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High-throughput sequencing-based study on bacterial community structure and functional prediction of fermented bean curd from different regions in China

Xue Feng✉, Zhao Dong & Teng Hao

In order to understand the bacterial community structure, microbial safety, and functional diversity of traditional fermented food—sufu (fermented bean curd) in China, high-throughput sequencing technology was employed to systematically analyze the bacterial community composition and functional profiles of sufu samples from eight different regions in China (Zunyi, Guizhou; Xiushan, Chongqing; Chengdu, Sichuan; Xinhua, Hunan; Ji'an, Jiangxi; Mouding, Yunnan; Guilin, Guangxi; and Shilin, Kunming). The results revealed that the predominant bacterial phyla in sufu included Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota, with key genera such as *Pseudomonas*, *uncultured Enterobacteriaceae*, and *Lactococcus*. The sample from Ji'an, Jiangxi (JXR5), exhibited the highest bacterial species richness, while the sample from Shilin, Kunming (KMR8), showed the highest species diversity, indicating significant impacts of geographical location and production processes on microbial communities. Functional analysis demonstrated that the bacterial communities in sufu were primarily involved in metabolic pathways such as chemoheterotrophy, aerobic chemoheterotrophy, and fermentation. The sample from Xiushan, Chongqing (CQR2), had the highest proportion of fermentation-related functions, contributing to the formation of a finer texture and richer flavor. The sample from Guilin, Guangxi (GXR7), exhibited the highest aromatic compound degradation function, which may enhance flavor or reduce undesirable odors. Additionally, significant variations were observed in functions such as nitrate reduction, nitrogen respiration, and nitrate respiration among different samples, suggesting that microbial diversity in nitrogen metabolism may influence the safety and quality of sufu. This study highlights the regional characteristics and functional diversity of bacterial communities in sufu, providing a scientific basis for optimizing production processes and developing region-specific products.

Keywords Sufu, Bacterial community, High-throughput sequencing, Functional prediction

Fermented bean curd is a traditional fermented soybean product in China, and it has the laudatory title of "Oriental cheese". It is widely popular in Asia and globally due to its unique flavor and nutritional value[1]. The fermentation process of fermented bean curd mainly depends on the action of the microbial community, among which bacteria play a key role in the formation of the quality, the generation of flavor substances, and the guarantee of safety of fermented bean curd[2]. However, the fermentation process of fermented bean curd is affected by various factors, including raw materials, production processes, fermentation environments, etc. The differences in climatic conditions, cultural traditions, and craftsmanship in different regions may lead to significant differences in the structure and function of the microbial community in fermented bean curd. For example, in the cold and dry climate of northern China, low-temperature salting and slow fermentation are adopted. The dominant bacteria, Mucor and Rhizopus, secrete proteases and lipases, endowing fermented bean curd with a rich and mellow umami taste. In the warm and humid southern China, the fermentation cycle is short. Mucor and Monascus purpureus work in concert. Monascus purpureus produces pigments such as monascin and amylase, etc., making the fermented bean curd sweet, fresh, and rich in ester aroma. Due to the unique plateau environment and ethnic craftsmanship of the fermented bean curd in Mouding, Yunnan Province,

Guilin Tourism University, Guilin 541000, Guangxi, China. ✉email: 1739877528@qq.com

Bacillus subtilis produces a variety of enzymes, and combined with local spices, it forms a rich, spicy flavor[3]. Internationally, natto in Japan relies on Bacillus subtilis natto and has health-care functions; the microorganisms in French cheese are complex, and the joint action of lactic acid bacteria, yeasts, and molds, etc., forms a unique flavor and texture, which is significantly different from Chinese fermented bean curd[4]. Therefore, systematically analyzing the bacterial community structure and its function of fermented bean curd in different regions of China is of great significance for revealing its fermentation mechanism and improving product quality.

In recent years, the application of high-throughput sequencing technology has advanced research on the microbial communities of sufu. Studies have shown that sufu primarily contains Lactobacillus, Bacillus and Saccharomyces, which play key roles in flavor formation and quality control[4]. Additionally, significant differences in microbial composition have been observed in sufu from different regions. For example, southern sufu tends to have a higher abundance of Lactobacillus, while Bacillus is more common in northern sufu[5]. However, most existing research focuses on single regions or specific microbial groups, lacking systematic comparative studies. Although the diversity of microbial communities in sufu has been partially revealed, the functional potential of bacterial communities, particularly genes related to flavor, protein degradation, and safety, remains underexplored[5]. Furthermore, the ecological driving factors, such as environmental parameters and process characteristics, influencing microbial community structure have not been comprehensively analyzed[6].

Based on this, this study systematically analyzes the bacterial communities of fermented bean curd from 8 different regions through high-throughput sequencing technology. It can accurately identify the core bacterial groups and their functional genes in fermented bean curd from different regions, clarify the influence mechanism of environmental parameters and process characteristics on the structure of the microbial community, fill the gap in the systematic comparative study of the microbial communities of fermented bean curd in multiple regions, provide a scientific basis for constructing standardized production, quality control, process optimization, and new product development of fermented bean curd based on regional characteristics, and promote the development of the fermented bean curd industry towards standardization and scientificization.

Materials and methods

Samples

To ensure the representativeness and broadness of research samples, fermented bean curd samples were systematically sampled from the JD Mall platform in March 2024. The specific sampling method was as follows: Entering the keyword "fermented bean curd" in the platform search bar, screening out the top 50 products by sales volume ranking, and preferentially selecting commodities from major fermented bean curd production areas in Southwest, Central - South, and Southeast China. That is, from the products of 8 typical fermented bean curd manufacturers in Zunyi, Guizhou; Xiushan, Chongqing; Chengdu, Sichuan; Xinhua, Hunan; Ji'an, Jiangxi; Mouding, Yunnan; Guilin, Guangxi; Shilin, Kunming and other regions, one commercially available product with sales ranking within the top 10 and good reviews was randomly selected from each manufacturer. It was ensured that only 1 brand and 1 product were selected from each production area, and there was no repeated sampling.

The selected samples have a wide geographical distribution, coming from different climate and ecological environment zones, and all adopt local raw materials and traditional craftsmanship to ensure that their flavors, textures, and fermentation characteristics can reflect local characteristics, such as the spicy flavor of Zunyi, Guizhou, and the numb - spicy flavor of Chengdu, Sichuan. Meanwhile, these samples are all representative products circulating in the local market, in line with consumers' taste preferences and eating habits, and have the dual representativeness of cultural inheritance and market demand. They also fully reflect the differences in microbial community characteristics and production processes of fermented bean curd products in various regions.

All samples were packaged in glass bottles, with each specification being 250 g×2 bottles, totaling 8 samples. During the procurement process, products with similar production dates were strictly screened to ensure the freshness and comparability of the samples. In addition, the selected samples covered two main types: dried fermented beancurd and oil-preserved fermented beancurd, with processing methods including traditional natural fermentation and artificial inoculation fermentation. The detailed information of the samples is shown in Table 1, and the geographical distribution of the samples is shown in Fig. 1.

Reagents and equipment

Fast Deoxyribonucleic Acid (DNA) Spin Kit for Soil: MP Biomedicals, USA. Agarose (biochemical reagent): Solarbio Biotechnology Co., Ltd. QIAGEN DNeasy mericon Food Kit DNA genome extraction kit: QIAGEN, Germany. Polymerase Chain Reaction (PCR) cleaning kit: Axygen Biotechnology (Hangzhou) Co., Ltd. Centrifuge 5417R refrigerated centrifuge: Eppendorf, Germany. Nanodrop 2000 ultra-micro spectrophotometer: Thermo Scientific, USA. Agilent 2100 bioanalyzer: Agilent, USA. Illumina MiSeq sequencer: Illumina, USA.

Methods

DNA extraction

In this study, the MP Biomedicals FastDNA™ Spin Kit for Soil was used to extract the total DNA of microorganisms in fermented bean curd. Targeted pretreatment was performed based on the matrix differences between oil-preserved fermented bean curd (high-fat) and dried fermented bean curd (high-salt): for oil-preserved fermented bean curd samples, a defatting buffer containing 0.1% Triton X-100 was used for centrifugation to remove the lipid layer, and the process was repeated until the interface became clear; for dried fermented bean curd samples, after desalting with 0.5 M EDTA (pH8.0), 10 mg/mL lysozyme (Sigma L6876) was added and treated at 37℃ for 30 min. Subsequently, DNA extraction was carried out using a standardized procedure, and the integrity and concentration of DNA were detected by 1% agarose gel electrophoresis and an ultra-micro spectrophotometer.

Serial number	Sample name	Place of origin	Sodium (mg/100 g)	Amino nitrogen content, (g/kg)	Water-soluble proteins (g/100 g)	Type	Processing method
1	GZR1	Zunyi City, Guizhou	3340	15.07	17.22	Dry fermented bean curd	Traditional natural fermentation
2	CQR2	Xiushan County, Chongqing	3487	13.67	14.24	Dry fermented bean curd	Traditional natural fermentation
3	SCR3	Chengdu City, Sichuan	2950	17.44	19.72	Dry fermented bean curd	Traditional natural fermentation
4	HNR4	Xinhua County, Hunan	3200	14.27	15.33	Dry fermented bean curd	Traditional natural fermentation
5	JXR5	Ji'an City, Jiangxi	3800	11.87	13.12	Oil fermented bean curd	Artificial inoculation fermentation
6	YNR6	Mouding County, Yunnan	2663	8.94	10.01	Oil fermented bean curd	Artificial inoculation fermentation
7	GXR7	Guilin City, Guangxi	3000	9.59	10.42	Oil fermented bean curd	Artificial inoculation fermentation
8	KMR8	Shilin County, Kunming City	2644	9.98	11.43	Oil fermented bean curd	Artificial inoculation fermentation

Table 1. Information about the samples tested in this study. Note: For the convenience of understanding, the sample numbers are represented by the initials of the pinyin of the provinces and cities where the samples are located. For example, GZ represents Guizhou, CQ represents Chongqing, SC represents Sichuan, etc., and other samples are arranged in this way.

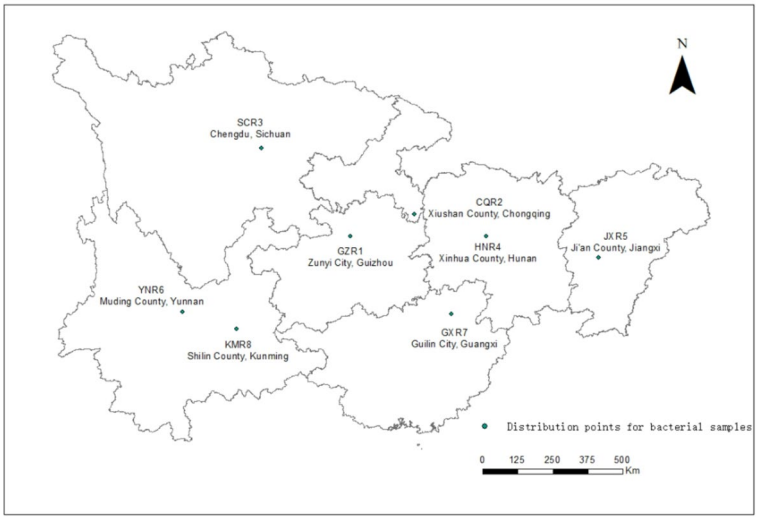

Fig. 1. Geographical distribution map of samples.

Amplicon sequencing

Using the extracted total genomic DNA as a template, the bacterial 16 S rDNA V3-V4 region was amplified by PCR using the universal primers 338 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR amplification conditions were as follows: pre-denaturation at 95 °C for 3 min, followed by 27 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s), then a final extension at 72 °C for 10 min, and finally stored at 10 °C.

The PCR amplification system is: 4 μL of buffer, 2 μL of 2.5 mmol/L deoxyribonucleoside triphosphates (dNTPs), 0.8 μL of the upstream primer (5 μmol/L), 0.8 μL of the downstream primer (5 μmol/L), 0.4 μL of Trans Start Fast Pfu DNA polymerase, 10 ng of template DNA, and ultrapure water to make up to 20 μL. The PCR products were purified, and the quality and quantity of the final products were evaluated by the Agilent 2100 bioanalyzer and Quantu Fluorometer. The NEXTflex Rapid DNA-Seq Kit was used for library construction. Shanghai Majorbio Bio-pharm Technology Co., Ltd. performed paired-end sequencing using the Illumina Mi Seq sequencing platform.

Analysis of high-throughput sequencing data

After the raw data was downloaded from the sequencing platform, the results were stored in Fastq format. The original sequencing data has been completely uploaded to NCBI's Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/Traces/sra) to achieve standardized storage and public access to the data.

The raw sequencing reads were quality-controlled using Fastp software[7], and the filtered sequences were assembled by FLASH[8]. After distinguishing the samples, sequence analysis was performed using the q2-dada2 plugin within the QIIME 2 framework. The DADA2 algorithm was employed to generate ASVs (Amplicon Sequence Variants) as a replacement for traditional OTU clustering[9,10]. Species annotation was performed by combining the Silva 138.1 database with the RDP classifier[11]. Diversity index analysis was conducted for the generated ASVs[12], and community structure statistical analysis was performed at different taxonomic levels based on taxonomic features. To ensure data reliability and reproducibility, three independent replicates were performed for key steps including DNA extraction, PCR amplification, and high-throughput sequencing of fermented bean curd samples from each region. During data analysis, ANOVA and PERMANOVA were used to evaluate differences between samples, and Tukey's HSD test was applied for multiple comparisons. Additionally, all hypothesis tests were corrected for multiple testing using the Benjamini-Hochberg method, with the adjusted FDR threshold set at 0.05.

Based on high-quality ASV data, PICRUSt2 and FAPROTAX software were used to predict bacterial functional potential[13], and COG functional annotation was executed via eggNOG-mapper[14]. This approach deeply explores the potential functional characteristics of bacteria during fermented bean curd fermentation, providing rich functional-level data support for subsequent research.

Results and analysis

Library construction and sequencing data analysis of Sufu samples

High-throughput sequencing technology was employed to analyze the bacterial diversity of eight sufu samples from different regions, yielding a total of 563,384 bacterial community sequences. The coverage of bacterial 16 S rRNA sequences for all samples exceeded 99.90%, indicating high representativeness of the microbial communities in the sequencing results. To assess the adequacy of the sequencing data volume, rarefaction curves were constructed by randomly sampling the sequences to plot the relationship between the number of OTUs (Operational Taxonomic Units) and the number of sequences.

The results (Fig. 2) showed that when the number of sequences approached 66,980, the number of OTUs tended to plateau, and the marginal contribution of increasing sequencing depth to the discovery of new OTUs significantly diminished. This suggests that the current sequencing data volume is sufficient to reflect the bacterial abundance information in the samples and adequately covers the species diversity present.

Analysis of bacterial α-diversity

α-diversity is used to characterize the abundance and diversity of microbial communities within samples. OTU, Chao index, and ACE index are used to characterize bacterial species richness, with values positively correlated with species richness. Simpson index and Shannon index are used to evaluate bacterial diversity, where the Simpson index is negatively correlated with diversity, while the Shannon index is positively correlated with diversity. The Coverage index reflects the library coverage, and a higher value indicates that the sequencing results can more truthfully represent the microbial composition of the sample. As shown in Table 2, the Coverage index of all samples exceeded 0.999, indicating that the sequencing depth was sufficient to well cover the vast majority of bacterial species in the samples, and the sequencing results were highly reliable. The JXR5 (Ji'an, Jiangxi, oil-preserved fermented beancurd, artificial inoculation fermentation) sample had the highest OTU, Chao index, and ACE index, with the richest bacterial species. The GXR7 (Guilin, Guangxi, oil-preserved

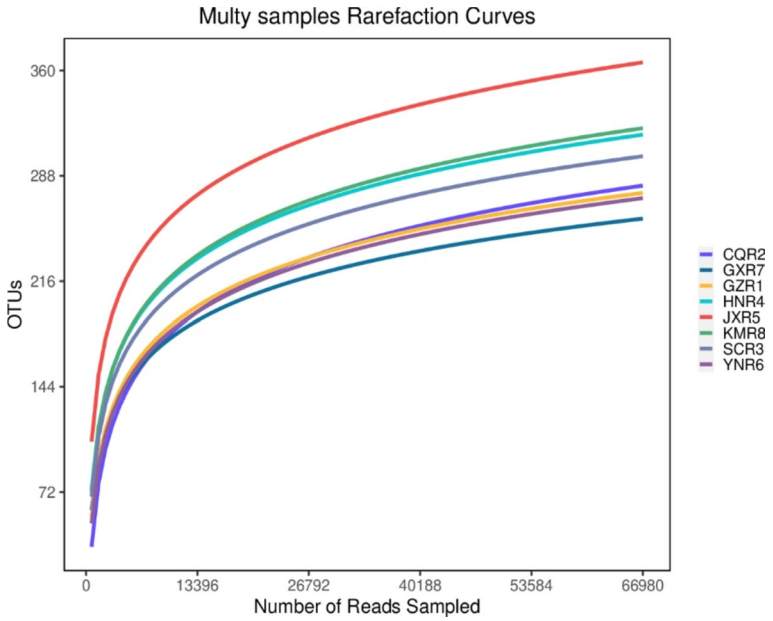

Fig. 2. Rarefaction curve of the samples.

	OTU Count	Chao1 Index	ACE Index	Shannon Index	Simpson Index	goods_coverage
GZR1	277	318.5769	313.6468	2.6785	0.8380	0.9993
CQR2	282	321.5161	317.3904	2.1896	0.9533	0.9993
SCR3	300	341.5769	333.4572	3.2074	0.6181	0.9993
HNR4	313	336.4286	339.4054	2.9249	0.8274	0.9994
JXR5	358	386.9565	378.2859	3.3512	0.6138	0.9994
YNR6	273	318.0000	316.3535	2.6693	0.8407	0.9993
GXR7	262	308.3000	311.5038	2.9251	0.8175	0.9992
KMR8	321	385.1667	365.5110	3.5085	0.5370	0.9992

Table 2. Alpha diversity index of bacteria in Sufu samples.

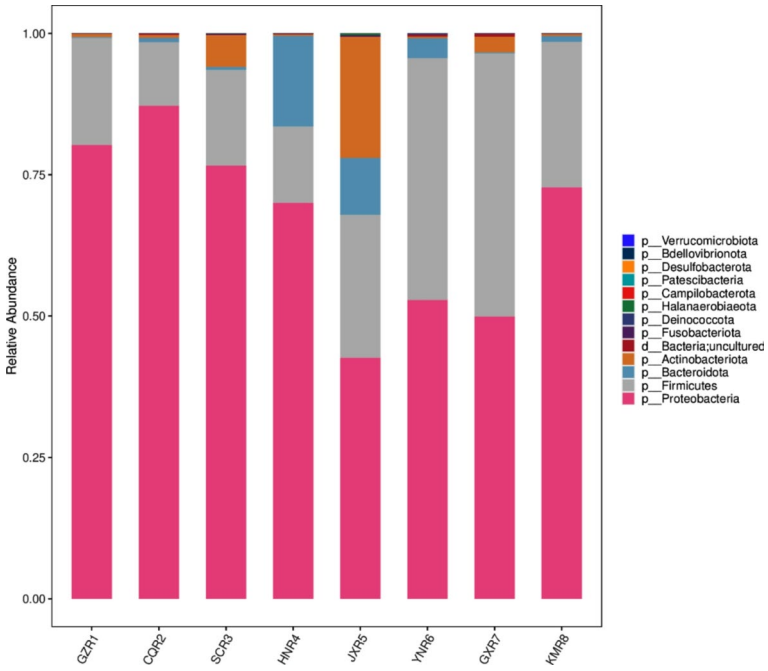

Fig. 3. Relative abundance of bacterial communities in fermented bean curd at the phylum level.

fermented beancurd, artificial inoculation fermentation) sample had the lowest bacterial species richness. The species richness of YNR6 and KMR8 samples, which were also oil-preserved fermented beancurd, was also at a low level, indicating significant differences in bacterial species richness among oil-preserved fermented beancurd. The KMR8 (Shilin, Kunming, oil-preserved fermented beancurd, artificial inoculation fermentation) sample had the lowest Simpson index (0.537) and the highest species diversity; the CQR2 (Xiushan, Chongqing, dried fermented beancurd, traditional natural fermentation) sample had the highest Simpson index (0.953) and the lowest species diversity. Comparing the two types of samples, dried fermented beancurd and oil-preserved fermented beancurd, the SCR3 (Chengdu, Sichuan, traditional natural fermentation) in dried fermented beancurd had a higher Shannon index, close to that of some oil-preserved fermented beancurd samples. In addition to KMR8, the JXR5 (Ji'an, Jiangxi, artificial inoculation fermentation) in oil-preserved fermented beancurd also had a higher Shannon index, indicating that the impact of processing methods (traditional natural fermentation and artificial inoculation fermentation) on bacterial diversity is not absolute and may also be related to factors such as origin. Overall, the bacterial community structures of the 8 different samples were significantly different.

Analysis of bacterial community structure

Analysis of bacterial community structure at the phylum level

The analysis of bacterial community structure at the phylum level (Fig. 3) revealed that the bacterial communities in sufu samples were primarily composed of Proteobacteria (66.50%), Firmicutes (25.13%), Bacteroidota (4.04%), and Actinobacteriota (3.97%), which together accounted for 99.64% of the total community. Although the dominant phyla were similar across the eight samples, their relative abundances exhibited significant differences ($p < 0.05$), This difference is closely associated with the origin, type, and processing methods of the samples, revealing distinct geographical distribution characteristics and technological influences. Proteobacteria exhibited significant spatial heterogeneity across different samples. The CQR2 sample (Xiushan County, Chongqing,

dried fermented beancurd, traditional natural fermentation) showed the highest abundance, reaching 87.16%, while the JXR5 sample (Ji'an, Jiangxi, oil-preserved fermented beancurd, artificial inoculation fermentation) had the lowest abundance at only 42.64%. During traditional natural fermentation, the random participation of environmental microorganisms and local environmental factors such as temperature and humidity might promote the massive proliferation of Proteobacteria rich microbes in Xiushan, Chongqing, during the fermentation of CQR2 fermented beancurd. In contrast, for JXR5 with artificial inoculation fermentation, the regulation of microbial community structure by inoculated strains led to a decrease in Proteobacteria abundance[15]. Firmicutes served as the second dominant phylum, with relatively high proportions in the GXR7 (Guilin, Guangxi, oil-preserved fermented beancurd, artificial inoculation fermentation) and YNR6 (Mouding, Yunnan, oil-preserved fermented beancurd, artificial inoculation fermentation) samples, reaching 46.54% and 42.81%, respectively. These bacteria can produce various organic acids such as lactic acid and acetic acid during fermentation, which not only regulate the pH of the fermentation system but also significantly impact the flavor and texture of fermented beancurd through metabolite accumulation. Therefore, the high abundance of Firmicutes may be one of the key driving factors for forming the unique flavor of fermented beancurd[16]. Bacteroidota showed low overall abundance (4.04%) in the samples, but its proportion significantly increased to 16.05% in the HNR4 sample (Xinhua, Hunan, dried fermented beancurd, traditional natural fermentation). In comparison, their abundances were only 0.14% and 0.29% in the GZR1 (Zunyi, Guizhou, dried fermented beancurd, traditional natural fermentation) and GXR7 samples, respectively. This notable spatial distribution difference may reflect the unique fermentation environment (e.g., water activity, substrate composition) or specific nutritional components in the raw materials of the HNR4 sample, which may promote the growth of Bacteroidota[17]. Actinobacteriota had relatively high abundances in the SCR3 (Chengdu, Sichuan; dry sufu; traditional natural fermentation) and JXR5 samples, accounting for 5.58% and 21.44%, respectively. Certain species of Actinobacteriota can produce antibiotics (e.g., streptomycin) and other bioactive compounds (e.g., vitamin B12). These secondary metabolites may not only regulate microbial community structure during fermentation but also contribute significantly to the flavor and functional properties of sufu[18].

Additionally, the average abundance of uncultured bacteria (Bacteria; uncultured) was 0.15%, with extremely low proportions in all samples (all below 0.5%). These unculturable microorganisms may depend on specific growth conditions, such as extreme pH or low-nutrient environments, making them difficult to isolate and cultivate under conventional laboratory conditions. Their potential biological functions during sufu fermentation and their impact on product characteristics require further investigation[19].

Analysis of bacterial community structure at the genus level

The analysis of the bacterial community structure at the genus level showed (Fig. 4) that the bacterial communities in different fermented bean curd samples were mainly composed of 12 dominant genera, including *Pseudomonas* (22.31%), *uncultured Enterobacteriaceae* (18.11%), *Lactococcus* (6.89%), *Acinetobacter* (6.86%), *Enterococcus* (5.14%), *Acetobacter* (3.74%), *Corynebacterium* (3.72%), *Lactobacillus* (3.72%), *Comamonas* (3.66%), *Tetragenococcus* (3.17%), *Stenotrophomonas* (2.95%), and *Sphingobacterium* (2.95%). The relative abundances of these genera differed significantly among different samples ($p < 0.05$), revealing the spatial heterogeneity of the microbial community in fermented bean curd. As the most dominant genus, *Pseudomonas*

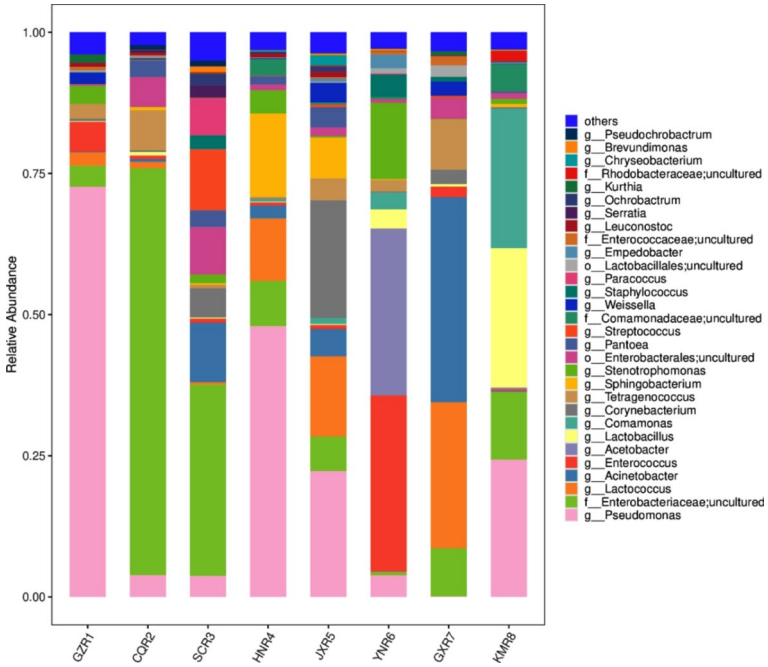

Fig. 4. Relative abundance of bacterial communities in fermented bean curd at the genus level.

had relatively high contents in the GZR1 (Zunyi, Guizhou) and HNR4 (Xinhua, Hunan) samples, accounting for 72.63% and 47.92% respectively. Some species of the genus *Pseudomonas* are opportunistic pathogens, and their high abundance in the samples may have an adverse impact on the safety and fermentation process of fermented bean curd. This phenomenon may be related to environmental factors such as the nutrient composition and pH of the samples[20].

The *uncultured Enterobacteriaceae* dominated in the CQR2 (Xiushan, Chongqing) sample (72.09%). Although some strains of the Enterobacteriaceae family have probiotic functions and are involved in the formation of flavor substances, their potential pathogenicity still needs to be concerned[21]. In the GXR7 (Guilin, Guangxi) sample, the relative abundances of *Lactococcus* and *Acinetobacter* were relatively high, accounting for 25.86% and 36.32% respectively. *Lactococcus* can produce acid, inhibit the growth of harmful bacteria, and participate in the formation of the flavor of fermented bean curd, while the high abundance of *Acinetobacter* may pose a potential threat to the safety of fermented bean curd[23]. In the YNR6 (Mouding, Yunnan) sample, the abundance of *Enterococcus* was the highest (31.15%). Some strains of the genus *Enterococcus* have probiotic characteristics, but the presence of virulence factors gives them a dual impact on the fermentation and safety of fermented bean curd[24]. The SCR3 (Chengdu, Sichuan) sample showed a relatively high diversity of the bacterial community, in which the abundances of *Acinetobacter* (10.56%) and *Streptococcus* (10.76%) were relatively high. Its fermentation environment provides suitable growth conditions for a variety of genera, and the complex interactions among different genera may jointly regulate the fermentation process of fermented bean curd[25]. In the JXR5 (Ji'an, Jiangxi) sample, the abundance of *Corynebacterium* was relatively high (20.82%). *Corynebacterium* is involved in the decomposition of proteins and fats, and may play a key role in the formation of the texture and flavor of fermented bean curd[26]. In the KMR8 (Shilin, Kunming) sample, the abundances of *Lactobacillus* (24.67%) and *Comamonas* (24.73%) were relatively high. *Lactobacillus* is a beneficial bacterium that participates in flavor formation and inhibits harmful bacteria; *Comamonas* has diverse metabolic pathways. Their high contents indicate that the environment of the KMR8 sample is conducive to the growth of beneficial bacteria, which may have a positive effect on the quality and safety of fermented bean curd[27].

PCoA analysis

Based on principal co-ordinates analysis (PCoA), the similarity and difference of bacterial community structures in 8 different fermented beancurd samples were analyzed. As shown in Fig. 5, the variation explanation rates of the first principal component and the second principal component were 23.99% and 20.56%, respectively. The results showed that SCR3 (Chengdu, Sichuan, dried fermented beancurd, traditional natural fermentation) and CQR2 (Xiushan, Chongqing, dried fermented beancurd, traditional natural fermentation) were completely identical in type and processing method. Moreover, Chengdu, Sichuan and Xiushan, Chongqing are geographically adjacent with similar climatic and environmental conditions, leading to similar types of environmental microorganisms involved in the traditional natural fermentation process, which makes the similarity of their bacterial community structures the highest. The bacterial community structures of four samples, GZR1 (Zunyi, Guizhou), HNR4 (Xinhua, Hunan), JXR5 (Ji'an, Jiangxi), and KMR8 (Shilin, Kunming), showed high similarity. In particular, the community structures of GZR1 and HNR4 were the closest, as they had the same type and processing method of fermented beancurd and are geographically adjacent. The bacterial community structures of GXR7 (Guilin, Guangxi) and YNR6 (Mouding, Yunnan) also showed certain similarity, but compared with SCR3 and CQR2,

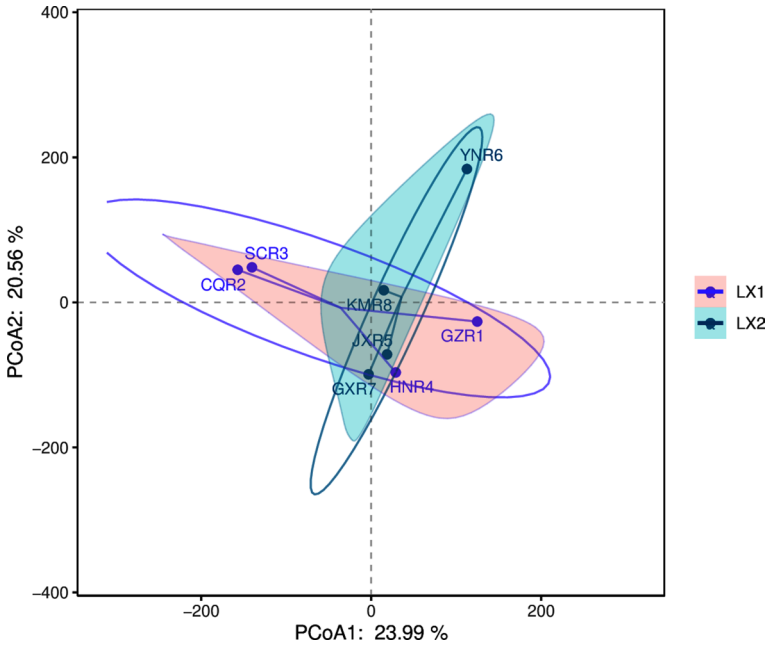

Fig. 5. PCoA clustering plot of bacterial communities in fermented bean curd.

their similarity was slightly weaker, possibly affected by different factors. From the above results, it can be seen that the bacterial community structure in fermented beancurd is affected by multiple factors such as origin, type, processing method, and raw material composition, showing obvious regional characteristics. Samples with close geographical location, similar raw material composition, and the same production process have high similarity in bacterial community structure, while different combinations of production conditions lead to significant differences in community structure[28].

Functional analysis of bacterial communities in Sufu

PICRUSt functional analysis

Functional prediction analysis of the sufu samples using PICRUSt2 software (Fig. 6), combined with Bray-Curtis distance clustering, revealed the functional characteristics of bacterial communities in various metabolic pathways and biosynthetic processes. The analysis showed that functional categories included amino acid metabolism, carbohydrate metabolism, energy metabolism, antibiotic biosynthesis, and coenzyme synthesis, among others.

The biosynthesis of ansamycins had the highest proportion, ranging from 1.43 to 3.92%, with the CQR2 (Xiushan, Chongqing) sample showing the highest value. This high proportion may reflect the ecological competition within the bacterial community of the CQR2 sample, where antibiotic synthesis is employed to inhibit the growth of other microorganisms[29]. Fatty acid biosynthesis exhibited relatively consistent proportions across samples, ranging from 1.65 to 2.10%. This suggests that the demand and synthesis capacity for fatty acids among bacteria in different sufu samples is similar, likely due to their fundamental roles in maintaining cell membrane structure and energy storage[30]. The biosynthesis of vancomycin group antibiotics ranged from 1.17 to 1.78%, with the YNR6 (Mouding, Yunnan) sample having the highest proportion. The antibacterial activity of vancomycin-group antibiotics may reflect the defensive mechanisms of the bacterial community in the YNR6 sample, maintaining ecological stability by inhibiting the invasion of foreign microorganisms. The biosynthesis and degradation of valine, leucine, and isoleucine accounted for 1.58–2.06% and 0.74–1.59%, respectively. These branched-chain amino acids play crucial roles in bacterial growth and energy metabolism, and the relative consistency in their metabolic levels suggests that bacterial communities in different samples have similar metabolic capacities in these functions[31]. Alanine, aspartate, and glutamate metabolism ranged from 1.24 to 1.39% across samples. These amino acids are key intermediates in microbial nitrogen and energy metabolism, and the similarity in their metabolic levels further confirms the conservation of these core pathways in bacterial communities across different samples[32]. Glycolysis/Gluconeogenesis ranged from 0.97 to 1.58%, with the GXR7 (Guilin, Guangxi) sample having the highest proportion. Glycolysis and gluconeogenesis are central pathways in bacterial energy metabolism, and the higher proportion in the GXR7 sample may reflect the advantages of its bacterial community in energy acquisition. Mismatch repair ranged from 1.03 to 1.53%, with the GXR7 (Guilin, Guangxi) sample again having the highest proportion. Mismatch repair is related to bacterial DNA repair capabilities, and its higher proportion may indicate the genomic stability advantages of the bacterial community in the GXR7 sample[28].

From the clustering results, the PICRUSt functions of the samples could be grouped into three clusters: JXR5 (Ji'an, Jiangxi), SCR3 (Chengdu, Sichuan), and CQR2 (Xiushan, Chongqing) formed one cluster; KMR8 (Shilin, Kunming), HNR4 (Xinhua, Hunan), and GZR1 (Zunyi, Guizhou) formed another cluster; and GXR7 (Guilin, Guangxi) and YNR6 (Mouding, Yunnan) formed the third cluster. The clustering results further validate

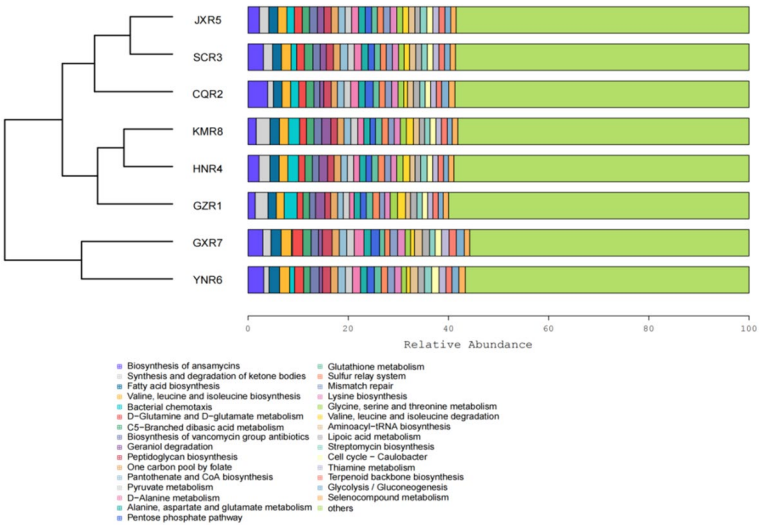

Fig. 6. Bray-Curtis cluster analysis and stacked bar plot of PICRUSt functional composition. (Note: On the left is the hierarchical clustering analysis of samples, and on the right is the PICRUSt functional bar plot of the samples. The x-axis represents the relative abundance of each function in the sample, while the y-axis represents the sample names. The figure displays the top 30 functions based on their relative abundance.)

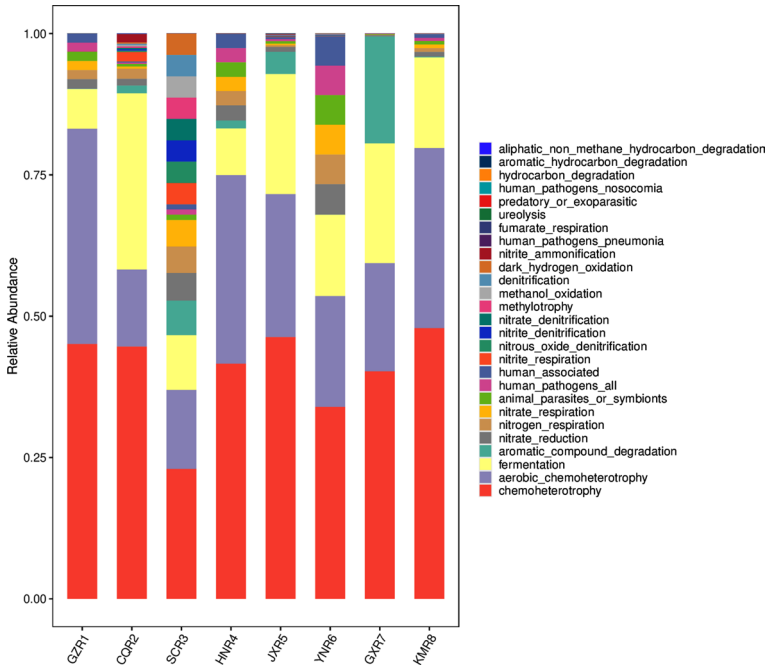

Fig. 7. Relative abundance of FAPROTAX functional profiles.

the similarities and differences in functional characteristics among bacterial communities in different samples, reflecting the potential influence of geographical region, production processes, and raw material selection on bacterial functional traits.

The functional prediction of PICRUSt2 relies on existing gene databases. This characteristic may lead to deviations between the prediction results and the actual metabolic activities. Therefore, the functional prediction results obtained based on PICRUSt2 in this study can only serve as a reference for understanding the functional characteristics of the bacteria community in fermented bean curd. In the follow-up, experimental methods such as metagenomic sequencing and metabolomic analysis need to be combined to verify the key functions, so as to more accurately reveal the true metabolic activities and functional characteristics of the bacteria community in fermented bean curd.

FAPROTAX functional analysis

Through the FAPROTAX functional analysis (Fig. 7), the functional profiles of the eight fermented bean curd samples covered various microbial metabolic pathways and specific functions such as chemoheterotrophy, aerobic_chemoheterotrophy, fermentation, aromatic_compound_degradation, nitrate_reduction, nitrogen_respiration, and nitrate_respiration. Chemoheterotrophy had the highest proportion in each sample (22.97%-47.87%), being the most dominant function, and it had the highest proportion in the KMR8 (Shilin, Kunming) sample. This indicates that the microorganisms in fermented bean curd mainly obtain energy and carbon sources by decomposing organic compounds such as proteins and sugars, which is in line with the characteristics of microbial growth and metabolism during the fermentation process of fermented bean curd, and provides a basis for the substance transformation during the fermentation of fermented bean curd, thereby affecting its flavor and quality[33]. There were significant differences in the proportions of the aerobic_chemoheterotrophy function in each sample. The proportion in the GZR1 (Zunyi, Guizhou) sample was as high as 38.08%, while that in the CQR2 sample was only 13.63%. This difference reflects the dynamic changes in the oxygen content during the fermentation process of fermented bean curd, which may lead to differences in the dominant microbial populations in different samples, thus affecting the quality and flavor of fermented bean curd[34]. The fermentation function had a relatively high proportion in each sample (7.01%-31.16%), and it was the highest in the CQR2 sample. Fermentation is the core process of making fermented bean curd, which can change its texture and endow it with a unique flavor. The high fermentation function of the CQR2 sample may lead to the generation of more enzymes and metabolites, thus forming a finer texture and a richer flavor[35]. There were significant differences in the aromatic_compound_degradation function among different samples. The proportion in the GXR7 sample was the highest (18.92%), while that in the YNR6 sample was the lowest (0.029%). This function is related to the removal of some off-flavor substances or the generation of special flavor substances in fermented bean curd. The high degradation ability of the GXR7 sample may help to improve the flavor or reduce the unpleasant odor, which is of great significance for optimizing the production process and improving the product quality[36].

There were significant differences in the proportions of functions such as nitrate_reduction, nitrogen_respiration, and nitrate_respiration in each sample. Among them, the nitrate_reduction function had the highest proportion in the YNR6 sample (5.38%) and the lowest proportion in the GXR7 sample (0.24%). The

differences in these functions reflect the diversity of microbial nitrogen source metabolism, which may affect the quality and safety of fermented bean curd. The nitrate reduction function may be related to the formation and transformation of nitrite, and under certain conditions, nitrite may react with amines in fermented bean curd to form nitrosamines, thus affecting the product safety[38].

Discussion

Through multi-dimensional analysis and functional prediction, this study systematically revealed the composition, structure, and functional characteristics of bacterial communities in sufu samples from 8 different geographical origins, providing a scientific basis for further understanding the fermentation mechanism of sufu and its quality regulation. Alpha diversity analysis showed that in terms of species richness, JXR5 (Ji'an, Jiangxi Province, oil sufu, artificially inoculated fermentation) had the richest bacterial species, which is related to the introduction of a relatively high number of bacterial strains through artificial inoculation fermentation, and the high sodium and high amino nitrogen contents provide a rich nutritional substrate for the bacterial community. In terms of species diversity, KMR8 (Shilin, Kunming, oil sufu, artificially inoculated fermentation) had the highest species diversity, which is closely related to the high abundance of *Lactobacillus* and *Comamonas* at the genus level, and the synergistic effect of these two genera enhances the stability of the community. CQR2 (Xiushan, Chongqing, dry sufu, traditional natural fermentation) had the lowest diversity, with the dominance of a single genus resulting in a simple community structure, which is consistent with the conclusion of Li et al. in their study on the reconstruction mechanism of microbial communities by inoculated fermentation, which stated that inoculated fermentation can introduce 11–15 exogenous strains[38].

The bacterial community in fermented bean curd is dominated by Proteobacteria and Firmicutes, with the main genera including *Pseudomonas*, *Lactococcus*, and *Acinetobacter*. Among them, the abundance of Proteobacteria varies significantly among different samples. For example, its abundance in the CQR2 (Xiushan, Chongqing) sample is as high as 87.16%, while in the JXR5 (Ji'an, Jiangxi) sample, it is only 42.64%. This is consistent with the study on fermented soybean products in southern China by Zhang, Q., Chen, X. Y., Wang, Q., et al., which pointed out that both Proteobacteria and Firmicutes occupy dominant positions in naturally fermented and artificially inoculated fermented bean curd, but the ratio of the two is significantly affected by the fermentation environment and raw material characteristics[36].

Firmicutes account for a relatively high proportion in the oil-fermented bean curd samples of GXR7 (Guilin, Guangxi) and YNR6 (Mouding, Yunnan), reaching 46.54% and 42.81% respectively. These two samples not only belong to the same type but also have relatively low water-soluble protein content. Referring to the research on fermented products by Zheng X, et al., in a low-protein environment, the Lactobacillus genus in Firmicutes can enhance the lactic acid fermentation pathway to utilize residual nutrients. In the fermentation of bean curd, this metabolic activity not only regulates the pH value of the fermentation system but also produces flavor substances such as lactic acid and acetic acid, laying the foundation for the formation of the unique flavor of fermented bean curd[39].

There are significant differences in the relative abundance of dominant bacterial genera at the genus level. *Pseudomonas* has the highest proportion in GZR1 and HNR4. Some strains of this genus are conditional pathogens, and their high abundance may be related to the nutritional components and pH of the samples, so attention should be paid to their impact on fermentation safety[40]. *The uncultured Enterobacter* genus accounts for 72.09% in CQR2. Although some strains are involved in flavor formation, their high abundance may bring pathogenic risks, which is also the main reason for the low diversity of this sample. In GXR7, *Lactococcus* and *Acinetobacter* have prominent proportions. *Lactococcus* inhibits harmful bacteria by producing acid and contributes to flavor, while the high abundance of *Acinetobacter* requires vigilance against safety risks[41]. SCR3 shows high diversity due to the coexistence of various genera such as *Acinetobacter* (10.56%) and *Streptococcus* (10.76%), and the synergistic effect between the bacterial communities may endow it with richer flavor layers.

In the PCoA analysis, the dried fermented bean curd samples SCR3 (Chengdu, Sichuan) and CQR2 (Xiushan, Chongqing), which are geographically adjacent, have the highest similarity in bacterial community structure. This may be due to their similar climatic conditions and traditional natural fermentation processes, which enable the massive enrichment of Proteobacteria microorganisms in the environment. The abundance of Proteobacteria in the CQR2 sample is as high as 87.16%. In contrast, the high-sodium sample JXR5 of oil-fermented bean curd, which is fermented by artificial inoculation, has a Proteobacteria abundance of only 42.64%. This is consistent with the view put forward by Chen et al. that "the high-salt environment inhibits the growth of aerobic flora of Proteobacteria". At the same time, the specific strains introduced by artificial inoculation may also inhibit the growth of Proteobacteria, reflecting the regulatory effect of processing methods on the microbial community structure[37]. In addition, GZR1 and HNR4 are both traditionally fermented dried fermented bean curd and are geographically close, so their community structures are also similar; although JXR5 and KMR8 are of the same type, they are far apart in origin but still cluster into one group due to similar processing methods. The similarity between GXR7 and YNR6 is weak, which may be affected by the differences in the microbial environment of the producing areas. These results confirm from the perspective of β diversity that the bacterial community structure of fermented bean curd is the result of the combined action of multiple factors such as the climate, type, technology, and raw materials of the producing area, reflecting the adaptive response of the microbial community to the environment[41].

Functional analysis showed that chemoheterotrophy and fermentation were the core functions of the sufu microbiota. Chemoheterotrophy had the highest proportion (47.87%) in the KMR8 (Shilin, Kunming) sample. In terms of sufu types, the average proportion of chemoheterotrophic functions in oily sufu samples reached 41.2%, which was significantly higher than that in dry sufu (35.6%). The high-fat environment of oily sufu may prompt microorganisms to rely more on protein decomposition (such as the lipoproteinase activity of Acinetobacter), which is consistent with the data of high water-soluble protein (11.43 g/100 g) in the KMR8

sample[42]. It also indicates that sufu microorganisms mainly obtain energy and carbon sources by decomposing organic compounds such as proteins and carbohydrates, a process that provides the material basis for the formation of sufu flavor and texture. Li, H., et al. also emphasized the importance of chemoheterotrophic functions in the formation of sufu flavor[34]. The fermentation function had the highest proportion (31.16%) in the CQR2 (Xiushan, Chongqing) sample, possibly related to its high abundance of lactic acid bacteria, which may significantly affect sufu flavor by producing acids and inhibiting the growth of harmful bacteria, further indicating the key role of fermentation functions in regulating sufu's sour taste and texture. This is consistent with the conclusion of Wang, Y., Li, C., et al. that the fermentation function is the core process of sufu production[35]. In addition, the nitrate reduction function varied significantly, with the highest proportion (5.38%) in the YNR6 (Mouding, Yunnan) sample. These differences may be related to the diversity of regional environments, production processes, and raw material selection. This function may be associated with the formation and accumulation of nitrite, increasing the risk of nitrosamine formation and posing a potential threat to the safety of sufu[43]. Moreover, the YNR6 sample had the lowest sodium content (2663 mg/100 g), and low-salt conditions may accelerate the proliferation of nitrate-reducing bacteria (such as Enterobacteriaceae). It is recommended to optimize salt control in local processes or add nitrite antagonists (such as vitamin C) to reduce the risk of nitrosamines[44]. Additionally, for sufu with high nitrate reduction function proportions, the nitrite content can be key monitored during online sales, and businesses should be required to clearly mark relevant risk warnings to ensure online food safety.

From the perspective of different types of fermented bean curd, dried fermented bean curd (GZR1, CQR2, SCR3, HNR4) are all traditionally naturally fermented, with significant differences in species richness and diversity: SCR3 has the highest diversity, with a synergistic effect between *Pseudomonas* and *Streptococcus*; CQR2 has the lowest diversity, dominated by Enterobacter. At the phylum level, Proteobacteria is the main group, while at the genus level, *Pseudomonas* and *uncultured Enterobacter* are the dominant groups. Functionally, chemoheterotrophy and fermentation are the core. The high fermentation function of CQR2 is related to its unique flavor, and the high abundance of *Bacteroides* in HNR4 may endow it with a special texture.

Oil-fermented bean curd (JXR5, YNR6, GXR7, KMR8) are all artificially inoculated, with JXR5 having the highest richness and GXR7 the lowest. At the phylum level, Firmicutes account for a prominent proportion in GXR7 and YNR6; at the genus level, *Lactococcus*, *Acinetobacter*, and *Lactobacillus* are the dominant groups. Functionally, they have stronger chemoheterotrophic capabilities. The high proportion of chemoheterotrophic capacity in KMR8 is related to the metabolic activity of its *Lactobacillus* and *Comamonas*.

From the perspective of health impacts, differences in microbial community structures and functions have varying effects on consumer health. High-sodium samples such as JXR5, although carrying the risk of excessive sodium intake, contain a relatively high abundance of Actinobacteria that can produce beneficial substances like vitamin B12, which may alleviate the potential cardiovascular hazards of high sodium to some extent; while the low-sodium sample YNR6, despite its advantages in sodium intake, cannot ignore the nitrosamine risks brought by its high nitrate reduction function. This indicates that the health attributes of sufu are the result of multiple factors. In the future, artificially inoculating specific functional microbial strains can be explored to enhance the safety and health value of sufu while maintaining its flavor.

Conclusion

This study systematically analyzed the composition, diversity, and functional characteristics of the bacterial communities in sufu samples from eight regions in China. It identified the main bacterial phyla and dominant genera in sufu and confirmed that there were significant differences in the bacterial community structure and species diversity among samples from different regions. The study further revealed the core functions of the sufu bacterial community and the expression differences of these functions in different samples. These functions are closely related to the flavor formation, texture control, and safety assurance of sufu. By summarizing the regional characteristics and functional diversity of the bacterial communities in sufu, this study provides an important scientific basis for optimizing the production process and developing regional characteristic products.

However, this study also has certain limitations: The sample scope and research depth still need to be further expanded. For example, multiple experimental analyses of samples from different batches of the same manufacturer should be strengthened to improve the reliability of the data. In addition, the fermentation of sufu is the result of the synergistic action of bacteria and fungi. Future research needs to combine the analysis of the fungal community to deeply explore the interaction mechanism between bacteria and fungi. At the same time, multivariate analysis methods should be adopted to systematically evaluate the comprehensive effects of geographical factors, production processes, and physical and chemical properties on the microbial community, so as to comprehensively analyze the driving mechanism of the microbial community during the fermentation of sufu. These follow-up studies will provide a more solid theoretical support for optimizing the production process, improving product quality, and promoting the sustainable development of the sufu industry.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.Should any reader or researcher wish to request the data from this study, they may contact the corresponding author, Dr. Xue Feng, directly at the following email address: 1739877528@qq.com.

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Author contributions

Xue Feng wrote the main manuscript text, Zhao Dong andTeng Hao was responsible for the polishing of the article. All authors reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to X.F.

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