthermore, *Halococcus*, *Lactobacilus*, *Clostridium*, and *Terrisporobacter* were predominated bacterial genera in the third year of fermentation (TH2018), *Lactobacilus*, *Clostridium*, *Terrisporobacter,* and *Acinetobacter* were predominated bacterial genera in the fourth year of fermentation (F2017).

Microbial richness and diversity

Alpha diversity is an ecological index of how many taxonomic groups are present within each sample and whether the abundance of these groups is evenly distributed. The number of species observed and the alpha-diversity indexes (Chao1, Shannon index) on the basis of the 97% identity OTUs are shown in Figure 4A-C. Through the box diagram, the Wilcox rank sum test was used to analyze whether there were significant differences in species diversity between groups. The research found that the number of OTUs, Chao1 and Shannon indices increased with the increase of years, and OTUs index of the original samples (Z2021) was significantly different from the other four samples in different fermentation years. In addition, similar results were observed in Chao1 index of the original samples (Z2021), which was significantly lower than other four samples. But there were differences between the fourth year of fermentation (F2017) and the other four years in Shannon index.

To characterize the microbial community composition among different fermentation years, the Bray Curtis distance was used to calculate beta diversity. The principal coordinate analysis (PCoA) of sequencing data showed that the microbial community structure of each treatment group (PERMANOVA $P < 0.001$) was significantly separated. The contribution rates of the first and second principal components were 59.26% and 13.8%, respectively, indicating the difference of community structure in different fermentation periods. If the sample distance is closer, the species composition structure is more similar, so the samples with high community structure similarity tend to be clustered

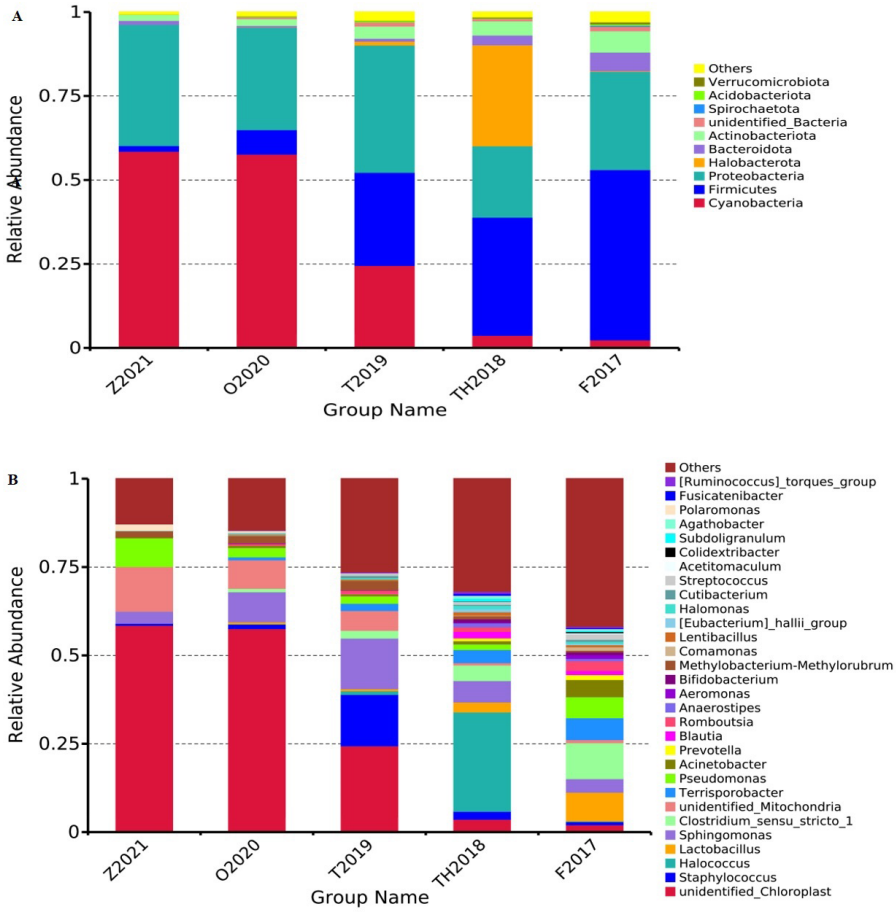

Figure 3. Bacterial community structure succession at the phylum level (A), Bacterial community structure succession at the genus level (B).

together, and the samples with large community differences will be far separated. We observed that distribution of microbial communities was widely during different fermentation periods. As the fermentation progressed, the samples in the scoring plot moved from quadrant 1 to quadrant 3. As illustrated in Figure 4D, there was a clear separated between T2019 and TH2018 of the samples, indicating that Dazu Dongcai fermentation progressed very rapidly in the third year of fermentation. The samples from first year (O2020) and second year (T2019) were relatively close among the six samples, showing that the community structure had a strong similarity. But overall, the community structure varies greatly among the five samples.

Analysis of microbial diversity

The integration of the PCoA analysis and the hierarchical cluster result indicated that microbial community structures in the rhizosphere had a marked difference among the five samples (Figure 4D). The community heatmap identified ten main clusters that illustrating bacterial community differences. We found that the similar colony structure between F2017 and Th2018, so are O2020 and Z2021. Moreover, T2019 was correlated with the other four groups of colonies. To explore variations in composition of microorganism among these samples, we performed LEfSe analysis to identify taxa that were either enriched or depleted in treated groups (Figure 5A and 5B). Thirty types of microorganisms were significantly different in different fermentation years. Z2021 contains six microorganisms, T2019 contains eight microorganisms, TH2018 contains four microorganisms, F2017 contains Twelve microorganisms (LDA>4, P<0.05) (Figure 5C).The abundance difference between 2020 and the other four years is not obvious, so the data of this year is not shown in this diagram. At the phylum level, whole fermentation process of sample were enriched in the *Firmicutes* and *Proteobacteria*, but the samples in the third year of fermentation enriched separately *Halobacterota*. At the genus level, whole fermentation process of sample enriched the genus *Lactobacillus*, *Terrisporobacter* and *Romboutsia* with the control. But the samples in the first two years of fermentation were enriched in unique *Sphingomonas* compared with the control. These results demonstrated that microbial community composition was different during fermentation period.

3.3 Correlation between microbial abundance and physicochemical indexes

To further evaluate the potential associations between microorganisms and the fermentative properties of Dazu Dongcai (Figure 6), the correlation coefficient were evaluated between microbial species contents and biochemical compound

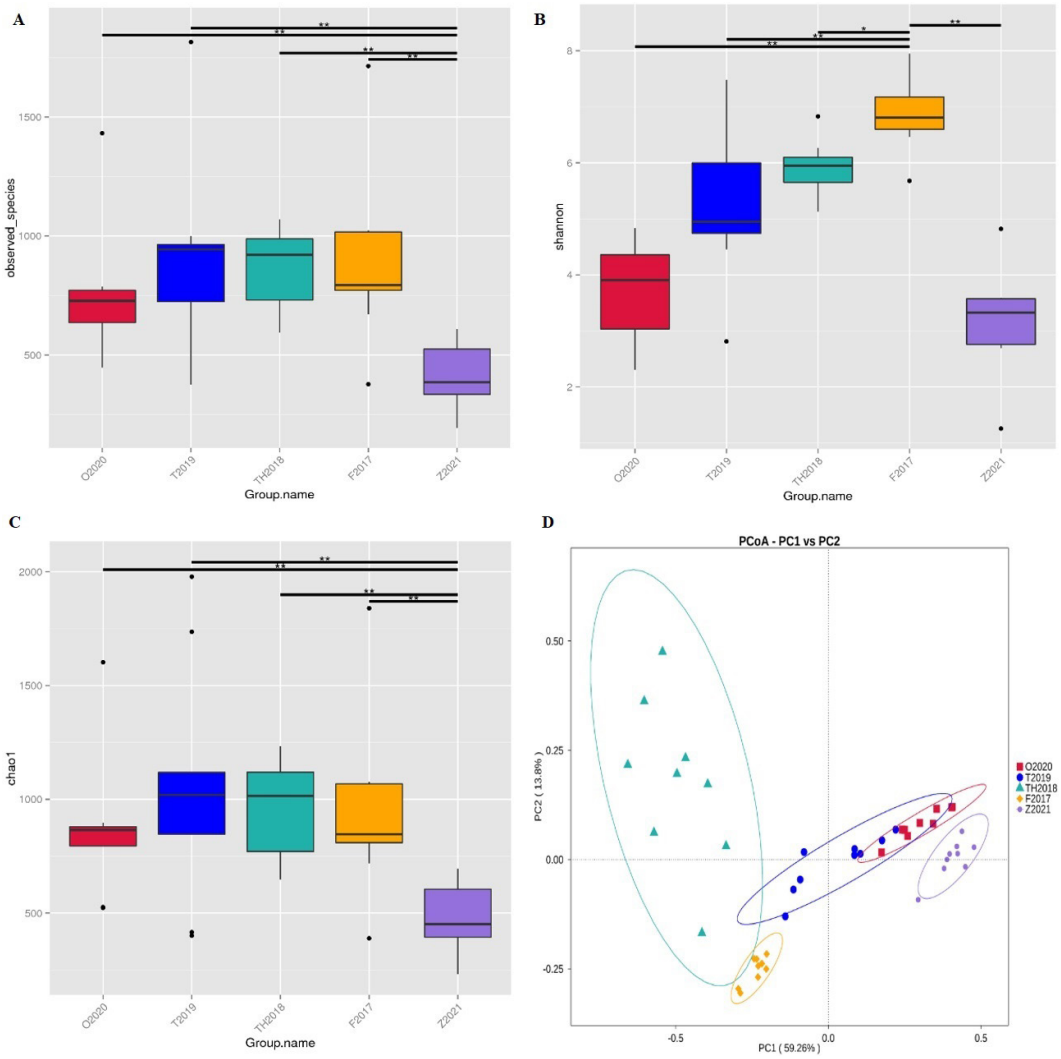

Figure 4. Bacterial biodiversity in Dongcai at different fermentation stages. (A-C) Variation in observed OTUs, Chao1, and Shannon index diversity metrics throughout fermentation. Principal coordinates analysis of Bray-Curtis distances of bacterial at different fermentation stages (D). Only those taxonomic groups with >0.01% relative abundances are shown.

contents (nitrite, total sugar, crude fiber, protein, amino acids) in fermented vegetables using Spearman's correlations. A total of 27, 5, 24 and 26 species ($|\rho| \geq 0.7, p<0.05$) were significantly correlated with protein, crude fiber, nitrite and total sugar during four years of fermentation, respectively. In particular, *Lactobacillus*, *Prevotella*, *Streptococcus* and *Romboutsia* were highly negative correlated with variation in protein ($|\rho|>0.9$).

But few bacteria were highly associated with crude fiber($|\rho| \geq 0.7, p<0.05$). Through the chart, we found that *Lactobacillus*, *Terrisporobacter*, *Prevotella*, *Romboutsia* and *Streptococcus* were highly negative correlated to variation in nitrite ($|\rho| > 0.9$), while these five bacteria are positively correlated with total sugar. Meantime, Bacteria were negative correlation with crude fiber, these bacteria are positively correlated with total sugar. Among the 16 FAAs, *Lactobacillus*, *Clostridium_sensu_stricto_1*, *Terrisporobacter*, *Prevotella*, *Blautia*, *Romboutsia*, *Bifidobacterium*, *Cutibacterium*, *Streptococcus* and *Agathobacter* abundances exhibited high negative correlations with the contents of the aforementioned FAAs. *unidentified_Chloroplast*, *Staphylococcus*, *Halococcus*, *unidentified_Mitochondria*, *Methylobacterium Methylorubrum*, *Acetitomaculum* and *Polaromonas* abundances exhibited high positive correlations with the contents of the aforementioned FAAs. the concentrations of Asp, Glu, Gly, His and Arg exhibited strong correlations with more than 20 species ($\rho > 0.7$). Significant correlations were not observed for taxa and the contents of Ile, as well as cadaverine and spermine.

4 Discussion

Fermented vegetables are popular globally due to their enriched nutritional value, increased organoleptic quality, and their unique health-promoting properties (Marco et al., 2017). Dazu Dongcai is a traditional product of vegetables fermentation that is produced by spontaneous fermentation with autochthonous microbiota originated from raw materials and environment around. In particular, the nutritional and organoleptic properties of the end product of spontaneous

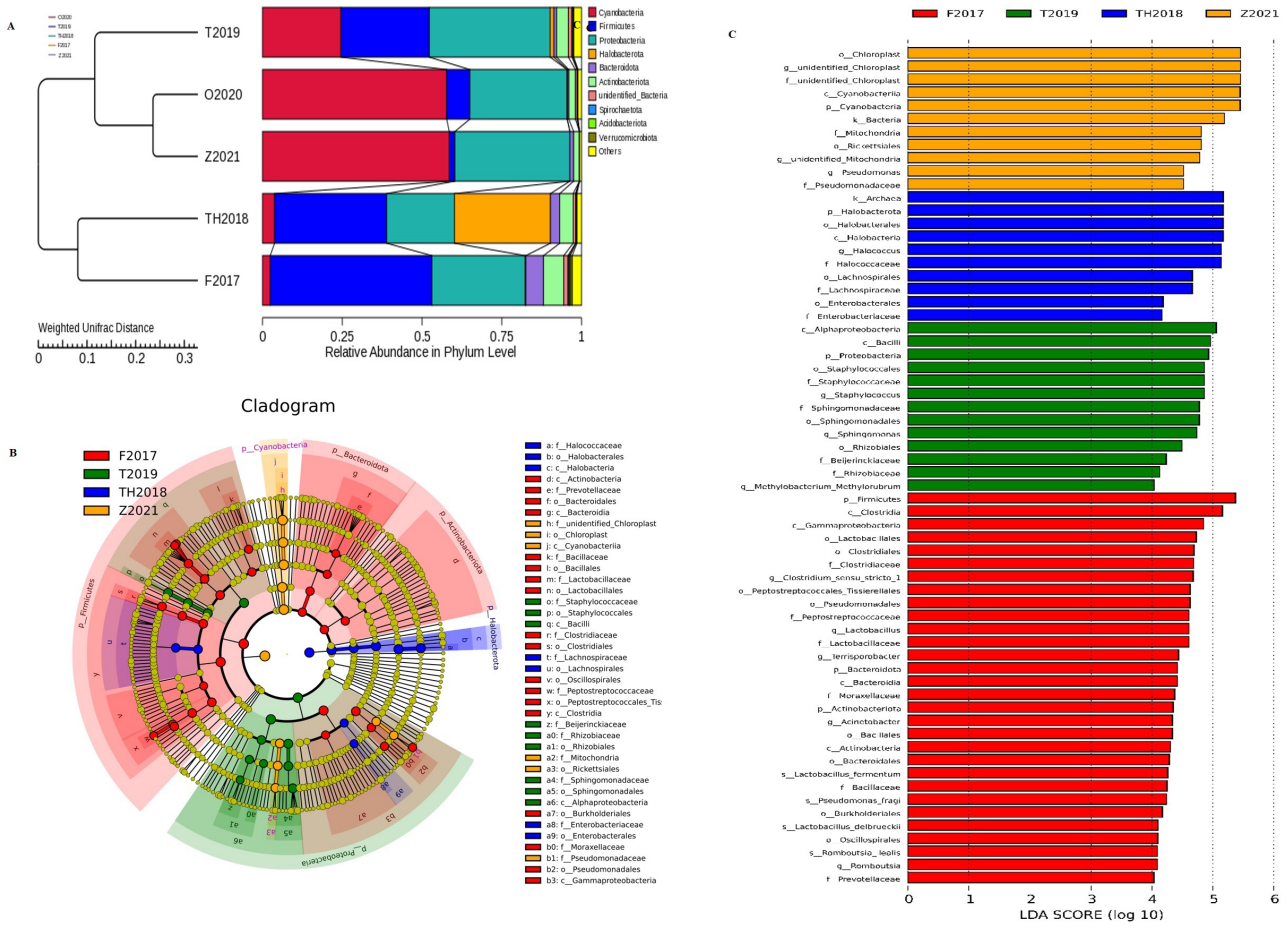

Figure 5. UPGMA cluster tree (A), LEfSe analysis evolutionary branch graph during Dazu Dongcai fermentation (B). LDA score determined the degree of differentiation (C).

fermentation mostly rely on a community of autochthonous microorganisms (Torres et al., 2020).

Raw vegetable is the major source of autochthonous microbiota in vegetable fementations (Song et al., 2020). However, The presence of common pathogenic and spoilage microorganisms at the beginning of sample fermentation, including *Sphingomonas*, *Pseudomonas* and *Agathobacter*, but these groups rapidly disappeared during the early fermentation stage, possibly due to niche selection (He et al., 2017). Fermented vegetable products are highly dependent on naturally occurring Lactobacillus. Accordingly, the relative abundances of *Lactobacillus* increased with Dazu Dongcai fermentation, the its abundance increased from the initial 0.03% to the final 8.17% (Leech et al., 2020).

Bacteria account for the major proportion of microbiota involved in vegetable fermentation. For example, metagenomics analyses have shown that fermentation vegetables mostly contain bacterial taxa, with bacteria comprising 96.1% of the communities based on phylum-level relative abundances (Guan et al., 2020). Bacterias were also identified during Dazu Dongcai fermentation in this study and included *Staphylococcus*, *Halococcus*, *Sphingomonas*, *Lactobacillus, Terrisporobacter, Prevotella, Bifidobacterium* and *Streptococcus* et.al, which contain beneficial and pathogenic bacteria during fermentation. *Lactobacillus* and *Terrisporobacter* can simultaneously consume glucose and other carbohydrates to decompose lactic acid and acetic acid, which are the major acid products of fermented vegetables. Additionally, according to the analysis of the correlation between the content of physical and chemical indicators and the flora, a large amount of lactic acid was produced with the increase in the content of *Lactobacillus*. *Prevotella* produce acetic acid and succinic acid. *Romboutsia* separate a variety of sugars to produce butyric acid, and the generated acid promoted the fermentation process. The antibacterial substances of *Terrisporobacter* have broad-spectrum bactericidal activity, which can prevent pollution of other bacteria.

Total sugar, crude fiber and nitrite contents are important chemical indices of fermented vegetables, which are correlated with the microbial fermentation process. Specifically, microorganisms consume not only energy but also secondary metabolism during fermentation. In this study, the crube fiber contents decreased from 12.07% to 10.47% and the total sugar contents increased from 3.61% to 5.59%. Concomitantly, the relative abundance of *Lactobacillus*, *Terrisporobacter*, *Prevotella*, *Bifidobacterium* and *Streptococcus* increased with fermentation. These changes of microorganisms displayed significantly negative correlation with protein and nitrite, while a significant positive correlation with total sugar content. These results suggest that these five genera can

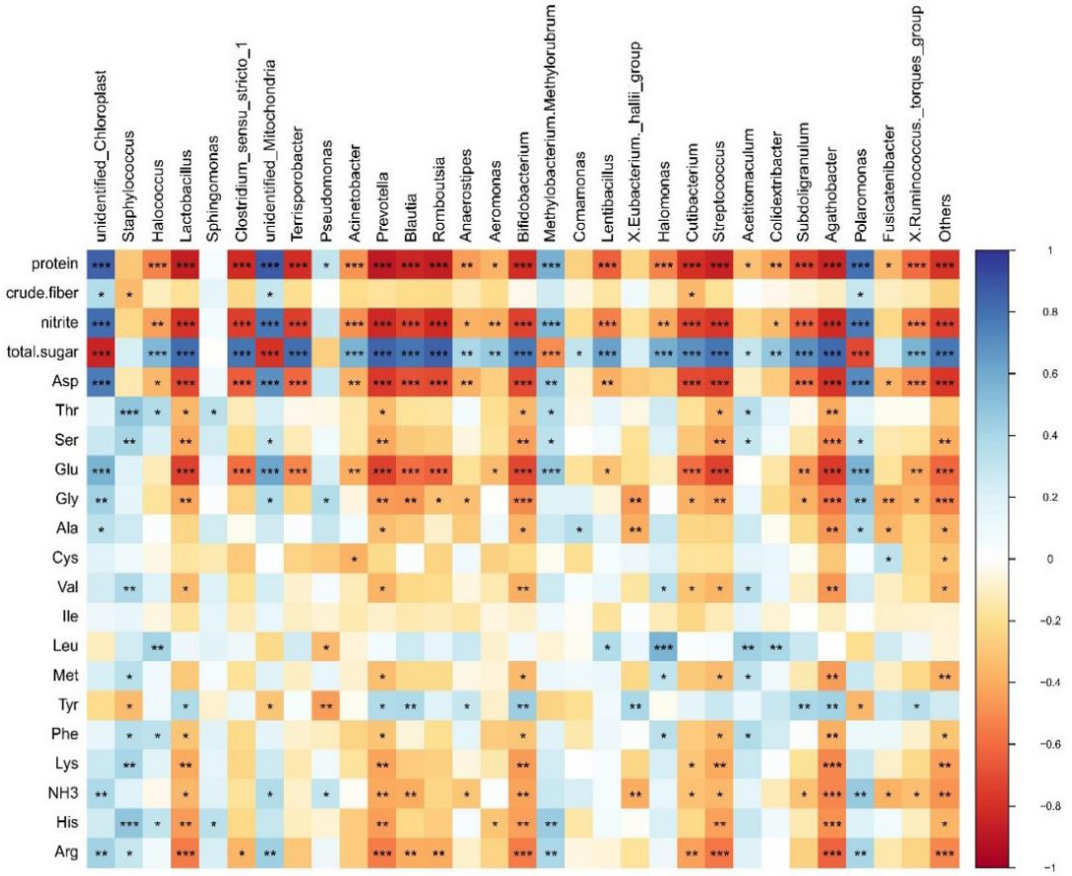

Figure 6. Heatmap of Spearman correlations between microbial species (35 bacterial species with >1.0% relative abundances) with protein, crude fiber, nitrite, total sugar and Seventeen free amino acids during Dongcai fermentation. Levels of significance are indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

continuously adapt to the curing environments and may have an impact on physical and chemical indicators, which makes great contributions to the fermentation of Dongcai. Besides, the initial spike in nitrite content was likely due to the reduction of nitrate by microbial nitrate reductases. As fermentation progresses, the environment becomes anaerobic and supports the growth of lactic acid bacteria. Increased production of lactic acid lowers the pH and impairs nitrate reductase activity, thereby reducing the nitrite content (Pi et al., 2021).

Pickles produced by fermentation exhibit enhanced nutritional and organoleptic properties due to the transformation of substrates and the formation of microbial metabolites such as amino acids and protein (Li et al., 2022). In this study, we directly monitored variation in the concentrations of these compounds during Dongcai throughout fermentation, and observed that fermented Dongcai contained significantly different biochemical profiles than raw mustard samples. For examples, clear increases of lactic acid bacteria can produce large amounts of lactic acid, which contribute to the unique acidity, tastes and flavors of fermented products, as previously reported in pickles (Dai et al., 2019). It was found that the contents of crude protein and total amino acids decreased by 30% and 48%, respectively, but the proportion of essential amino acids increased from 27% to 34%, which improved the nutritional structure. Furthermore, the contents of FAAs that absorbed unami (i.e.,Asp and Glu) and sweet-tasting (i.e., Gly) characteristics were generated during fermentation, which contributed to the unique sensorial properties of the end products.

Microbiota might play important roles in the production of distinctive characteristics of fermented Zhacai. The utilization of sugars as substrates for the production of lactic acid, ethanol, and acetic acid is common in all food fermentation processes due to Lactobacillus-related specie (Oguntoyinbo et al., 2018). In this study, *Staphylococcus, Lactobacillus*, *Terrisporobacter*, *Blautia*, *Romboutsia*, *Bifidobacterium* and *Streptococcus* dominated the whole fermentation process, which also have important functions during fermentation. These bacteria can provide energy for other microorganisms by decompose carbohydrates, and secrete a variety of metabolites. The generated volatile fatty acids such as acetic acid can effectively inhibit the growth of harmful bacteria and create an acidic environment for the growth of *Lactobacillus* to promote fermentation. Besides, *Staphylococcus* can also secrete enzymes to promote the formation of color, texture and flavor during fermentation. Free amino acids (FAAs) are presented in different combinations in fermented foods and are important contributors to the distinctive taste profiles of traditional Chinese pickles (Charve et al., 2018; Xiao et al., 2020). The abundances of the *Lactobacillus*, *Terrisporobacter*,

Prevotella, *Bifidobacterium* and *Streptococcus* were all negatively correlated with FAAs contents (|ρ|>0.7). Previous studies have shown that FAAs are generated through primary proteolysis of raw materials by proteases of lactic acid bacteria during food fermentation (Zhao et al., 2016).

5 Conclusions

This study investigated the biochemical changes and microbial community dynamics associated with spontaneous fermentation of Dazu Dongcai. The results indicate that the *Staphylococcus*, *Lactobacillus*, *Terrisporobacter*, *Blautia*, *Romboutsia*, *Bifidobacterium* and *Streptococcus* dominate the whole fermentation process, which were strongly correlated with the physical and chemical indexes. These flora have promoting function during fermentation and may affect the quality of Dongcai. Moreover, these predominant microorganisms associated with Dongcai fermentation and exhibited strong correlations with the contents of nitrite, protein, amino acids, crude fiber and total sugar, suggesting critical contributions to the formation of these compounds. These findings will be helpful to establish a further understanding of Dazu Dongcai fermentation, potentially helping to improve its product quality.

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References

Charve, J., Manganiello, S., & Glabasnia, A. (2018). Analysis of umami taste compounds in a fermented corn sauce by means of sensory-guided fractionation. *Journal of Agricultural and Food Chemistry*, 66(8), 1863-1871. http://dx.doi.org/10.1021/acs.jafc.7b05633. PMid:29397710.

Dai, C. Y., Chen, J. H., Guo, Z., Zhang, Z. D., Ma, L., & Lei, M. (2019). The effect of lactic acid bacteria from Zaoyang physalis water on the fermentation quality of kimchi and kimchi water. *China Brewing*, 5, 54-58.

Deng, L. (2014). Isolation and identification of phenyllactic acid-producing strains from Sichuan winter vegetables. *China Brewing*, 33(4), 97-100.

Dong, L., Pu, B., Ao, X. l., Zhang, X. P., Zheng, Y. k., & Li, X. l. (2012). Composition and diversity of bacterial communities in Sichuan winter vegetables. *Acta Microbiology*, 4, 519-525.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, 10(10), 996-998. http://dx.doi.org/10.1038/nmeth.2604. PMid:23955772.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics (Oxford, England)*, 27(16), 2194-2200. http://dx.doi.org/10.1093/bioinformatics/btr381. PMid:21700674.

Guan, Q., Zheng, W., Huang, T., Xiao, Y., Liu, Z., Peng, Z., Gong, D., Xie, M., & Xiong, T. (2020). Comparison of microbial communities and physiochemical characteristics of two traditionally fermented vegetables. *Food Research International*, 128, 108755. http://dx.doi.org/10.1016/j.foodres.2019.108755. PMid:31955789.

He, P. H., Qian, Y., Wang, M., Yang, J. T., Zhao, T. T., Jiang, Y. L., Che, Z. M., & Rao, Y. (2017). Isolation, identification and growth characteristics of membrane-producing bacteria from corrupt fermented vegetables. *Shipin Kexue*, 38(10), 92-97.

Itoh, H., Navarro, R., Takeshita, K., Tago, K., Hayatsu, M., Hori, T., & Kikuchi, Y. (2014). Bacterial population succession and adaptation affected by insecticide application and soil spraying history. *Frontiers in Microbiology*, 5, 457. http://dx.doi.org/10.3389/fmicb.2014.00457. PMid:25221549.

Kang, J. Y., Hong, J., Gao, Y., Yang, Y., Chen, M., Yi, X. X., & Gao, X. Z. (2019). Analysis of bacterial diversity during fermentation of naturally ermented vegetables in Shanxi. *Shipin Kexue*, 40(10), 106-111.

Kioroglou, D., Mas, A., & Portillo, M. C. (2020). High-throughput sequencing approach to analyze the effect of aging time and barrel usage on the microbial community composition of red wines. *Frontiers in Microbiology*, 11, 562560. http://dx.doi.org/10.3389/fmicb.2020.562560. PMid:33013793.

Leech, J., Cabrera-Rubio, R., Walsh, A. M., Macori, G., Walsh, C. J., Barton, W., Finnegan, L., Crispie, F., O'Sullivan, O., Claesson, M. J., & Cotter, P. D. (2020). Fermented-food metagenomics reveals substrate-associated differences in taxonomy and health associated and antibiotic resistance determinants. *mSystems*, 5(6), e00522-20. http://dx.doi.org/10.1128/mSystems.00522-20. PMid:33172966.

Li, T., Wu, R. N., Zhang, Q. S., Wang, F. F., Liu, C. l., Ding, R. X., & Wu, J. R. (2022). Research progress on the relationship between microorganisms in pickled vegetables and product flavor quality. *Food Industry Science and Technology*, 14, 475-483.

Liu, J. (2021). Comparison of Kjeldahl determination of protein in food. *Food Safety Guide*, 2021(12), 147-149.

Ma, W., Wu, Y., Wei, Y., Zou, W., Yan, Y., Xue, J., Tian, G., Wang, L., Wang, W., & Pan, H. (2018). Microbial diversity analysis of vineyards in the xin jiang region using high-throughput sequencing. *Journal of the Institute of Brewing*, 124(3), 276-283. http://dx.doi.org/10.1002/jib.501.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligne, B., G¨anzle, M., Kort, R., Pasin, G., Pihlanto, A., Smid, E. J., & Hutkins, R. (2017). Health benefits of fermented foods: microbiota and beyond. *Current Opinion in Biotechnology*, 44, 94-102. http://dx.doi.org/10.1016/j.copbio.2016.11.010. PMid:27998788.

Oguntoyinbo, F. A., Cnockaert, M., Cho, G. S., Kabisch, J., Neve, H., Bockelmann, W., Wenning, M., Franz, C., & Vandamme, P. (2018). Halomonas nigrificans sp. nov., isolated from cheese. *International Journal of Systematic and Evolutionary Microbiology*, 68(1), 371-376. http://dx.doi.org/10.1099/ijsem.0.002515. PMid:29231158.

Pi, J. T., Liu, D. M., Wang, J. H., Mou, M. W., Fang, F., Ning, J. H., & Zhang, Y. S. (2021). The mechanism and application of lactic acid bacteria to degrade nitrite in pickles. *Shipin Yu Fajiao Gongye*, 24, 301-307. http://dx.doi.org/10.13995/j.cnki.11-1802/ts.027488.

Song, C. (2019). The traditional production process of Tianjin winter vegetables. *Rural Know-How*, 20, 44-45.

Song, H. S., Whon, T. W., Kim, J., Lee, S. H., Kim, J. Y., Kim, Y. B., Choi, H., Rhee, J., & Roh, S. W. (2020). Microbial niches in raw ingredients determine microbial community assembly during kimchi fermentation. *Food Chemistry*, 318, 126481. http://dx.doi.org/10.1016/j.foodchem.2020.126481. PMid:32126467.

Torres, S., Ver'on, H., Contreras, L., & Isla, M. I. (2020). An overview of plant autochthonous microorganisms and fermented vegetable foods. *Food Science and Human Wellness*, 9(2), 112-123. http://dx.doi.org/10.1016/j.fshw.2020.02.006.

Wang, F. M., & Ma, L. B. (2016). Effects of metabolites of non-Saccharomyces cerevisiae on wine quality during fermentation. *China Brewing*, 12, 12-17.

Xiao, Y., Huang, T., Huang, C., Hardie, J., Peng, Z., Xie, M., & Xiong, T. (2020). The microbial communities and flavour compounds of Jiangxi yancai, Sichuan paocai and Dongbei suancai: three major types of traditional Chinese fermented vegetables. *Lebensmittel-Wissenschaft + Technologie*, 121, 108865. http://dx.doi.org/10.1016/j.lwt.2019.108865.

Yang, X., Hu, W., Xiu, Z., Jiang, A., Yang, X., Sarengaowa, Ji, Y., Guan, Y., & Feng, K. (2020). Microbial dynamics and volatilome profiles during the fermentation of Chinese northeast sauerkraut by Leuconostoc mesenteroides ORC 2 and Lactobacillus plantarum HBUAS 51041 under different salt concentrations. *Food Research International*, 130, 108926. http://dx.doi.org/10.1016/j.foodres.2019.108926. PMid:32156375.

Yang, Y., Zhang, Y., Wu, Z., Shi, X., He, D. Z., Yang, D. Z., & Huang, Y. (2016). *Research and application of key technologies for processing and by-product utilization of dazu winter vegetables.* Retrieved from https://kns.cnki.net/KCMS/detail/detail.aspx?dbname=SNAD&filename=SNAD000001626662

Yao, Y. Z., Dong, L., Pan, S., & Zhu, Y. (2016). Changes in Sichuan winter dishes. *Xiandai Shipin Keji*, (2), 205-210. http://dx.doi.org/10.13982/j.mfst.1673-9078.2016.2.031.

Zhang, C. C., Zhang, J. M., & Liu, D. Q. (2021a). Biochemical changes and microbial community dynamics during spontaneous fermentation of Zhacai, a traditional pickled mustard tuber from China. *International Journal of Food Microbiology*, 347, 109199. http://dx.doi.org/10.1016/j.ijfoodmicro.2021.109199. PMid:33878643.

Zhang, J., Zhang, C., Wu, W., Lv, X., Xin, X., Liu, D., Hu, H., & Guo, S. (2021b). Correlation of the bacterial communities with umami components, and chemical characteristics in Zhejiang xuecai and fermented brine. *Food Research International*, 140, 109986. http://dx.doi.org/10.1016/j.foodres.2020.109986. PMid:33648221.

Zhang, J., Zhao, Z. F., Wang, Z. J., Xiao, Y. C., & Zhong, D. L. (2021c). Research progress on the composition, flavor and quality and safety of Sichuan pickles. *Chinese Condiments*, 10, 178-182.

Zhang, Y. Q., Ye, S. J., Zhou, R., Zhang, J., & Liu, S. P. (2021d). Fermented food flavor substances and related microorganisms. *Brewing Science and Technology*, 2, 85-96. http://dx.doi.org/10.13746/j.njkj.2020168.

Zhao, C. J., Schieber, A., & G¨anzle, M. G. (2016). Formation of taste-active amino acids, amino acid derivatives and peptides in food fermentations: a review. *Food Research International*, 89(Pt 1), 39-47. http://dx.doi.org/10.1016/j.foodres.2016.08.042. PMid:28460929.

Zhou, L. B., Jiang, C. Y., Shi, J. P., & Jiang, K. X. (2022). Gray pattern recognition and grey system cluster analysis of multi-indexes of five kinds of tea. *Fresenius Environmental Bulletin*, 31(3), 2510-2521.

Zhu, R. (2019). *Optimization of key production processes and study on volatile components of L. japonica* (Master's thesis). Guangxi University, Guangxi.