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Analysis on microbial communities and characteristic flavor metabolic of PXDB-meju by partially substituting wheat flour with soybean flour and gluten flour

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ABSTRACT

Pixian Douban (PXDB) is one of the most popular condiments in China due to its unique flavor. Wheat flour that contains abundant nutrients is an important raw material in producing flavors during PXDB fermentation. In this study, wheat flour was substituted with soybean flour and gluten flour that have higher proteins in portions of 10.4% and 4.2% (F1), 8.9% and 7.2% (F2), 9.6% and 5.8% (F3). The results indicated that the substitutions increased the amino acid nitrogen content and improved flavor quality compared with traditional group (CT). Especially, the key amino acids including spartate, glutamic, arginine and lysine, and the phenylacetaldehyde as one of the most important volatile compounds exhibited preferable higher contents in F2 group than those in CT group. Metagenomic analysis showed that the abundances of predominant bacteria, including *Kosakonia_cowanii, unclassified_f_Enterobacteriaceae* and *unclassified_g_Enterobacter*, were higher in the F2 compared to the CT. *Lupinus_albus* and *Plutella_xylostella* were the top two fungi in relative abundance, with higher growth rates in F2 than in CT. Furthermore, metabolism pathway analysis revealed higher relative abundance of enzymes producing key amino acids and phenethylaldehyde in the F2 compared to the CT. Meanwhile, these enzymes were exclusively annotated to the *Kosakonia_cowanii*, *Bacillus_velezensis* and *Escherichia_coli* in F2. This study provided a theoretical foundation for improving PXDB flavor quality in industry production.

1. Introduction

Pixian Douban (PXDB) is a traditional fermented food in China and an essential seasoning in Sichuan cuisine (Ding et al., 2021b). It is honored as "the soul of Sichuan cuisine" due to its distinctive flavors, which forms by the metabolism of microbial communities during fermentation (Lin et al., 2018; Lin et al., 2019). Using broad bean, flour and chili as raw ingredients, the manufacturing of PXDB mainly includes the broad bean-to-Meju, chili-to-moromi, and the mixed fermentation of 3 months to 1 year (Lu et al., 2019). During the PXDB-Meju fermentation stage, raw broad beans and wheat flour were mixed in proportion to make koji, and then transformed into meju (Li et al., 2016). In this process, polypin-tides and free amino acids (FAAs) are obtained by hydrolyzing the proteins of raw ingredients with the microorganism, peptidases, and glutaminases in the fermentation system, forming the unique fresh, sweet, bitter, and salty flavor of PXDB (Lu et al., 2019; Tan et al., 2021). Amino acid nitrogen, which formed by combining the free amino acids with some nitrogen elements, is of great importance for measuring the quality grade of PXDB product. Therefore, by increasing the protein content of raw material has considered to be a great potential to improve the quality of PXDB.

In the previous study, soybean flour and gluten flour, which have higher protein content, were severally used for partially substituting wheat flour in PXDB-meju fermentation. Comparing with traditional

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meju fermentation, the addition of soybean flour or gluten flour could increase the amino acid nitrogen, the content of key aroma substances and organoleptic quality (Zhao et al., 2024). Previous studies have shown that in the early stage of PXDB fermentation (0-30d), the degradation of alcohol-soluble protein was dominating. Afterwards, water-soluble protein played a dominant role in degradation. Generally, soybean flour is composed of more water-soluble protein (Lin et al., 2022). On the other hand, gluten flour contained a bigger portion of alcohol-soluble protein. Therefore, the combination of soybean flour and gluten flour in partially substituting wheat flour might be more efficient in promoting the quality of PXDB-meju.

Flour is one of the most important ingredients for microorganisms growing and metabolizing, and the application of substitutions for flour changes the growth environment of microorganisms to some extent. The changes of environment like temperature and oxygen contents would influence the composition and metabolism of microbial communities during food fermentation (Ding et al., 2021b; P. Liu et al., 2020; Xian et al., 2022). By utilizing gluten and soybean in PXDB-meju fermentation, previous study only concentrated on the changes analysis for physicochemical indexes. There lacks a deep investigation for the differences in microorganism composition and their metabolism by substituting the wheat flour with gluten and soybean.

In the early studies, traditional isolation method based on selective culture media was applied to analyze the microbial composition in PXDB fermentation system. Subsequently, high-throughput sequencing technology has been developed and employed for revealing the structure and diversity of microbial community during PXDB fermentation at the genus level (Guan et al., 2017; Li et al., 2017; P. Liu et al., 2020; Lu et al., 2020). Nevertheless, only part of microbial are concerned based on these methods, limiting the overview for the entire information of microbial community. Thereby, to avoid the problem above, metagenomic sequencing, a technique that involves the direct extraction of genetic information for all the microorganisms in samples, has been put forward (Chen and Pachter, 2005; Tian et al., 2020; Xian et al., 2022). Currently, metagenomic sequencing has been widely employed in the study of microbial communities and their functions in fermented foods (Liu et al., 2019), including *fine flavour cocoa* (Fernández-Niño et al., 2021), *Pu-erh tea* (Lin et al., 2018), *Toddy* (Das and Tamang, 2023), *Sieng* (Tamang et al., 2023). These studies provided a theoretical basis for using metagenomic sequencing to comprehensively reveal the microbial community and metabolism mechanism in PXDB-meju fermentation system.

Therefore, in this study, the flavor quality changes of PXDB-meju by the substitution of wheat flour with both of gluten and soybean flour were firstly explored. Meanwhile, metagenomic sequencing technology was applied to comparatively reveal the differences of microbial communities and metabolic pathways related to the formation of key flavor compounds during the fermentation of PXDB-meju.

2. Materials and methods

2.1. Materials

The broad beans (*Vicia faba* L.) were sourced from the local market in Hongguang Town, (Chengdu, China). Flour, soybean flour, and gluten flour were procured from Wal Mart supermarket (Chengdu, China). The strain Hu Niang 3.042 of *Aspergillus Oryza* was obtained from Chengdu Qu Fu Biotechnology Co., Ltd. Chemical reagents were purchased from Chengdu Divelep Technology Co., Ltd. (Chengdu, China). All chemicals reagents are chromatographic grade or analytical grade.

2.2. Sample preparation

The sample preparation process was performed following our previously established methods. Firstly, broad beans were blanched in hot water for 2 min after manually selection. Subsequently, flour was mixed with bean petals according to the composition detailed in Table 1.

Table 1
Four groups of auxiliary material ratio of PXDB-Meju.

Category	Flour		
	Wheat flour	Soybean flour	Gluten flour
CT	25%	–	–
F1	10.4%	10.4%	4.2%
F2	8.9%	8.9%	7.2%
F3	9.6%	9.6%	5.8%

Afterwards, *Aspergillus Oryzae* was added at a ratio of 0.5% to the mixture. Next, koji was made at 30 °C for 3 days. Finally, a 40-days sealing fermentation at 45 °C was conducted for the mixture of the prepared koji and brine (16% NaCl). Samples were collected at 0, 10, 20, 30, and 40 days during the fermentation process, and were tested in triplication to ensure reproducibility.

2.3. Non-volatile compounds analysis

The analysis of organic acids (OAs), amino acid nitrogen, free amino acid contents were conducted following the procedures described in our previous study. Amino acid nitrogen was measured by titration based on the methods of (Chen et al., 2015). The analysis of organic acids (OAs) used the E 2695 HPLC system [Waters, Maple Technology (Beijing) Ltd., Beijing, China] equipped with an Amine HPX-87H Ion Exclusion column. The free amino acid contents were analyzed using a SYKAM amino acid analyzer (Chengdu Weir Co, Chengdu, China) equipped with an LCA K06/Na column and gradient temperature control, ranging from 58 °C to 74 °C, and a UV detector (at 570 nm and 440 nm) (Tan et al., 2021).

2.4. Volatile compounds analysis

The extraction of volatile compounds from PXDB-Meju samples was performed using the HS-SPME method (Ding et al., 2022). To elaborate, 5.000 g of mashed PXDB-Meju sample was introduced into a 20 mL headspace vial, and subsequently, 10 μL of 1,2-dichlorobenzene (100 μg/mL in methanol) was added as an internal standard. The vial was securely sealed and allowed to equilibrate at a temperature of 55 °C for 45 min. As for desorption, it was conducted in GC syringe at a temperature of 250 °C for 1 min. The GC × GC-MS analysis was conducted following a previously reported method with some adjustments (Wang et al., 2021). Volatile compounds were subjected to analysis using a GC × GC-MS system (QP2020; Shimadzu Co., Kyoto, Japan) equipped with a DB-Wax column (polar; 36.4 m × 0.25 mm × 0.25 μm; Agilent Technologies Inc., California, The United States of America) and a DB-17ms column (non-polar; 1.2 m × 0.18 mm × 0.18 μm; Agilent Technologies). Initially, the initial column was held at 40 °C for 2 min, followed by a heating rate of 5 °C/min to 230 °C and a subsequent 4-min hold. The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV and a scan range spanning 41–330 m/z. The temperatures at the inlet and outlet of the hot zone differed from the oven temperature by 70 °C and 160 °C, respectively. The upper temperatures of the inlet and outlet hot zones were 260 °C and 320 °C. Retention indices (RIs) were calculated using the C7–C30 n-alkane series (≥99%; Sigma-Aldrich Co., Ltd, Shanghai, China) under the same chromatographic conditions. Identification of volatile compounds was accomplished by comparing their mass spectra with those stored in the National Institute of Standards and Technology library (NIST20). The OAV value was calculated by the ratio of the compound concentration to the odor threshold obtained from the literature. Compounds with OAVs ≥1 were considered to be important contributors to the aroma characteristics of bean paste (Ding et al., 2021a).

2.5. DNA extraction and metagenomic sequencing

DNA extraction was extracted using the E.Z.N.A.® Soil DNA Kit. The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, TBS-380). The integrity of the DNA was assessed through 1% w/w agarose gel electrophoresis. DNA libraries were prepared on the Illumina platform following the manufacturer's guidelines. Each sample utilized 1 μg of DNA as input material for the DNA sample preparation. Sequencing libraries were created using the NEXTflex Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA) after fragmenting the DNA to an average size of approximately 400 bp using the Covaris M220 instrument (Gene Company Limited, China). Paired-end sequencing was carried out using Illumina NovaSeq Reagent Kits (Illumina Inc., San Diego, CA, USA) on the NovaSeq 6000 S4 Reagent Kit v1.5 (300 cycles). This sequencing procedure was performed by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China), adhering to the manufacturer's protocols available at https://www.illumina.com. The initial raw reads obtained from metagenome sequencing were subjected to analysis on the Majorbio Cloud Platform (cloud.majorbio.com) to obtain clean reads. These clean and high-quality reads were subsequently assembled into contigs using the MEGAHIT software (https://github.com/voutcn/megahit). Open reading frames (ORFs) within the contigs were identified using Prodigal (version 2.6.3, https://github.com/hyattpd/Prodigal). To create a non-redundant gene catalog, CD-HIT was employed with a threshold of 90% identity and 90% coverage. After quality control, the reads were aligned to the representative genes with a 95% identity using SOAPaligner (http://soap.genomics.org.cn/), and the abundance of genes within each sample was assessed.

2.6. Statistical analysis

All the experiments were conducted in triplicates. The data pertaining to volatile organic compounds and key flavor substances are presented as mean ± standard deviation (X ± SD). These data were subjected to analysis using Duncan's multiple comparison method (Duncan) through SPSS Statistics 21.0 software to ascertain significant differences at a P value of less than 0.05. Moreover, Venn plots were employed to enumerate common and unique gene types in the samples, referring to a previous study (Zhao et al., 2018). For functional annotation, BLASTP (BLAST Version 2.2.28+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) was utilized. Subsequently, functional annotation annotation of genes were performed using KOBAS 2.0 (KEGG Orthology-Based Annotation System). To assess the connection between selected microorganisms and key flavor substances, Spearman's rank correlation coefficients were computed using R (pheatmap package), and the resulting significant correlations were visualized using heatmaps (Zhu et al., 2018). The statistical analyses and visualizations of all microbial communities was based on the Majorbio online platform (https://www.majorbio.com/majorbio/index).

3. Results and discussion

3.1. Changes of non-volatile profiles during fermentation

3.1.1. Analysis of amino acid nitrogen content

During the fermentation of PXDB-Meju, amino acid nitrogen is formed by the hydrolysis of the protein in the raw materials with the participation of microorganisms and enzymes (Lijie Zhang et al., 2020). The contents of amino acid nitrogen is regarded as one of the most important indicators to determine the maturity of PXDB-Meju and also the quality of PXDB-Meju. Higher amino acid nitrogen content usually indicates better quality of PXDB (Lijie Zhang et al., 2020). As illustrated in Fig. 1, it could be found that the contents of amino acid nitrogen in the four groups (CT, F1, F2 and F3) gradually rose as fermentation time progressed. Clearly, obvious differences could be obsered between F1, F2 and F3 groups with the CT group ($P < 0.05$). At the initial stage of fermentation (Day 0), the amino acid nitrogen contents for CT, F1, F2, and F3 were 0.35, 0.31, 0.35, and 0.27 g/100g, respectively. After the 40 days of fermentation, the contents of amino acid nitrogen increased to 0.53, 0.61, 0.64, and 0.6 g/100g. Notably, compared to the CT group, the amino acid nitrogen contents in the F1, F2, and F3 groups increased by 15.1%, 20.8%, and 13.2%, respectively. The results obtained above manifested that the combined approach of soybean flour and gluten flour significantly contributed the accumulation of amino acid nitrogen in PXDB-Meju, particularly in Group F2, in which the wheat flour is partially substituted with 7.2% gluten flour and 8.9% soybean flour. There exists tight correlation between the content of amino acid nitrogen and the contents of free amino acids (FAAs) that mainly grant to the taste flavor of PXDB (Lijie Zhang et al., 2020). Therefore, in order to futher explore the effect of combination fermentation on the taste quality of PXDB-meju, the contents of FAAs in F2 and CT groups were analyzed subsequently.

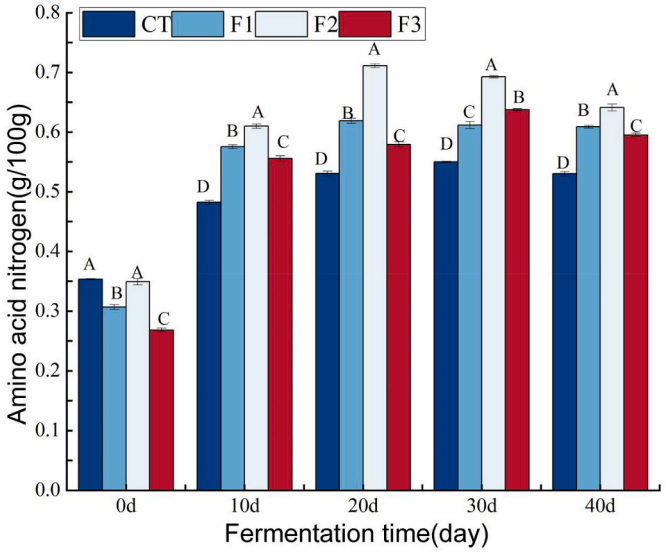

Fig. 1. Changes of amino acid nitrogen content during the fermentation of PXDB-meju.

3.1.2. Analysis of free amino acids contents

Free amino acids (FAAs) are considered as key contributors to the distinctive flavor of PXDB (Jiang et al., 2007). Based on the differences in taste flavor, FAAs are mainly categorized into umami, sweet, bitter, and tasteless amino acids (Lin et al., 2018). According to the result derived from the analysis of amino acid nitrogen content, the CT group and F2 group were selected for the determination of FAAs. A total of 17 amino acids were detected in the samples and their contents were shown in Table 2. Obvious increases of the FAAs contents were observed with the fermentation time extending in both F2 and CT groups. At the end of fermentation, the contents of all the 17 FAAs in F2 group were higher than those in CT group. It could obtain from the data in Table that the contents of umami amino acids increased by 21.0% in F2 group when compared with the CT group. Although the umami FAAs in PXDB was lower than that of bitter and sweet FAAs, the taste of PXDB was still primarily characterized by spicy, salty, and umami, rather than bitter and sweet taste. This might be attributed to the lower threshold of umami FAAs (Chen et al., 2015; Liu et al., 2018).

3.1.3. Organic acids content

Organic acids and free amino acids are essential non-volatile flavor substances in PXDB, produced during the protein hydrolysis process (Lin et al., 2022). Organic acids have a significant impact on the flavor balance, chemical stability, pH, and sample quality of PXDB (Lin et al.,

Table 2
Changes of free amino acids during fermentation of PXDB-Meju.

Taste	FAAs	Content (g/kg)			
		0d		40d	
		CT	F2	CT	F2
Umami	Asp	0.070 ± 0.001^{B}	0.130 ± 0.001^{A}	2.960 ± 0.010^{b}	3.480 ± 0.010^{a}
	Glu	0.207 ± 0.002^{A}	0.250 ± 0.002^{A}	3.410 ± 0.020^{b}	3.800 ± 0.030^{a}
	Subtotal	0.276 ± 0.003^{B}	0.380 ± 0.002^{A}	6.654 ± 0.037^{b}	8.051 ± 0.024^{a}
Sweet	Thr	0.185 ± 0.001^{B}	0.311 ± 0.005^{A}	1.400 ± 0.010^{b}	1.705 ± 0.005^{a}
	Ser	0.065 ± 0.001^{B}	0.155 ± 0.002^{A}	1.550 ± 0.010^{b}	2.225 ± 0.025^{a}
	Gly	0.024 ± 0.001^{B}	0.058 ± 0.000^{A}	0.765 ± 0.005^{b}	3.800 ± 0.030^{a}
	Pro	0.032 ± 0.001^{B}	0.044 ± 0.001^{A}	1.015 ± 0.015^{b}	1.330 ± 0.000^{a}
	Ala	0.083 ± 0.002^{B}	0.143 ± 0.000^{A}	1.645 ± 0.015^{b}	1.945 ± 0.035^{a}
	Lys	0.196 ± 0.001^{B}	0.262 ± 0.004^{A}	1.735 ± 0.015^{b}	2.165 ± 0.015^{a}
	Subtotal	0.582 ± 0.005^{B}	0.972 ± 0.0110^{A}	8.110 ± 0.070^{b}	13.170 ± 0.110^{a}
Bitter	Val	0.119 ± 0.001^{B}	0.190 ± 0.006^{A}	1.560 ± 0.010^{b}	1.895 ± 0.025^{a}
	Met	0.060 ± 0.001^{B}	0.085 ± 0.000^{A}	0.271 ± 0.004^{b}	0.366 ± 0.000^{a}
	Ile	0.089 ± 0.001^{B}	0.153 ± 0.002^{A}	1.375 ± 0.005^{b}	1.770 ± 0.030^{a}
	Leu	0.166 ± 0.001^{B}	0.267 ± 0.005^{A}	2.175 ± 0.015^{b}	2.825 ± 0.025^{a}
	Tyr	0.072 ± 0.001^{B}	0.124 ± 0.001^{A}	0.995 ± 0.015^{b}	1.445 ± 0.015^{a}
	Phe	0.091 ± 0.001^{B}	0.154 ± 0.003^{A}	1.260 ± 0.000^{b}	1.655 ± 0.015^{a}
	Lys	0.196 ± 0.001^{B}	0.262 ± 0.004^{A}	1.735 ± 0.015^{b}	2.165 ± 0.015^{a}
	His	0.032 ± 0.001^{B}	0.156 ± 0.006^{A}	2.100 ± 0.020^{b}	2.515 ± 0.035^{a}
	Arg	0.305 ± 0.000^{A}	0.314 ± 0.005^{A}	1.510 ± 0.010^{b}	2.060 ± 0.020^{a}
	Subtotal	1.126 ± 0.010^{B}	1.703 ± 0.015^{A}	11.246 ± 0.078^{b}	14.531 ± 0.165^{a}
Tasteless	Cys	0.074 ± 0.002^{A}	0.076 ± 0.002^{A}	0.1395 ± 0.001^{b}	0.182 ± 0.006^{a}
Total		1.862 ± 0.001^{B}	2.868 ± 0.0270^{A}	26.149 ± 0.185^{b}	36.115 ± 0.242^{a}

Note: Different letters in the same line (A, B) indicated significant differences at 0d, and (a, b) indicated significant differences at 40d (Duncan's test,$P < 0.05$).

2018). As indicated in Fig. 2, a total of seven acids, including oxalic acid, tartaric acid, malic acid, lactic acid, citric acid, fumaric acid, and succinic acid were identified. Obvious increases of the organic acids contents could be observed in CT, F1, F2 and F3 groups. At the 40th day of fermentation, the organic acid content of CT, F1, F2, and F3 reached 23.620, 16.758, 25.466, and 26.615 mg/g, respectively. Evidently, the F2 and F3 groups exhibit higher levels of organic acid content. This may be due to the higher content of soybean flour in the F1 group compared to the other groups. According to our previous study on PXDB by the substitution with soybean flour or gluten flour, groups with higher soybean flour content had lower organic acid levels than other groups, including the CT group (Zhao et al., 2024). Among them, the lower alcohol-soluble protein content in soybean flour may lead to the changes of microbial community, which in turn affects the content of organic acids.

Among the 7 organic acids, the contents of lactic acid, and succinic acid were crucially dominated, and all of them demonstrated a notable increase during the fermentation process, which played significant role in providing the natural sour flavor of PXDB. On the other hand, the citric acid, as an essential acid flavor regulator in PXDB, exhibited

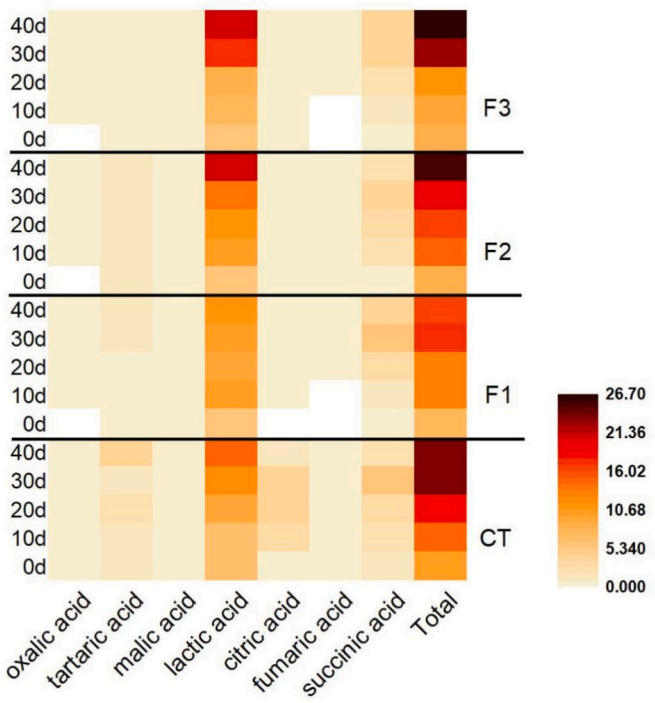

Fig. 2. Changes of organic acids during fermentation of PXDB-meju.

unapparent changes in the F1, F2, and F3 groups during fermentation, while clear increase of citric acid content was discovered in CT group, which might be related to the changes of the fermentation environment and raw material typesin F1, F2 and F3 (Choi and Bajpai, 2010; Lin et al., 2018; Xiang et al., 2024).

3.2. The profiles of volatile components

As shown in Fig. 3A, after a 40-day fermentation period, volatile compounds including alcohols, aldehydes, acids, esters, phenols, ketones, ethers, alkanes, olefins and others were detected by GC × GC-MS analysis in PXDB-meju samples. Fig. 3B presented the contents map of all the volatile compounds in CT, F1, F2 and F3 groups. It could clearly find that the volatile compounds in F1, F2 and F3 groups were more than those in CT group. This result might be ascribed to the higher protein content in soybean flour and gluten flour. Proteins can be broken down by enzymes into peptides or amino acids, as well as forming flavor compounds (Cai et al., 2020).

Among the hundreds of volatile compounds in PXDB-meju, only a small part of them contribute to its overall aroma profile. According to the corresponding odor threshold, the odor activity value (OAVs) of the aroma substances was calculated, and 13 flavor compounds were identified as the key flavor compounds. As illustrated in Fig. 4A, except for the blue area, all other regions show OAVs greater than 1. These key flavor compounds included linalool, 3-methylthio propanol, phenylacetaldehyde, 3-methylation-propanal, nonyl aldehyde, isovaleraldehyde, ethyl palmitate, β-Laurene, 2-acetyl pyrrole, tetramethylpyrazine, nonanal, benzaldehyde, 1-octen-3-ol. The key volatile compounds and their contents map were illustrated in Fig. 4B. The cumulative contents of key aromatic compounds in the CT, F1, F2, and F3 groups were 296.95, 585.91, 850.81, and 583.90 ng/g, respectively. In comparison to the CT process, F1, F2 and F3 exhibited increase in the total content of key aromatic compounds. Moreover, F2 group contained the highest content of key volatile compounds. Meanwhile, the phenethylaldehyde in the F1, F2, and F3 groups was found higher than those in CT group. The phenethylaldehyde and phenethyl alcohol could mutually convert under the action of aryl-alcohol dehydrogenase

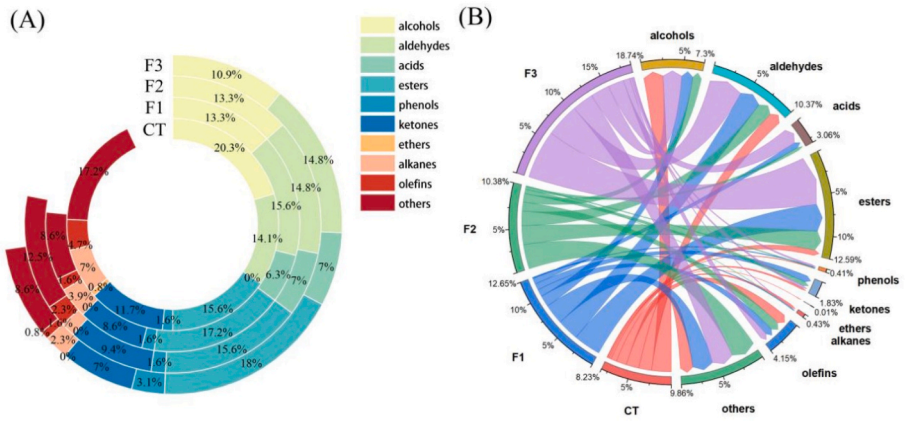

Fig. 3. Flavor maps for the 40th days fermentation of four different groups. (A) type map; (B) content map.

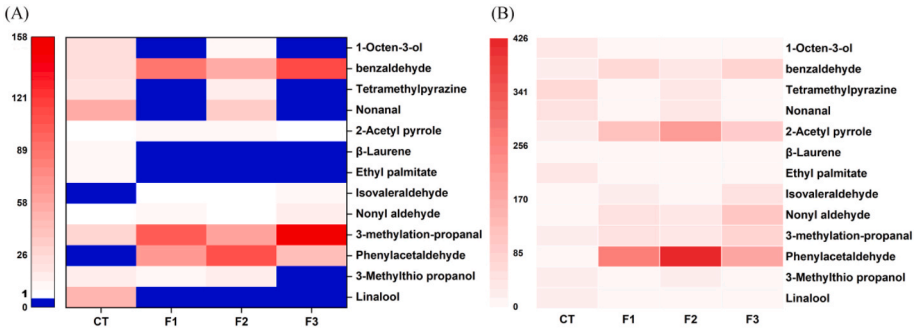

Fig. 4. The main volatile substances of samples. (A) Odor activity values (OAV) of key aroma substances in PXDB-meju. (B) Heat map analysis of the key aroma substances.

(EC1.1.1.90). Previous studies have indicated that phenethyl alcohol is one of the most important key aroma compounds in fermented broad bean mash (Yang et al., 2022; Zhao et al., 2024). Meanwhile, phenethylaldehyde, a crucial aroma compound in traditional fermented foods, exhibited significantly higher levels in the F2 group compared to the other groups (Lee et al., 2013). Therefore, the F2 group also demonstrates the most favorable effect.

3.3. Genome sequencing and assembly quality analysis of samples

According to the results obtained above, generally, F2 achieved preferable quality profiles than F1 and F3. Hence, F2 was selected for metagenomic analysis to further reveal the influences of partially using soybean flour and gluten flour on the fermentation microorganisms in PXDB-meju. In order to determine the information of the microbiota in PXDB, a total of 6 samples were analyzed using metagenomic sequencing. During this procedure, a raw read count of 9.36×10^8 (an average of 7.80×10^7 reads for the brine samples) were generated. After quality processing and the removal of host genes, the average sequence utilization rate was 98.16%. These sequences were assembled using Megahit, and 2,598,442 Contigs were produced. The sample Contigs counts were ranging from 90,180 to 329,412. N90 provides an accurate representation of sequence splicing efficiency, and a longer N90 value signifies a superior sample assembly. Across all samples, the N90 values ranged from 341 bp to 408 bp, indicating the suitability of this information for subsequent analysis (Table 3). ORF prediction was performed on the results. Subsequently, the predicted gene sequences from the samples were clustered using CD-HIT software to construct a non-redundant gene set. This process yielded a total of 566,080 non-redundant gene sets. The Venn diagram illustrates that there are significant differences in gene species between the CT 0d and CT 40d samples, as well as the F2 0d and F2 40d samples (Fig. 5A,C). The CT 0d

Table 3
Statistics profiles of the metagenome.

Sample	Raw reads	Clean reads	Clean base(bp)	Percent in raw reads(%)	Contigs	Contigs bases(bp)	N90(bp)
CT0d_1	71328740	69942998	10545194068	98.05724593	329412	224453015	346
CT0d_2	71583166	70325360	10594573850	98.24287459	343280	222878688	341
CT0d_3	78432492	76709176	11554628153	97.80280346	334584	233570139	346
CT40d_1	80938046	79476966	11964796637	98.19481681	92638	90603354	394
CT40d_2	73545348	72385904	10893450872	98.42349784	90180	85356678	390
CT40d_3	72184340	70835702	10654785857	98.1316751	90192	82865861	385
F0d_1	73128718	71539444	10775071067	97.82674434	311583	308328266	387
F0d_2	115024900	113007170	17025732059	98.24583199	356613	353710374	385
F0d_3	68581072	67188898	10123260557	97.97003173	297714	313135775	398
F40d_1	74741404	73510506	11068886303	98.35312433	114456	111691854	398
F40d_2	70246086	69120740	10409172638	98.39799473	117395	117954790	407
F40d_3	86432126	84944424	12789434688	98.27876269	120395	124197895	408

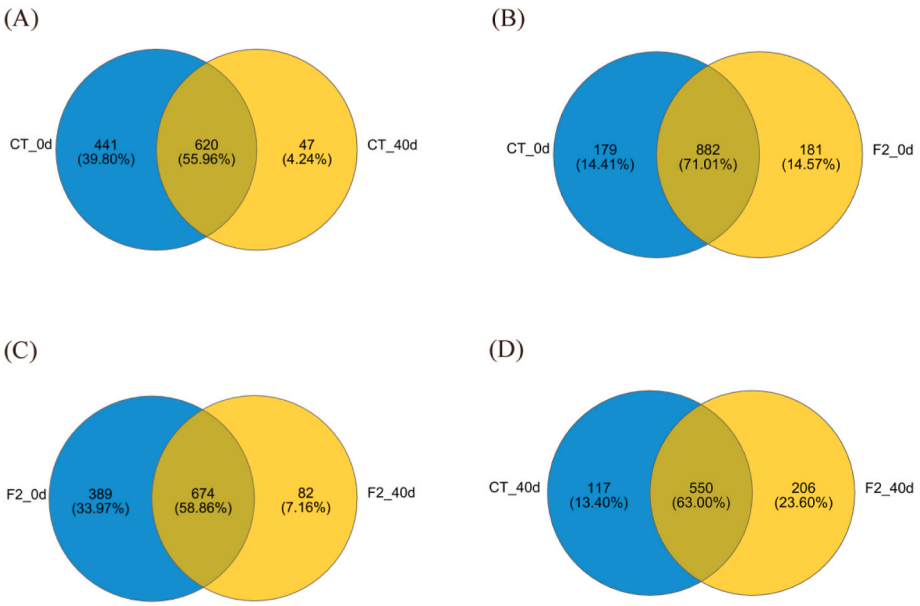

Fig. 5. Venn diagrams of the distribution of gene species between (A) CT_0d and CT_40d; (B) F2_0d and F2_40d; (C) CT_0d and F2_0d; (D) CT_40d and F2_40d.

and F2 0d samples show almost no difference in gene species, because both groups had not started fermentation (Fig. 5B). In contrast, the gene species differ between the CT 40d and F2 40d samples, indicating the impact of the completed fermentation process (Fig. 5D).

3.4. Analysis of microbial community in different fermentation processes

3.4.1. Analysis of microbial compositions

The relative abundance variations for bacteria and fungi at genus level were presented in Fig. 6. It could be discovered that the top 5 bacteria in the two processes were similar, including *Bacillus_f_Bacillaceae, Enterobacter, Kosakonia, unclassified_f_Enterobacteriaceae, Pantoea*. Most of these bacteria presented consistent variation trends during a 40d fermentation procedure. *Enterobacter* showed sharp increases in both CT and F2 group at the end of fermentation. In the F2 group, it increased by 40.3%, while in the CT group, it increased by 37.7%. At the 40d, the content of *Enterobacter* in both processes exceeded 50%. This suggested that *Enterobacter* is the dominant bacterial species in PXDB fermentation. According to previous study, *Enterobacter* has the ability to break down compounds into sugars, alcohols, organic acids, and other substances through oxidative processes, influencing the overall flavor of PXDB (X.-F. Liu et al., 2020). Meanwhile, *unclassified_f_Enterobacteriaceae* also increased obviously in both fermentation groups. It is identified as a dominant acid-producing bacterium (Chen et al., 2023). The relative abundance increase in the F2 group was 18%, whereas in the CT group, it was only 12%. Consistently, the organic acids contents in F2 group was found higher than that in CT group.

As for fungi (Fig. 6B), *Aspergillus* was dominated in CT 0d and F2 0d, followed by *Trifolium Pisum, Medicago, Glycine, Lupinus, Cicer, Vicia, Mucuna*, and *Plutella*. The relative abundance of *Aspergillus* in F2 0d (92.98%) was nearly three times higher than that in CT 0d (31.71%). At the end of fermentation, the relative abundance of *Aspergillus* decreases to 7.8% and 4.87% for F2 and CT group. a decrease rate much higher than that in the traditional group. These results suggest that, compared to CT fermentation, F2 fermentation has a more significant impact on fungal succession than on bacterial succession. At the end of fermentation, *Lupinus* and *Plutella* became the predominant fungi in the CT and F2 group. Specifically, the relative abundance of *Lupinus* increased significantly from 1.2% to 50.5% for F2 group, and from 2.8% to 57.3% for CT.

As shown in Fig. 7, a total of 6309 microorganisms were identified at the species level during the fermentation process of PXDB-Meju. Among them, the top 50 abundant species accounted for more than 90% of the species abundance, representing the characteristic microorganisms in

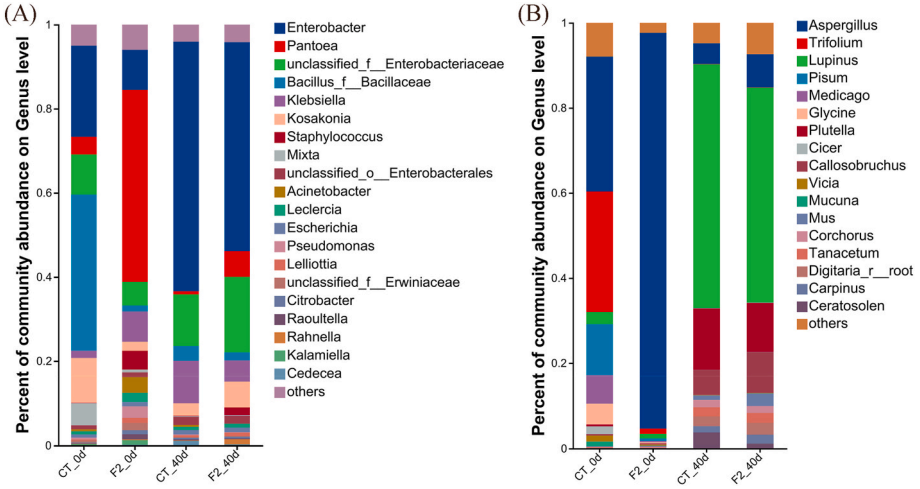

Fig. 6. Distribution of (A) bacterial and (B) fungi community abundance at the genus level.

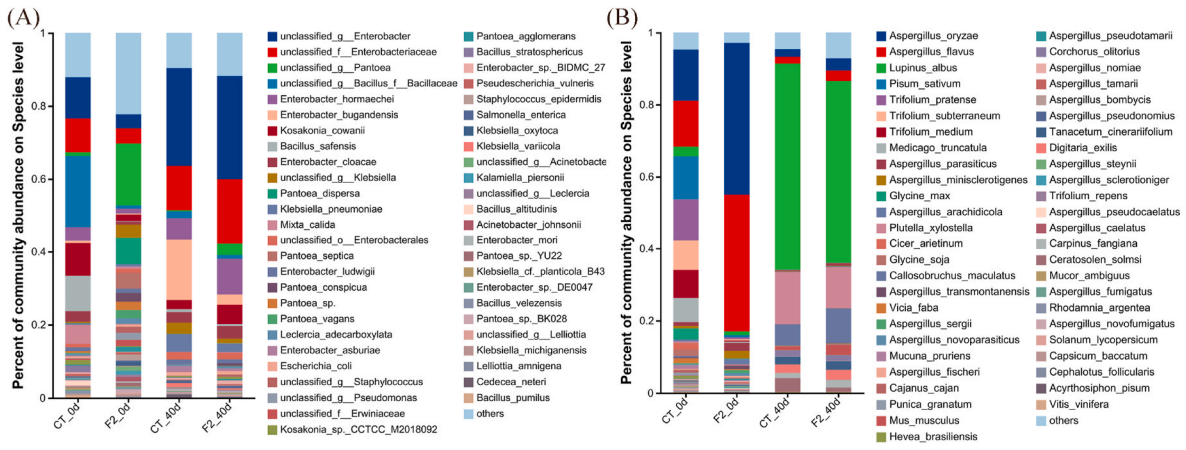

Fig. 7. Distribution of (A) bacterial and (B) fungi community abundance at the species level.

the PXDB-Meju. Among them, *unclassified_g_Bacillus_f_Bacillaceae, unclassified_g_Enterobacter, Bacillus safensis, unclassified_f_Enterobacteriaceae, Kosakonia_cowanii* had the comparably highest relative abundance of the microbial community, respectively (Fig. 7A). *Unclassified_f_Enterobacteriaceae* and *unclassified_g_Enterobacter*, in both of the CT and F2 groups, increased with the extension of fermentation time. This suggests that these two species might be the dominant bacterial strains in the PXDB-Meju fermentation process for both groups. Furthermore, at the 40th day of fermentation, the abundances of these species in F2 group were significantly higher than those in CT group. Meanwhile, *Kosakonia_cowanii* was found a increase trend with fermentation time extending in the F2 group. Conversely, it decreased in CT group with the time prolonging. Previous study had revealed that *Kosakonia_cowanii* had the ability to produce lipase and hydrolyze fats, which might contribute to the flavor forming in the F2 group (Ren et al., 2020).

For fungal community, the total relative abundance of the top 5 fungal species in CT and F2 are *Aspergillus_oryzae*, *Aspergillus_flavus*, *Pisum_sativum*, *Trifolium_pratense,* and *Trifolium_subterraneum* (Fig. 7B). Among these species, *Lupinus_albus* and *Plutella_xylostella* significantly increased in both of the CT and F2 groups with fermentation time prolonging, indicating that these two fungi might be dominant fungi in fermenting PXDB-Meju. In contrast, the abundance of *Aspergillus_oryzae* and *Aspergillus flavus* displayed a decrease trend with the extension of fermentation time in both of CT and F2 groups. This might be attributed that the organic acids contents were increased during fermentation and would inhibit the growth of *Aspergillus flavus* (Tao et al., 2014; Zhang et al., 2017).

3.5. Correlation analysis between flavor compounds and representative microbial

As most of the flavor compounds are mainly attributed the metabolism of microbial communities in fermented food, it is important to reveal the potential key microbial that contribute mostly to the formation of flavor by correlation analysis. In this study, Spearman correlation analysis was conducted to assess the relationships between the top 50 microorganisms at the species level with 7 organic acids, 17 free amino acids, and 15 key volatile compounds. A significant correlation threshold was set at $|R| \geq 0.5$.

Lactic acid acts as an important part in adjusting the balanced sour flavor and fermented environment of fermented food. The content of Lactic acid increased with the fermentation extending and achieved the highest among all the organic acids of PXDB-meju in this study. As illustrated in Fig. 8A, in CT fermentation PXDB-meju, 13 microorganisms including *Enterobacter_asburiae, Enterobacter_bugandensi, Enterobacter_hormaechei, Enterobacter_kobei, Enterobacter_ludwigii, Enterobacter_mori, Enterobacter_roggenkampii, Enterobacter_sp._BIDMC_27, Enterobacter_sp._BIDMC_29, Escherichia_coli, Klebsiella_oxytoca, unclassified_f_Enterobacteriaceae, unclassified_g_Enterobacter, unclassified_o_Enterobacterales* were positively related with the contents of organic acids. However, in F2 fermentation group (as shown in Fig. 8B), more microorganisms like *Acinetobacter_johnsonii, Aspergillus_flavus, Aspergillus_oryzae, Enterobacter_mori* were found negatively related to the contents of organic acids. This might be caused by the higher level of lactic acid accumulated in F2 group that would inhibit the growth of some microorganisms.

Succinic acid are important acidifying agents that could blend sour flavor and inhibit the growth of stray bacteria in PXDB (Xiang et al., 2024). The content of succinic acid is second only to lactic acid among organic acids in this study. As illustrated in Fig. 8A, microorganisms in CT group including *Enterobacter_asburiae, Enterobacter_bugandensis, Enterobacter_kobei, Enterobacter_ludwigii, Enterobacter_mori, Enterobacter_roggenkampii, Enterobacter_sp._BIDMC_29, Escherichia_coli, Klebsiella_oxytoca, Klebsiella_pneumoniae, Salmonella_enterica, unclassified_f_Enterobacteriaceae, unclassified_o_Enterobacterales, unclassified_g_Klebsiella, unclassified_g_Enterobacterdisplayed* strongly positive correlations with succinic acid. On the other hand, as presented in Fig. 8B, there were 45 microorganisms in F2 fermentation group related to the contents of organic acids, in which *Enterobacter_asburiae, Enterobacter_bugandensis, Enterobacter_cloacae, Enterobacter_hormaechei, Enterobacter_sp._BIDMC_27, Escherichia_coli, Klebsiella_pneumoniae, Kosakonia_cowanii, Kosakonia_sp._CCTCC_M2018092, Salmonella_enterica, Staphylococcus_gallinarum, unclassified_f_Enterobacteriaceae, unclassified_o_Enterobacterales were strongly positive with* succinic acid. Interestingly, it could be found that these microorganisms that correlated with organic acids in CT and F2 were significantly different. This might be owing to the partially substitution of flour with soybean flour and gluten flour, which changed the environment of microorganisms. Generally, the total organic acids contents in F2 were higher than those in CT group. Therefore, the microbial that highly correlated with the organic acids require further exploite to make a better control of the fermentation enviroment and the natural sour flavor of final product.

Glutamic acid (Glu) is one of the most important amino acids in fermented paste (Ding et al., 2021b; Liang et al., 2020). The contents of Glu were the highest in both of CT and F2 groups. As shown in Fig. 8A, *Cedecea_neteri, Enterobacter_roggenkampii, Klebsiella_variicola, Salmonella_enterica* in CT group were found positively related with the contents of Glu.

Otherwise, according to the result in Fig. 8 (B), in F2 group, *Enterobacter_asburiae, Enterobacter_cloacae, Enterobacter_hormaechei, Klebsiella_pneumoniae, Kosakonia_cowanii, Kosakonia_sp._CCTCC_M2018092, Staphylococcus_gallinarum, unclassified_f_Enterobacteriaceae, unclassified_g_Enterobacter* and *unclassified_o_Enterobacterales* had positive

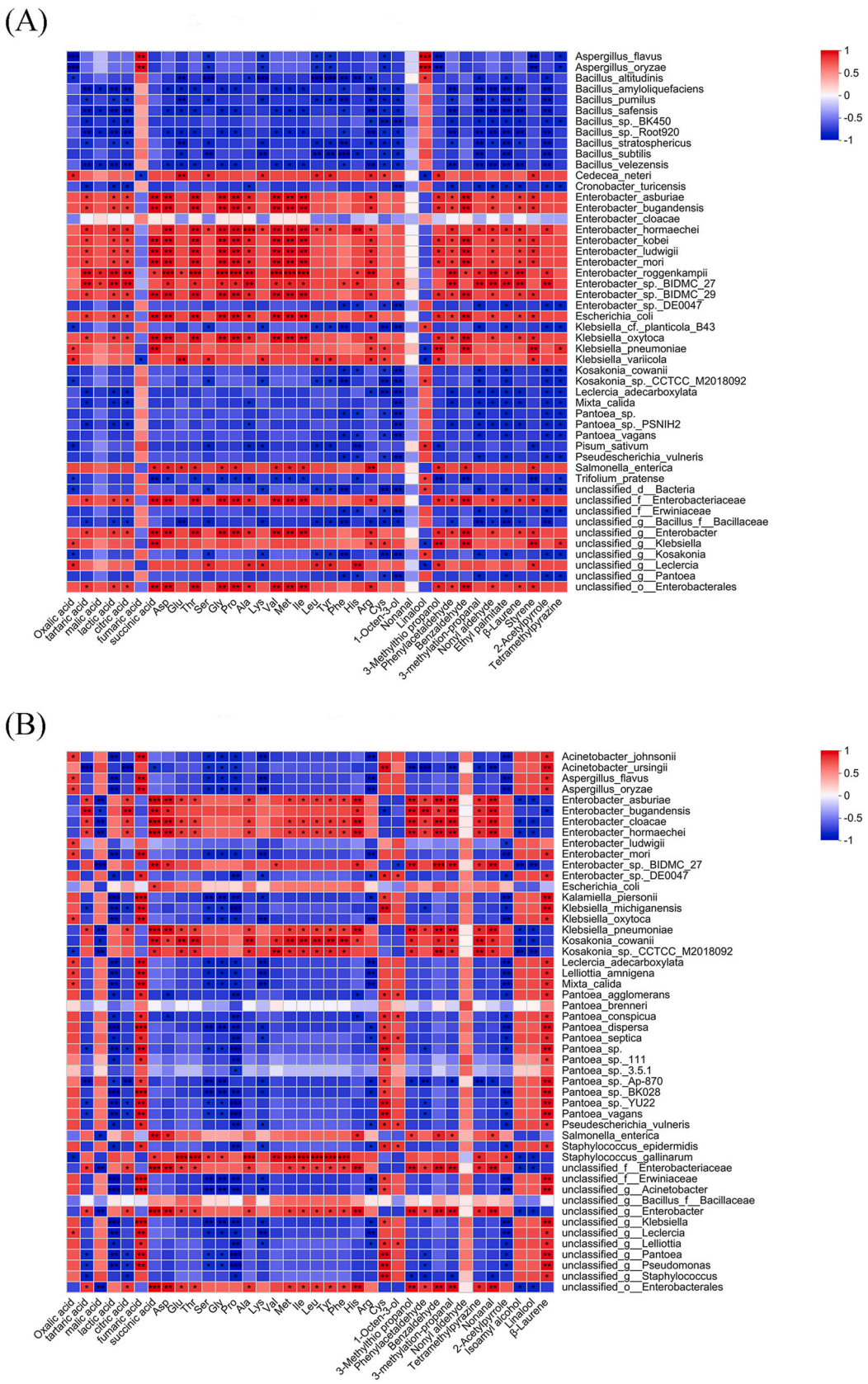

Fig. 8. Correlation analysis of flavor substances and microorganisms: (A) CT and (B) F2.

relation with the contens of Glu. Similarly with the finding in organic acids, the key microorganisms that greatly contributed to Glu were different. Futhermore, more Glu could be obtained by the fermentation process of F2 based on the results above. This could be mainly attributed to the higher protein conatined in soybean and gluten than flour (Zhao et al., 2024).

Regarding to volatile flavor, totally microbial species in CT group were related to the 13 compounds. *Enterobacter_asburiae, Enterobacter_bugandensis, Enterobacter_hormaechei, Enterobacter_kobei, Enterobacter_ludwigii, Enterobacter_mori, Enterobacter_roggenkampii, Enterobacter_sp._BIDMC_27, Enterobacter_sp._BIDMC_29, Escherichia_coli, Klebsiella_oxytoca, unclassified_f_Enterobacteriaceae, unclassified_g_Enterobacter, unclassified_o_Enterobacterales* had positive relation with the contens of phenylacetaldehyde. This volatile compounds mainly provide rose and hyacinth aroma flavor to PXDB-meju, meanwhile, it had the highest contents among all the 13 key volatile compounds. On the other hand, in F2 group, different microorganisms including *Enterobacter_asburiae, Enterobacter_bugandensis, Enterobacter_cloacae, Enterobacter_hormaechei, Klebsiella_pneumoniae, unclassified_f_Enterobacteriaceae, unclassified_g_Enterobacter, unclassified_o_Enterobacterales* were found positively related with the contents of phenylacetaldehyde. Flowery aroma of phenylacetaldehyde is able to generate complex, concentrated, and layered aromas of PXDB (Lin et al., 2019).

3.6. Metabolic pathways of major flavor substances

During fermentation, monosaccharides, free amino acids (FAAs) and fatty acids in the raw materials could be firstly broken down with the enzymes produced by microorganisms, which is called primary metabolic pathways. Subsequently, these primary metabolic compounds, acting as flavor precursors, participate in further metabolic processes, leading to the production of the secondary metabolites (Ye et al., 2022; Zhang et al., 2023). To further explore the potential synthesis pathways of key flavor compounds during the fermentation of PXDB, the sequencing data and KEGG functional databases were combined for analysis. Metabolic pathway prediction network for the key flavor compounds such as aspartate, glutamate, lysine, arginine, phenylacetaldehyde, and phenethyl alcohol were constructed. A total of 71 enzymes were collated in both of CT and F2 groups. Meanwhile, 32 microorganisms among the top 50 species in terms of abundance were annotated that were associated with the corresponding enzymes (Fig. 9).

During the fermentation process of broad beans, starch and cellulose would be hydrolyzed by corresponding enzymes and the glucose produced in the hydrolysis was used as crucial energy source for microbial growth. 9 enzymes that related to starch degradation were annotated, and were highlighted in red (Fig. 9). In this process, starch was catalytic hydrolyzed by alpha-amylase (EC 3.2.1.1) and isoamylase (EC 3.2.1.68), forming maltose and dextrin. Isoamylase was not annotated to any relevant microorganisms in CT group, while in the F2 group, five microorganisms were annotated, including acid-producing *Klebsiella pneumoniae*. It has been reported that *Klebsiella pneumoniae* could utilize glucose to generate pyruvic acid through the sugar fermentation pathway. Pyruvic acid, acted as a major precursor, could produce substances such as 2,3-butanediol, acetic acid, and ethanol (Tao et al., 2019).

Lactic acid is a major organic acid in PXDB, and it has greatly influence on the flavor profiles and shelf life of fermented food, and worked as the precursor of some desirable esters in fermented broad beans.The lactic acid metabolic pathway is shown in green in Fig. 9. L-Lactate and D-Lactate are stereoisomerism and could be mutually transformed. L-lactate acid is widely used in the food industry and is beneficial in improving the acidic and stimulating taste of acetate and can be further converted to other flavor compounds such as ethyl lactate (Xiang et al., 2024). Lactaldehyde dehydrogenase (EC 1.2.1.22) had the highest abundance in producing L-lactate acid. Among the microorganisms annotated with lactaldehyde dehydrogenase (EC 1.2.1.22) in the CT group, there were 3 species: *Pantoea_dispersa, Enterobacter_hormaechei,* and *unclassified_o_Enterobacterales*. In the F2 group, 6 relevant microorganisms were annotated, *Kosakonia_cowanii* was exclusively annotated in the F2 group. Research suggests that utilizing *Kosakonia_cowanii* for the decomposition of cotton and coffee waste can yield a significant amount of lactic acid, aligning with the higher lactate content in the F2 group compared to the CT group in this study (El-Sheshtawy et al., 2022).

Succinic acid played a crucial role in the acidification and flavor enhancement of PXDB, ranking second only to lactic acid in organic acid content in this study. The lactic acid metabolic pathway is shown in brown in Fig. 9. Succinyl-CoA synthetase alpha subunit (EC:6.2.1.5) was the most abundant of the three enzymes. It could be found that the relative abundance of EC:6.2.1.5 in F2 group was higher than that in CT group at the 40th day.

Aspartic acid and glutamic acid are of great importance in broad bean paste fermentation system. Studies indicate that aspartic acid and glutamic acid play a crucial role in the freshness taste of fermented bean products (Yang et al., 2021). From the results of free amino acids, it is evident that these two amino acids content in the F2 group was higher than that in the CT group.

As shown in Fig. 9, four enzymes related to L-aspartic acid were annotated in purple. They are asparaginesynthase (EC 6.3.5.4), glutaminase (EC 3.5.1.38), L-asparaginase (EC 3.5.1.1), and aspartate aminotransferase (EC 2.6.1.1). Among them, the gene abundance of L-asparaginase (EC 3.5.1.1) in the F2 group is higher than that in the CT group. It was annotated to 2 microorganisms in CT group and 7 microorganisms in F2 group. The *Bacillus_velezensis* in F2 has been demonstrated to effectively enhance the flavor of fermented broad beans products (Luo et al., 2023).

L-glutamate is associated with 9 relevant enzyme classes. There were three main pathways for L-glutamate synthesizing as indicated in light blue in Fig. 9. EC 1.2.1.88 had the highest relative abundance in the glutamate synthesis pathway. At the end of fermentation, F2 group had higher relative abundance of genes related to 1-pyrroline-5-carboxylate dehydrogenase (EC 1.2.1.88) than CT group.

Arginine is crafted through a series of enzymatic reactions with glutamate as its precursor substance. This amino acid holds significant importance in the context of fermented food products (Chen et al., 2024; Liu et al., 2022; Majumdar et al., 2016). In this study, the arginine content in the F2 group was higher than that in the CT group. Two biochemical pathways have been identified for the generation of arginine, as illustrated in Fig. 9 and noted in pink.

Lysine was identified as a key savory amino acid in broad bean samples, imparting bitter taste to the broad bean paste and enriching the flavor of the product (Liang et al., 2020). As shown in Fig. 9 with orange annotation, lysine biosynthesis mainly involved two pathways. In the synthesis pathway of lysine, aspartate kinase (EC:2.7.2.4) had the highest abundance compared with the other enzymes. In this study, the lysine content in the F2 group was higher than that in the CT group. Among them, *Escherichia coli* that was exclusively found in F2 group was annotated by aspartate kinase (EC:2.7.2.4). Although *Escherichia coli* is a conditional pathogenic bacterium, research has found that using *Escherichia coli* to produce amino acids (lysine, methionine, tryptophan, threonine, valine, and arginine) can enhance amino acid fermentation performance, simplify fermentation process control, and reduce production costs (Guo et al., 2021).

The amino acid metabolism in the fermentation process of broad bean paste could further generate a large number of flavor compounds, in which numerous metabolic enzymes and various microorganisms involved. In this study, phenethylaldehyde was the key flavor substance with the highest content. Phenethyl alcohol and phenethylaldehyde can undergo mutual conversion under the action of aryl-alcohol dehydrogenase (EC 1.1.1.90). Meanwhile, the phenethylaldehyde and phenethyl alcohol in the F2 groups were found higher than those in CT group. Therefore, the metabolic pathways of key aromatic compounds

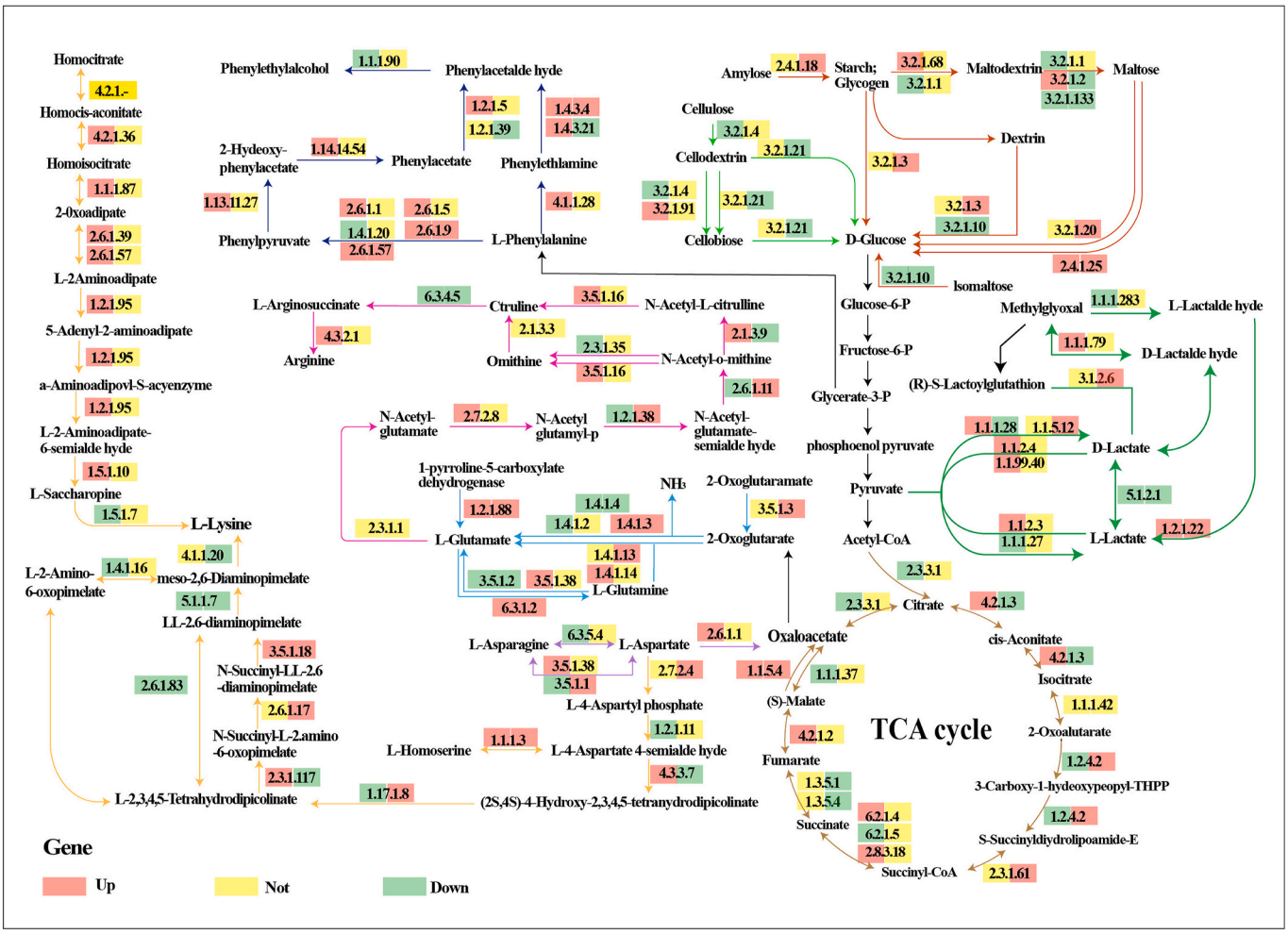

Fig. 9. Analysis of the metabolic pathways of relevant flavor compounds. For this figure, the rectangle chart represents the number of genes that related to enzyme, and the up and down expression of genes were represented by the F2 in relation to the CT.

including phenethylaldehyde and phenethyl alcohol were primarily annotated. As shown in Fig. 9 in deep blue, phenethylaldehyde and phenethyl alcohol are primarily synthesized from Glycerate-3-P as a precursor. Glycerate-3-P is derived from glucose decomposition, and it is involved in the interconversion with amino acids. In this study, the F2 group had a higher protein content due to the partial substitution of wheat flour with high-protein soybean flour and gluten flour. This led to a significantly higher amino acid content in the F2 group compared to the CT group, further promoting the synthesis of phenethylaldehyde and phenethyl alcohol, resulting in higher levels of phenethylaldehyde and phenethyl alcohol in the F2 group compared to the CT group. Aromatic-amino-acid transaminase (EC 2.6.1.57) has the highest abundance in this metabolic pathway. At 0d and 40d, the abundance of EC 2.6.1.57 in F2 group was higher than that in CT group. In both CT and F2 groups, three types of microorganisms related to aromatic-amino-acid transaminase (EC 2.6.1.57) were identified. Among them, *Escherichia coli* in F2 group is commonly found in the intestinal microbiota. In recent years, scholars have discovered that *Escherichia coli* has the function of catalyzing the degradation of aromatic flavor precursors (Long et al., 2021; Zhong et al., 2017), which may contribute to the synthesis of phenylacetaldehyde in the F2 group fermentation process.

4. Conclusions

In this research, wheat flour was partially substituted with soybean flour and gluten flour in portions of 10.4% and 4.2% (F1 group), 8.9% and 7.2% (F2 group), 9.6% and 5.8% (F3 group) during the PXDB-meju fermentation. The impacts of the substitution on the flavor quality and microbial community diversity of PXDB-meju were investigated. Generally, the amino acid nitrogen content increased by 15.1%, 20.8%, and 13.2% compared to the CT group. The F2 and F3 groups also exhibited slightly higher levels of organic acids compared to the CT group. Aspartate, glutamic acid, arginine, and lysine as key amino acids in PXDB, and phenylacetaldehyde as key volatile substances were found higher in F2 than those in CT group. Metagenomic technology revealed predominant bacteria were *Kosakonia_cowanii, unclassified_f_Enterobacteriaceae,* and *unclassified_g_Enterobacter* in F2 and CT groups. These bacteria abundances in F2 group were higher than those in CT group. *Lupinus_albus* and *Plutella_xylostella* were dominant fungi species in fermenting PXDB-Meju. In addition, metabolic analysis explored key flavor compound synthesis, including key amino acids and phenylacetaldehyde, phenethyl alcohol. Enzyme abundance and associated microorganisms related to key flavor compounds significantly differed in the F2 group compared to CT. In the metabolic pathways of key amino acids and phenethylaldehyde, the abundances of EC 3.5.1.1, EC 1.2.1.88, EC:2.6.1.11, EC:2.7.2.4, and EC 2.6.1.57 were higher in F2 than in CT, *Kosakonia_cowanii*, *Bacillus_velezensis* and *Escherichia coli* were annotated to these enzymes in F2 group. This study explored the flavor differences among PXDB fermented with differences flour types and examined the relationship between microorganisms and flavor in order to stabilize and enhance the flavor and quality of PXDB.

CRediT authorship contribution statement

Min Xu: Data curation, Funding acquisition, Writing – original draft, Formal analysis, Software. **Yuxin Guo:** Data curation, Funding acquisition, Writing – original draft, Formal analysis, Software. **Xiaoyan Song:** Formal analysis, Data curation. **Ling Li:** Data curation, Methodology. **Zedong Xu:** Data curation, Methodology. **Jianhua Zhao:** Data curation, Methodology. **Jie Zhao:** Project administration, Supervision. **Hongbin Lin:** Project administration, Supervision. **Shirong Dong:** Resources, Methodology, Funding acquisition. **Jing Lu:** Resources, Methodology, Funding acquisition. **Wenwu Ding:** Resources, Methodology, Funding acquisition. **Ping Liu:** Writing – review & editing, Supervision, Project administration. **Jie Tang:** Methodology, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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