

Metagenomics and volatile metabolomics reveal microbial succession and its correlations with fruity flavor volatile compounds during Mianhua industrial processing

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ARTICLE INFO

Keywords:

Mianhua
Core microbes
Volatile compounds
Metabolome-microbiome correlation
Characteristic flavor
Fruity ester

ABSTRACT

Mianhua, a traditional fermentation-type staple food popular in northern China, undergoes dynamic microbial and volatile compound changes during industrial processing. 848 volatile compounds were identified using volatile metabolomics dominated by esters (18.51 %), notably hexanoic acid ethyl ester and octanoic acid ethyl ester, which confer fruity flavors. Metagenomics analysis revealed *Proteus* (25.93 %), *Fructilactobacillus* (16.63 %), *Lactobacillus* (10.16 %) and *Companilactobacillus* (7.14 %) as dominant genera. Mixing with traditional starters was critical for flavor development, driven by microbial succession and synergistic interactions between *Lactobacillaceae* (e.g., *Fructilactobacillus sanfranciscensis* and *Lactobacillus helveticus*) and *Kazachstania* during fermentation. Notably, *F. sanfranciscensis* and *L. helveticus* were significantly correlated with the formation of key esters with fruity characteristics, elucidating their roles in substrate conversion via carbohydrate metabolism and the esterification pathways. This study clarifies the microbial contributions to fruity flavor and provides insights into volatile-microbiota correlations, laying a foundation for future flavor-oriented research and industrial applications of microbiota regulation in Mianhua production.

1. Introduction

Mianhua is a traditional stable food made from arid alkaline wheat grown in the plain area of Bohai Bay. As an intangible cultural heritage of craftsmanship in Hebei Province, Mianhua is widely celebrated in northern China due to its attractive flavor and cultural symbolism. In recent years, with continuous progress in the intensive processing and comprehensive utilization of arid-alkaline wheat, the characteristics of this flour product have attracted more attention. Mianhua processing involves a fermentation process similar to that used in making Chinese steamed bread, utilizing traditional starters known as lao-mian. Chinese steamed bread represents a type of solid-state fermented grain products with many potential health benefits (Estruch & Lamuela-Raventos, 2023). As the essence of Mianhua, traditional starters were derived from prior fermentation processes and achieved through daily addition of appropriate ingredients to keep microorganisms metabolically active (Minervini et al., 2014).

For Mianhua production, Mianhua doughs were matured by continuous propagation using traditional starters and underwent a process of mixing and kneading, cutting decorations, fermenting and proofing, and finally steaming and cooling. Traditional starters are essential for the flavor of steamed bread, and the fermentation process plays a key role (Xu et al., 2020). While traditional craftsmanship endows Mianhua its unique character, it also results in a lack of standardization in processing and inconsistent quality. Limited research has been conducted in the industrialization application of traditional starters in Chinese steamed bread, particularly the microbial and metabolic variation in doughs during traditional starters fermentation (Wang et al., 2023). Microbial activities in traditional starters are crucial for the formation of volatiles during steamed bread production (Ma et al., 2021). As a traditional craft, Mianhua production using traditional starters depends significantly on empirically supported and lacks a quantitative foundation for consistent quality in cosmically production. Although some studies have examined the differences in volatiles of

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<https://doi.org/10.1016/j.fochx.2025.103446>

Received 9 July 2025; Received in revised form 11 December 2025; Accepted 25 December 2025

Available online 26 December 2025

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steamed bread from different regions, the microbiologic formation mechanisms of flavor substances throughout Mianhua processing remains limited.

Flavor is an essential quality of flour products in contributing to its popularity and acceptability (Teleky et al., 2020). Mianhua is also a fermented wheat product similar to steamed bread. Thus, studies on the flavor of steamed bread can be used as references for that of Mianhua. Many research focused on the identification and extraction of active flavor compounds from Chinese steamed bread, including the classification as alcohols, aldehydes, ethers, esters and ketones. Nevertheless, the contents of these compounds varied with separate geographical origin (Song et al., 2024; Zhang et al., 2022). Microorganisms are crucial for nutrient decomposition processes. Like carbohydrates, proteins and lipids from flour, contributing to whole flavor in fermented wheat foods. The breakdown produces small molecules like fatty acids, amino acids and a variety of microbial metabolites, which collectively attribute to sensory characters of final products (An et al., 2023; Bokulich et al., 2016). Cultivation properties and metabolic processes in the fermentation of plenty LAB, especially *Fructilactobacillus sanfranciscensis*, have been comprehensively examined (Fujimoto et al., 2019).

Emerging trends in studying microbial communities in traditional fermented foods present opportunities for innovative approaches. The integration of multi-omic approaches, coupled with relevance networks analysis, has been utilized to explore the correlation between metabolites and microbiota of fermented food in situ systems (Feng et al., 2024; Wang et al., 2019). Metabolomics offers more profound comprehension of flavor profiles, while metagenomics was employed to investigate the genetic composition and community function (Tan et al., 2022). In our previous research, higher content of ketones and acids were identified in Mianhua produced by wheat plants in arid alkaline than which in general land using GC-IMS (Y. Liu et al., 2024). Though these technologies were crucial for enhancing flavor of flour products (Sun, Yu, et al., 2025), microbiological mechanisms of Mianhua flavor formation are lacking. The formation of flavor substances in steamed bread depends on many factors, such as the flour, processing technology, microbial strains (Ran et al., 2024) and fermentation conditions. However, other than fermentation, the effects of other process steps on the flavor formation of steamed bread have rarely been studied (Ding et al., 2024). The microbial mechanisms underlying the fruity esters during processing of traditional starter-based flour products remain poorly understood

(Pradal et al., 2025). It is worthwhile to investigate the effects of different process steps on the volatile compounds of Mianhua to understand the formation of fruity flavor during its elaboration. Previous research had demonstrated that various elaboration steps have distinct impacts on bread aroma (Birch et al., 2013). Therefore, monitoring the dynamic alterations of volatiles in different processing steps could offer insights into Mianhua aroma formation.

In this study, our objective was to identify the key volatiles and core bacteria and fungi which contributed to the specific flavor of Mianhua processing. HS-SPME-GC-MS was employed for clarifying the volatile profiles during Mianhua processing, while metagenomics was applied to explore the microbial influence on metabolite transformation, with the goal of enhance comprehension of flavor formation throughout Mianhua processing.

2. Materials and method

2.1. Mianhua manufacturing process and sample collection

Mianhua was produced in Dijian Food Co. Ltd. using arid alkaline wheat from Huanghua, Cangzhou, Hebei Province, China (38.37° N, 117.33° E). Flow sheet for Mianhua production was illustrated in Fig. 1A. Recognizing the significant impact of traditional starters fermentation on Chinese steamed bread aroma (Wei & Regenstein, 2023; Xi et al., 2020), Mianhua samples were gathered from industrial production line. Specifically, at mixing, cutting, proof and cooling after steamed step, samples were randomly chosen and frozen into powered form and preserved at -80°C .

Preparation and fermentation of dough: The traditional starters and the flour were mixed in a kneading machine for 15 min to ensure homogenous incorporation. The resulting dough underwent mechanical pressing using a roller press machine for 4 consecutive passes to achieve uniform texture and gluten development. The doughs were divided into ~ 75 g subunits, afterwards pressed into molds and shaped for cutting decorations.

Proofing, steaming and cooling: After air-drying, the cut dough pieces were placed in a dough prover under controlled conditions (38°C , 45 % relative humidity) for 1 h to undergo proofing. Subsequently, the dough samples were transferred to a steamer containing a gauze layer positioned above boiling water for 19–20 min of steaming. Following this,

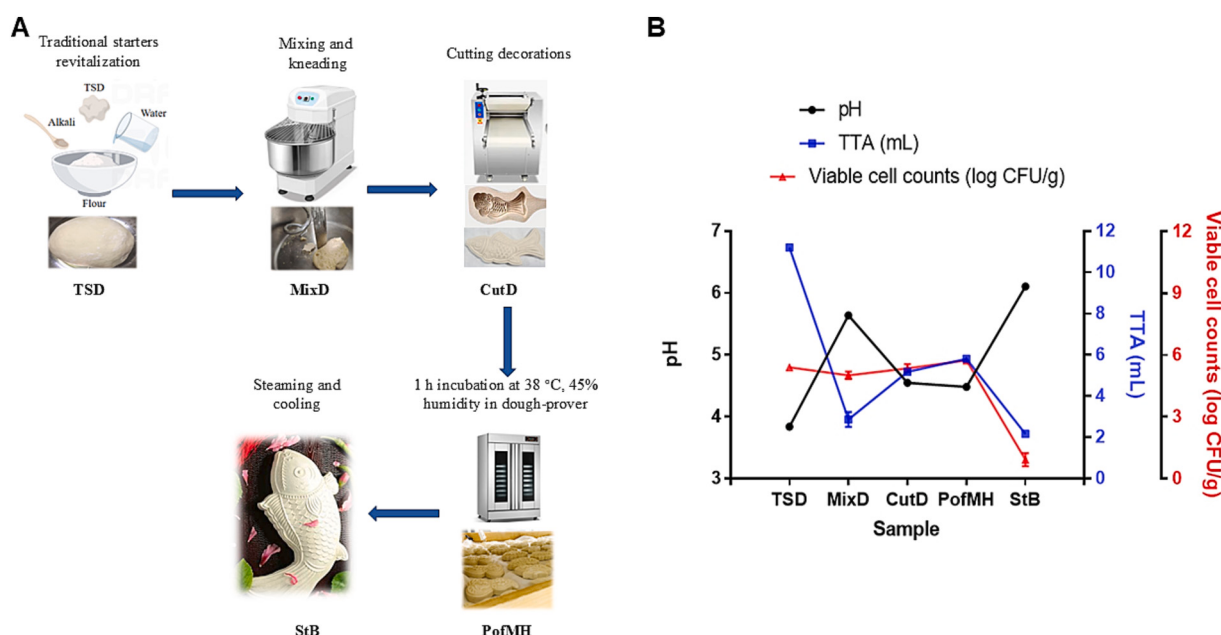


Fig. 1. Flowchart of Mianhua industrial processing and physicochemical properties (pH, TTA and viable cell counts) during processing.

the heat source was turned off, and the steamer was allowed to stand for 2 min before opening the lid. Finally, the finished Mianhua products were removed and allowed to cool to room temperature.

Sample collection: At every aforementioned step, samples were randomly selected and immediately converted to the drikold for rapid cooling. The collected samples from Mianhua processing steps were designated as the traditional sourdough (TSD), mixed and kneaded dough (MixD), cutted dough (CutD), proofed dough after incubation (PofMH) and cooled steamed bun (STB). Samples for each category (TSD, MixD, CutD, PofMH and STB) were taken immediately after completion of their respective operations to minimize additional fermentation or volatile loss. For microbial analysis, portions of each sample were promptly processed for DNA extraction to enable comprehensive examination of microbiota composition and functional characteristics. The rest were preserved in liquid nitrogen and kept at -80°C . Samples were ground to a powder and then freeze-dried for volatile extraction.

2.2. Determination of pH, total titratable acidity (TTA) and viable cell counts

To determine the acidity, 10 g of the sample was homogenized in 90 mL sterile water. The pH was determined by a pH meter (SevenDirect SD50, Mettler-Toledo, Switzerland). TTA was determined by titration method and expressed as the volume of 0.1 M NaOH consumed to reach a final pH of 8.5. Viable cell counts were measured as previously described (Suo et al., 2020).

2.3. HS-SPME-GC-MS widely targeted metabolomics

2.3.1. Extraction of volatile compounds

Volatile compounds were extracted using headspace solid-phase microextraction (HS-SPME). Briefly, a freeze-dried sample (500 mg) was swiftly transferred into a 20 mL headspace vial with a NaCl saturated solution to inhibit the enzyme reaction. The vials were meticulously sealed utilizing crimp-top caps and immersed in 60°C to attain a state of equilibrium. At the time of SPME, a 120 μm DVB/CWR/PDMS fiber was exposed to the headspace with continuous agitation.

2.3.2. GC-MS conditions

After SPME, thermal desorption of the volatiles captured by fiber coating was performed in the injection port at 250°C . The identification and quantification of volatile compounds were achieved via Agilent Model 8890 GC coupled with a 7000D mass spectrometer, equipped with a 30 m $\times$ 0.25 mm $\times$ 0.25 μm DB-5MS column. Helium served as the carrier gas with 1.2 mL/min linear velocity. The programmed column temperature started at 40°C for 3.5 min, increasing at $10^{\circ}\text{C}/\text{min}$ to 100°C , at $7^{\circ}\text{C}/\text{min}$ to 180°C , at $25^{\circ}\text{C}/\text{min}$ to 280°C , hold for 5 min. MS was run in electron impact mode, with a scan range of 50–5000 m/z . The temperatures for the quadrupole mass detector, ion source and transfer line were set at 150°C , 230°C and 280°C , respectively. All samples were analyzed in triplicates.

2.4. Identification and quantification of volatile compounds in Mianhua processing

Primary metabolomics results were identified using an automated MS system integrated into the MassHunter Workstation. This system compares fragmentation patterns, retention time and accurate m/z values with standards from the self-compiled MetWare database (Wuhan, China) and public database. The volatile compounds were semi-quantified as previous described (Yuan et al., 2022).

$$C_i = (C_{is} \times A_i) / A_{is}$$

where C_i represents the mass concentration of any component ($\mu\text{g}/\text{L}$), C_{is}

represents the mass concentration of internal standard ($\mu\text{g}/\text{L}$), A_i is the chromatographic peak area of any component, and A_{is} is the chromatographic peak area of the internal standard. The identified metabolites were subsequently mapped to the KEGG database.

2.5. Calculation of rOAVs

Relative odor activity value (rOAV) is a parameter for identifying key aroma compounds on the basis of sensory threshold of compounds. It was used to pinpoint each compound's contribution to the overall aroma profile of the sample. Recently, rOAV has been used by an increasing number of researchers to determine key flavor compounds in various foods (Majcher et al., 2020).

The rOAVs of the volatiles were determined utilizing the equation (Huang et al., 2022). Generally, the volatiles with $\text{rOAV} \geq 1$ were deemed characteristics and considered as the direct contributor to overall flavor of the sample (Guo et al., 2021).

$$\text{rOAV}_i = C_i / T_i$$

where rOAV was calculated based on the ratio of its relative content (C_i) to the established threshold value for that compound in water (T_i).

2.6. Metagenomic sequencing analysis

2.6.1. Total DNA extraction and assessment

Total genomic DNA was extracted using E.Z.N.A.® soil DNA Kit. The purity and concentration of DNA were evaluated by NanoDrop 2000 and Syngery HTX, respectively.

2.6.2. Metagenomic sequencing

DNA extract was fragmented to ~ 350 bp using Covaris M220 Ultrasonicator for library construction. Construction of the paired-end library was carried out using NEXTFLEX Rapid DNA-Seq. Paired-end sequencing was conducted by Illumina NovaSeq™ X Plus (Illumina Inc., San Diego, USA).

2.6.3. Processing of sequencing data

In brief, the raw sequencing reads were subjected to adapter trimming, and any low-quality reads those with a length less than 50 base pairs or an average quality value below 20, were eliminated from further analysis (Chen et al., 2018). The obtained data were assembled by MEGAHIT. Contigs with a length of at least 300 bp were selected as the final assembly result. Open reading frames (ORFs) were identified with Prodigal, and a non-redundant gene catalog was assembled using CD-HIT with 90 % identity and coverage. Gene abundance was estimated by SOAPaligner.

2.6.4. Gene prediction, taxonomy and function annotation

The best-hit taxonomy of non-redundant genes was obtained by NCBI alignment. Functional annotation of non-redundant genes was obtained. Based on the abundance of non-redundant genes, the Kruskal-Wallis test was used to perform the differential analysis.

2.7. Multivariate statistical analysis and visualization

All tests were performed three times and the data were presented as means $\pm$ standard deviations with the significant differences identified through Duncan's multiple range tests of variance by SPSS (version 27). Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were conducted. The HCA results are shown as heatmaps with dendrograms, while Pearson correlation coefficients (PCC) between samples were calculated within R-4.2.2 and presented as heatmaps.

For two-group analysis, differential volatile metabolites were identified by variable importance in projection (VIP) and fold change (FC). VIP value, including score and permutation plots, were extracted from

the OPLS-DA. Metabolites with $|\text{Log}_2\text{FC}| \geq 1.0$ and $\text{VIP} > 1$ were defined as differential metabolites. Significant differences were determined with a confidence level set at $p < 0.05$. Spearman's correlation analysis was used to examine the association between microbial composition and volatiles.

3. Results and discussion

3.1. pH, TTA and viable cell counts

In the manufacture process of steamed bread, physicochemical indicators are important in evaluation of quality and monitoring of craftsmanship. Traditional stater dough has a high total acidity with a pH below 4. After mixing and kneading, the total acidity decreased, accompanied by an increase in pH to 5.63 (Fig. 1B). During shaping and proofing, the fermented dough subjected to acidification due to the production of organic acids by LAB, experienced a pH shift to 4.48, meanwhile proteases were responsible for the primary proteolysis. The total plate counts of doughs were above $5 \log \text{CFU g}^{-1}$ throughout the processing, exhibiting a slight increase during fermentation stage. Moderate total acid and a large number of microbes contributed to the appealing flavor of the dough. Subsequent studies examined changes in flavor metabolites and the evolution of microbial communities during Mianhua production, aiming to uncover the drivers and pathways of flavor compound formation.

3.2. Identification of the volatile compounds in Mianhua processing

To investigate the concentration changes of volatiles in dough during

Mianhua processing, HS-SPME-GC-MS was utilized. The PCA of Mianhua dough samples showed distinct clustering between the doughs before kneading and after kneading, indicating significant differences in their metabolite profiles (Fig. 2A). It was further demonstrated by OPLS-DA (Fig. 2B), showing a clear separation in volatiles among samples at different process steps. HCA also highlighted significant differences in metabolite composition (Fig. 2D). The metabolites in TSD and MixD formed a distinct cluster, the metabolites in CutD and PofMH formed another cluster, indicating that kneading, molding and proofing steps had an effect on volatiles profiles of Mianhua dough. Steaming is a heating process that can lead to the breakdown of metabolites or lipid oxidation in STB (Cho & Peterson, 2013). 848 volatile compounds were identified across Mianhua processing, encompassing 157 esters, 133 terpenoids, 106 ketones, 95 alcohols, 82 heterocyclic compounds, 67 hydrocarbons, 52 aldehydes, 41 acids, 33 phenol, 28 aromatics, 19 amines, 14 nitrogen compounds, 13 ethers, 6 halogenated hydrocarbons, 2 sulfur compounds (Fig. 2C). Esters constituted the most predominant volatile compounds in Mianhua processing, representing 18.51 % of the total. In fermented foods, esters are mainly produced through enzymatic reactions between acids and alcohols. These esters imparted pleasant fruity, sweet, and floral aromas to Mianhua samples, establishing their flavor characteristics. The volatile profile of the dough undergoes significant changes during Mianhua processing, emphasizing the imperative to identify key flavor characteristics throughout the manufacturing process.

For further examining the differential metabolites in vary process steps, the metabolites were categorized into four comparison groups: A1 (MixD vs TSD), A2 (CutD vs MixD), A3 (PofMH vs CutD), A4 (StB vs PofMH). OPLS-DA effectively identified the key variables influencing a

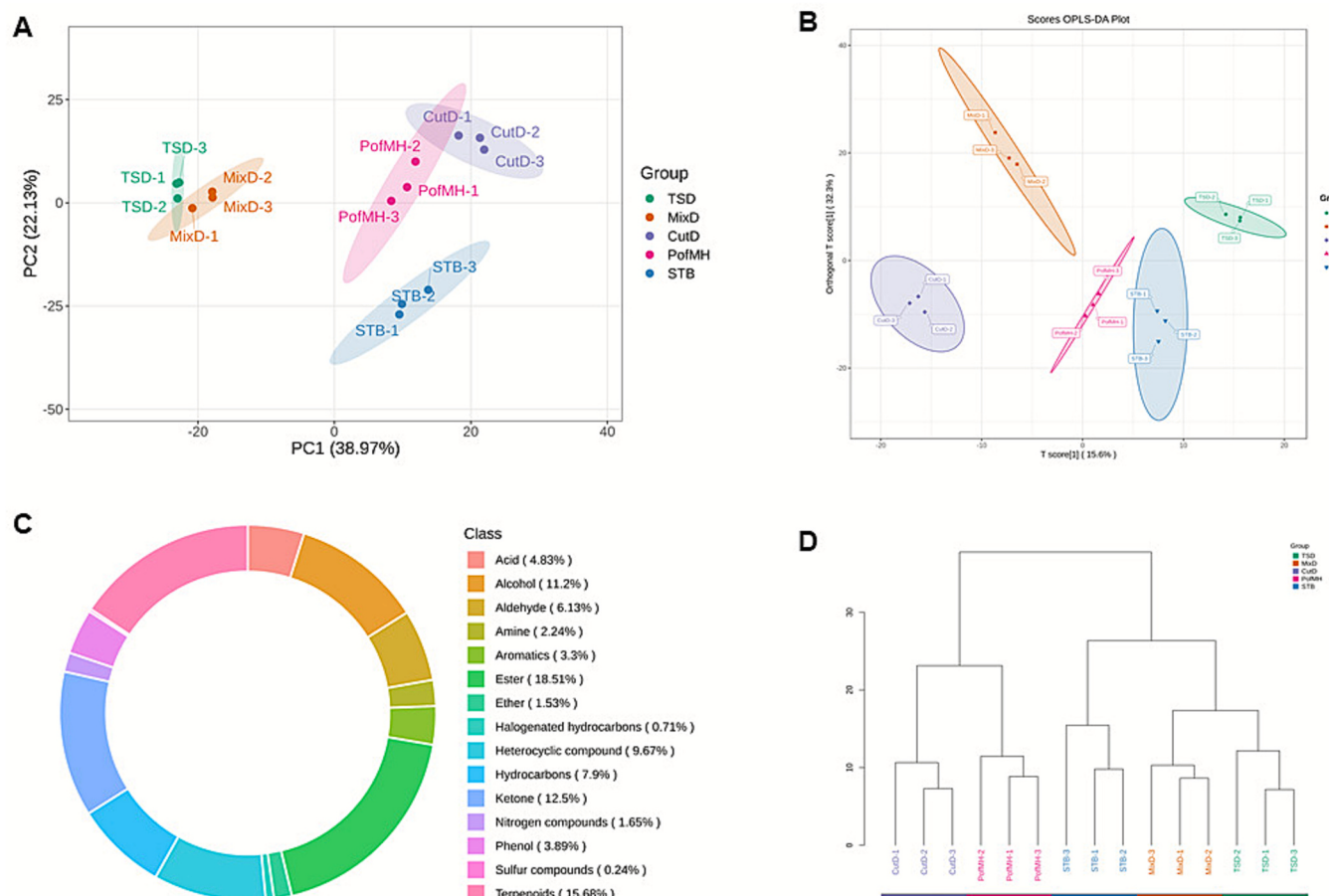


Fig. 2. Multivariate statistical analysis and classification of the volatile metabolites in samples from Mianhua processing. A, B, C and D represent PCA score plot, OPLS-DA score plot, classification, and clustering results of the volatile metabolites, respectively.

specific group. The variable importance factor (VIP) of Mianhua dough was analyzed by the model in Fig. S1 using GC-MS, with well-fitted parameters that confirm the model's accuracy. In the discriminant model, a variable is considered significant if VIP value exceeds 1. A total of 17 (5 up, 12 down), 220 (43 up, 177 down), 62 (36 up, 26 down), and 169 (107 up, 62 down) differential metabolites ($p < 0.05$) were identified among groups A1, A2, A3 and A4, respectively, as illustrated in Fig. 3A. The findings showed that the flavor of dough greatly changed during Mianhua processing. In particular, multiple dough pressing and kneading processes resulted in the aroma characteristics of the cut dough (CutD), which were different from those of the samples before molding. A key finding was the marked downregulation of most volatile compounds in CutD vs MixD comparison group. This could be attributed to the potential for volatilization of these compounds during vigorous mechanical working, or their enzymatic conversion into other metabolites as microbial competition shifted.

For each comparative group, differential metabolites meeting the screening thresholds were analyzed for their sensory annotations, with the top 10 most frequently characterized flavor attributes selected for radar chart visualization (Fig. 3B). Throughout Mianhua industrial manufacture, mixing with traditional starters emerged as a critical step that significantly affected flavor profiles, with fruity aroma compounds constituting the predominant class of differential metabolites.

In CutD vs MixD comparison group, all differential volatiles annotated with fruity characteristics (including 3-hexanone, 2-hexanone, ethyl octanoate, hexyl acetate, ethyl hexanoate, 1-hexanol, etc.) exhibited significant downregulation ($|FC| > 2$, $p < 0.05$). Concurrently, overwhelming majority of volatiles associated with sweet attributes also demonstrated reduced concentrations after dough mixing and kneading, while upregulated metabolites were predominantly annotated with woody, floral and minty attributes. This metabolic shift profile shown in Fig. 4 suggested that the differential regulation of fruity and sweet flavor components originated from the microbial community dynamics within the traditional starters during Mianhua manufacture. The mechanical action likely accelerated both enzymatic esterification and non-enzymatic oxidative pathways, leading to the rapid transformation or loss of these characteristic fruity esters and alcohols. Concurrently, the upregulation of metabolites with woody, floral, and minty characteristics suggests the activation of alternative lipid oxidation and degradation pathways under the new physicochemical conditions created by multiple-pass dough sheeting and pressing.

3.3. Evolution characteristic volatiles of Mianhua dough

The contribution of volatile compounds to flavor is closely linked to the rOAV, which influences the aroma profile of Mianhua. The distributions of metabolite rOAV were shown in Fig. 5A. 125 flavor compounds with rOAV > 1 was identified, indicating complex volatile compounds together constitute the flavor of Mianhua. Table S1 presents the rOAV (rOAV > 1) values of volatiles in different Mianhua processing stages. The concentration of each volatile was measured via semi-quantitative method. Among these 125 characteristic volatile compounds, the top 50 volatiles in relative content were detected as shown in Fig. 5B. The concentrations of 1-hexenol, 1-nonen-3-one, 1-octanol, geraniol, 1-octen-3-ol, propanoic acid pentyl ester, hexanoic acid ethyl ester, octanoic acid ethyl ester, etc. were highest in TSD and decreased throughout the processing. These volatiles were considered to be the characteristic flavor compounds that the traditional starters brought to Mianhua. After molding, the alcohols and esters decreased due to the loss of volatiles from enzymatic action variation during kneading (Martínez-Anaya, 1996). The variation in the intense of dough kneading could change the metabolic state of the microbial strains and enzymatic action, resulting in a faster consumption of aldehydes or an inhibition of the formation. Fig. 5C showed the content variation of the top 10 esters with fruity flavor, indicating the distinct characteristic fruity compounds enriched in different Mianhua process steps. The rOAV values

and relative content of most esters such as hexanoic acid ethyl ester, 3-mercaptopentyl acetate, octanoic acid ethyl ester, propanoic acid pentyl ester, decanoic acid ethyl ester in traditional starters (TSD) and mixed dough (MixD) were much higher than other samples. These esters were characterized by their fruity aromas, reflecting the fruity aroma profile of the fermented dough was yielded by the traditional starters. Additionally, hexanal and 1-hexanol with fruity and floral aroma, showed highest content in traditional sourdough.

3.4. Microbiota community characteristics

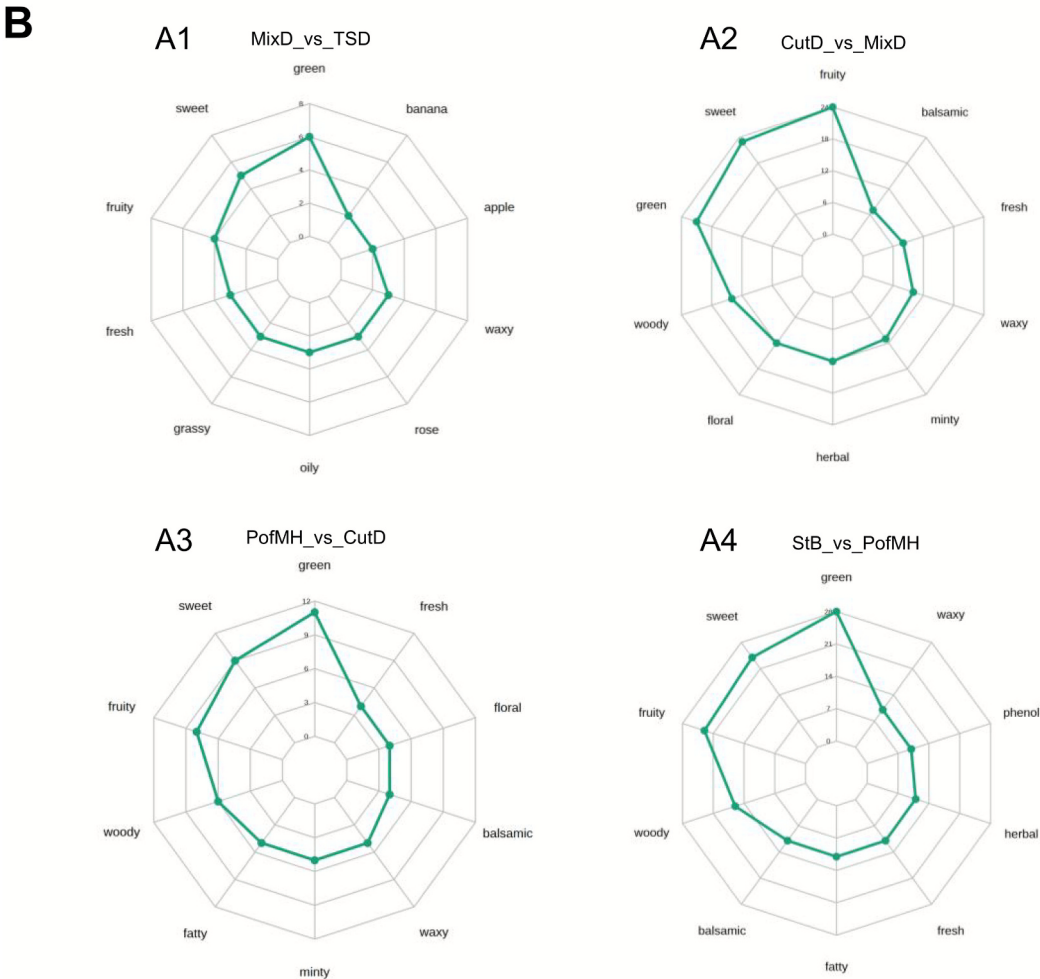
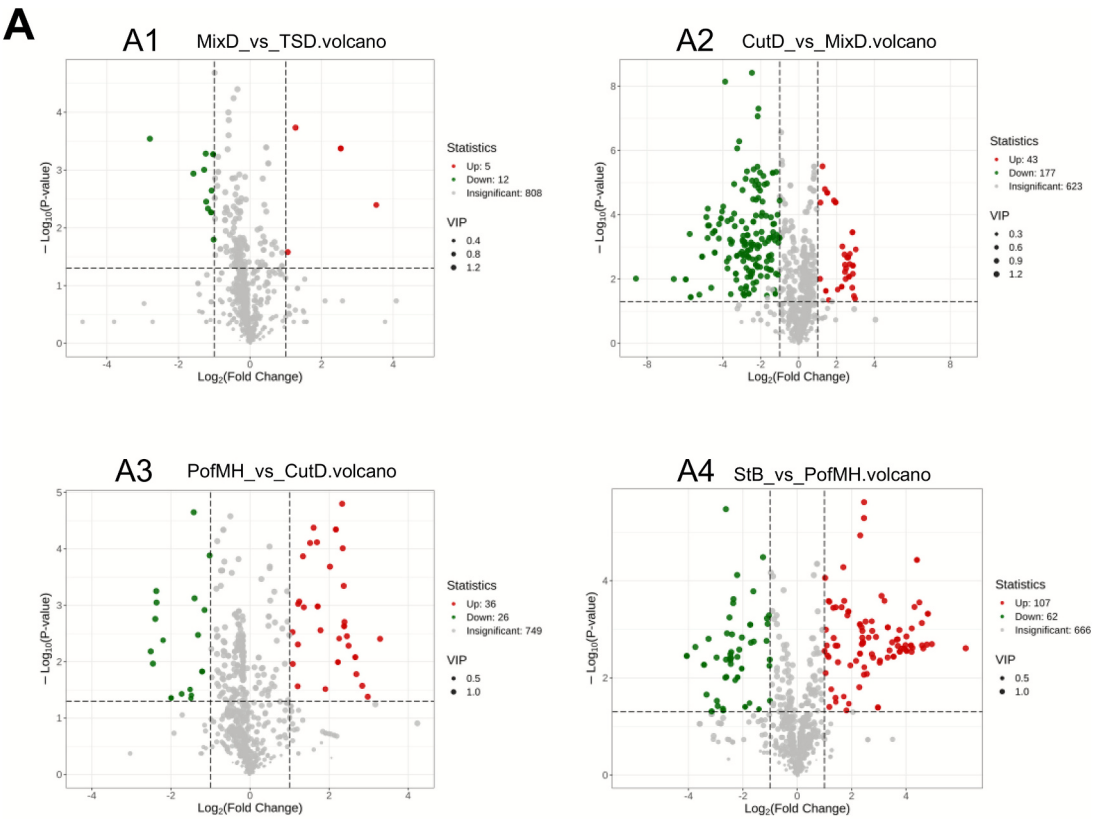
3.4.1. Microbial diversities

To assess the diversity of microflora, different α indexes were calculated as shown in Fig. 6A. Similar trend was noted in the Chao, Ace and Sobs indices, with the community richness declined firstly then increased by final proofing before steaming. PofMH were doughs gone through the proofing phase, which tended to have a higher community richness and diversity as compared to other samples. The levels of community richness and diversity varied across the groups, potentially reflecting the impact of traditional sourdough on the microbiota communities. During the initial MixD stage, microorganisms originating from the traditional sourdough (TSD) were primarily adapting to the new flour-water matrix. The subsequent mechanical stress from the pressing, cutting, and shaping operations in the CutD stage transiently suppressed certain bacterial populations (particularly dominant LAB species). In contrast, the proofing stage provided a relatively stable, warm and moderately humid environment. This stable incubation period created favorable conditions for microbial community restructuring and proliferation, thereby fostering an increase in taxonomic richness.

An analysis of β -diversity, employing the Bray-Curtis distance metric, unequivocally disclosed a substantial differentiation between the examined groups. To visually evaluate the similarities and differences among microbial communities of the Mianhua samples, PCA and HCA were shown in Fig. 6 BCE, samples within the same group clustered together and exhibited high similarity. Notably, TSD and PofMH clustered closer together and were clearly distinguishable from MixD and CutD. Microbial composition at genus level with relative abundance > 1 % was showed in Fig. 6D. *Proteus* (25.93 %), *Fructilactobacillus* (16.63 %), *Lactobacillus* (10.16 %) and *Companilactobacillus* (7.14 %) were the predominate genera (relative abundance > 5 %) during Mianhua production. LAB account for more than 35 % of the microbial community.

3.4.2. Alterations in microbiota dynamics and their functions during Mianhua fermentation

To elucidate the microbiota dynamics of Mianhua before steaming, the taxonomic distributions of the top 20 microbiota at order and genus level were depicted in Fig. 7A and B, respectively. *Lactobacillales* was the dominant microbial order throughout the Mianhua processing, exhibiting its highest relative abundance (64.62 %) during mixing phase in MixD. *Enterobacterales* emerged as the second most abundant order, with its peak relative abundance (54.42 %) observed during CutD phase. A critical contributing factor is that equipment surfaces, as well as manual handling, can act as reservoirs for environmental *Proteus*, from which they may be introduced into the dough. It was evident that *Fructilactobacillus*, *Lactobacillus*, *Companilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus* and *Lactiplantibacillus* were the dominant LAB during Mianhua processing from Fig. 8B, while *Kazachstania* was the dominant yeast genus. *Proteus* exhibited the highest relative abundance (40.77 %) in CutD, followed by *Fructilactobacillus* in MixD (35.96 %). *Lactobacillus* showed prominent richness in both TSD and MixD (14.33 %), while *Companilactobacillus* peaked at 14.28 % in PofMH after dough proofing. Heatmap analysis on species level was illustrated in Fig. 7C. *Companilactobacillus mindensis*, *F. sanfranciscensis* and *Lactobacillus helveticus*, as persistent dominant species throughout Mianhua process, originated from the traditional starters. These lactic acid bacteria experienced



(caption on next page)

Fig. 3. Screening and characterization of the differential volatiles during Mianhua process steps. (A) Volcano plot of the differential volatiles, A1: MixD vs TSD, A2: CutD vs MixD, A3: PofMH vs CutD, A4: StB vs PofMH. Red, green dots and gray dots represent the up-regulated, down-regulated differential metabolites and the non-differential metabolites. (B) Sensory characteristics radarchart of differential metabolites. The outer circle is sensory flavor characteristics, and the number corresponding to the green dot represents the number of differential metabolites annotated to the sensory characteristics. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

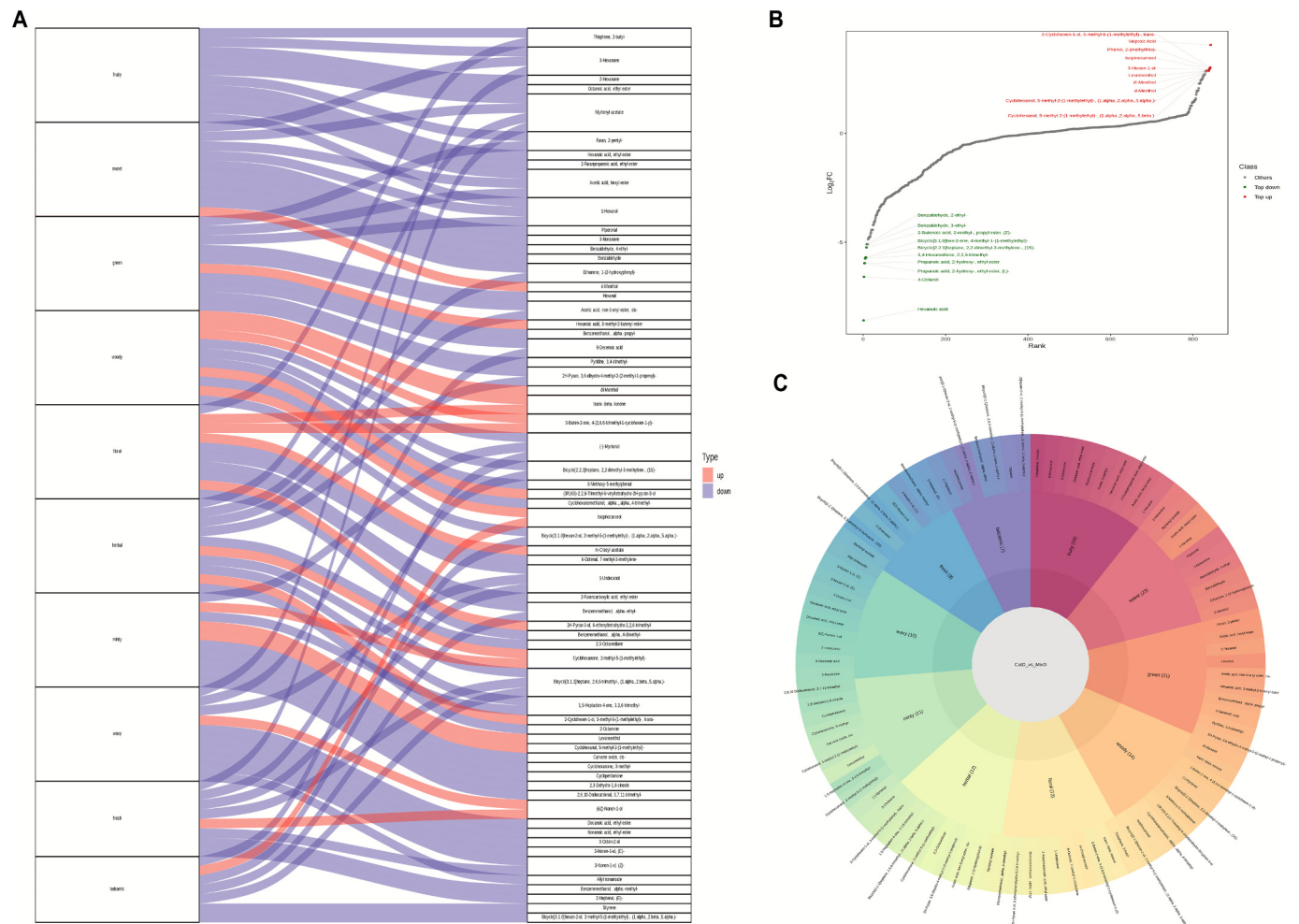


Fig. 4. Analysis of differential metabolites in CutD vs MixD. (A) Sankey diagram based on flavor characterizes of differential metabolites; (B) Differential metabolites distribution; (C) Molecular flavor wheel.

Note: In Fig.A, red streams indicate upregulated metabolites, blue streams indicate downregulated metabolites. In Fig.B, X-axis: cumulative compounds sorted by fold change (ascending), Y-axis: fold change ($\log_2$ -transformed). In Fig.C, bracketed numbers represent the number of differential metabolites annotated to top 10 sensory flavor characteristics. Top 10 VIP-ranked metabolites displayed when flavor characteristics associate with >10 differential compounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant reduction during dough pressing and carving (CutD), yet regained dominance after proofing (PofMH). Conversely, the sharp increase in *Proteus* abundance observed in the CutD stage corresponds to intensive mechanical processing operations, including rolling, cutting, and molding. Opportunistic species such as *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Escherichia coli* showed relatively enrichment in CutD, aligned with their adaption to oxygen-limited conditions and stress-tolerance. These mechanical actions likely generate localized anaerobic microenvironments that promote the proliferation of introduced or resident *Proteus* populations. The temporary reduction of LAB in CutD likely resulted from mechanical shear stress, while proofing reactivated their growth and metabolism.

Microbial interactions significantly impact the microbial community functions and structural stability, which can be preliminarily characterized by potential correlations among microorganisms (Fang et al., 2021). The co-occurrence networks of top 15 genus through spearman

correlation revealed that *Lactobacillus* demonstrated strong positive correlations with *Fructilactobacillus*, *Limosilactobacillus* and *Companilactobacillus* ($|r| > 0.6$, $p < 0.05$), but exhibited negative correlations with other genera (Fig. 7D). This synergistic relationship suggests that these dominant LAB genera could collaboratively metabolize the substances in dough to prevent the exogenous microorganisms, indicating their positive effects on Mianhua fermentation.

The KEGG functional contributions of microbial genera during Mianhua processing were illustrated in Fig. 7E. The KEGG pathway mainly includes metabolism, human diseases, genetic information processing at level 1, environmental information processing, organismal systems and cellular processes, and it was consistent with the results of steamed bread fermentation (Wei et al., 2024). The contribution of *Fructilactobacillus* to the 6 KEGG pathways of MixD was >20 %. *Fructilactobacillus*, *Lactobacillus* and *Companilactobacillus* collectively accounted for 71.44 % of predicted metabolic functions in MixD, with

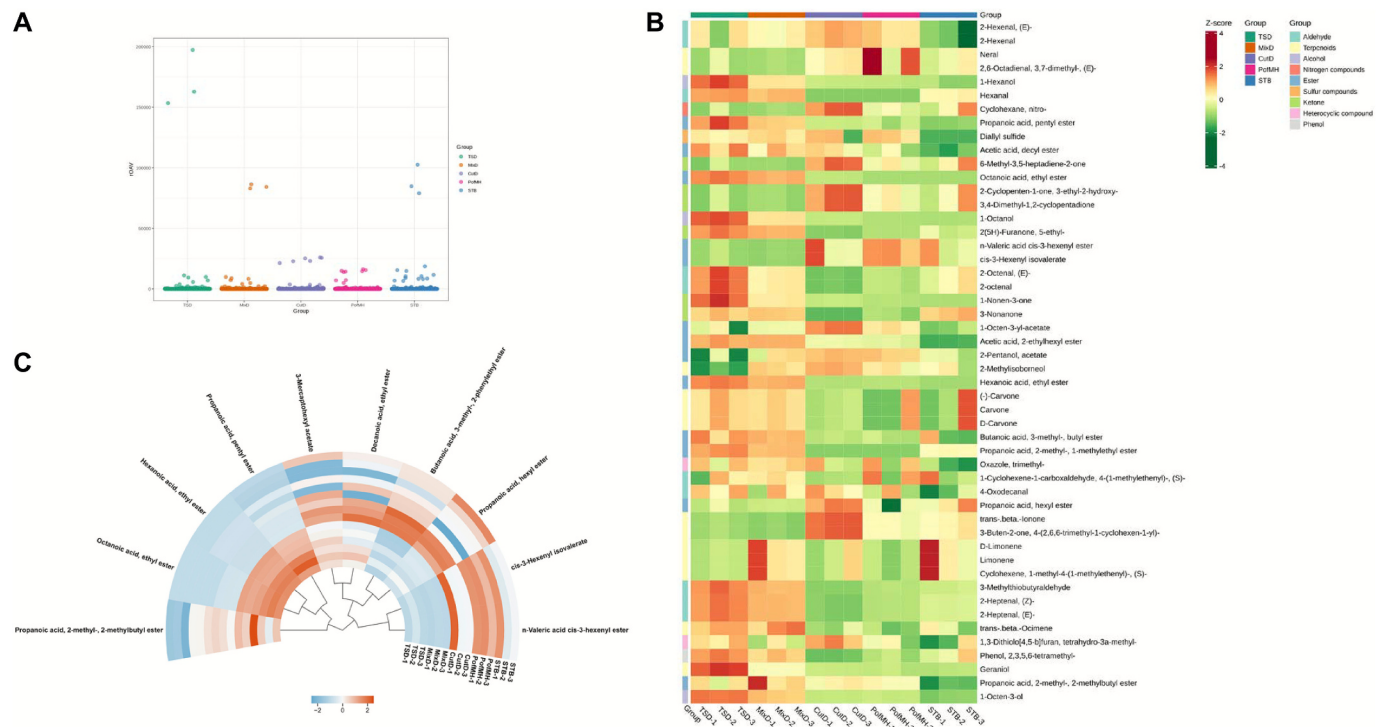


Fig. 5. (A) Metabolite rOAV scatter plot; (B) Heatmap of top 50 volatiles in relative content (rOAV >1); (C) Content dynamics of top 10 flavor-contributory esters annotated to fruity flavor.

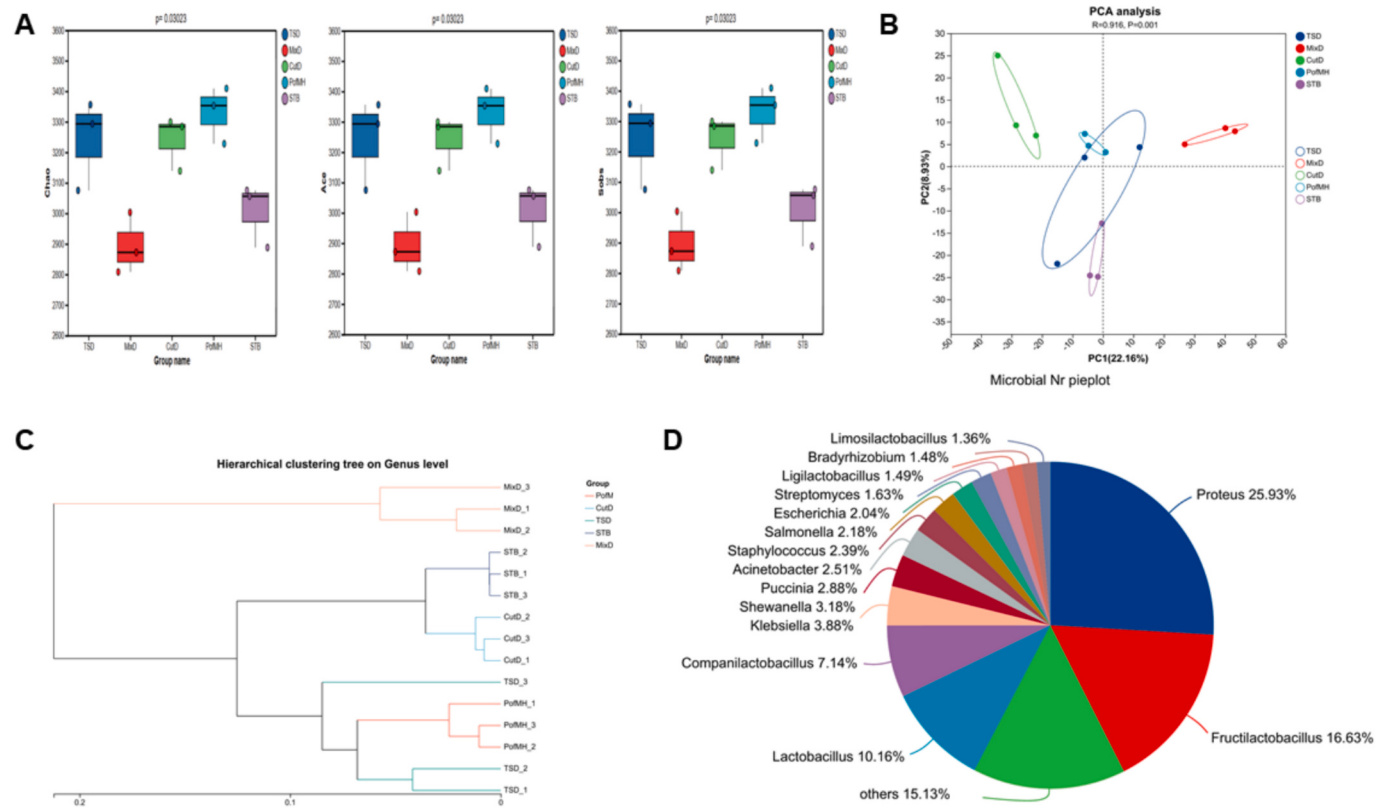


Fig. 6. (A) Bacterial alpha diversity indices (Chao index, Ace index, Sobs index) among 5 groups; (B) PCA, (C) hierarchical cluster and (D) microbial composition (relative abundance >1 %) on genus level.

contributions exceeding 45 % in both TSD and PufMH phases. This functional dominance in metabolism highlights their critical role in

driving enzymatic activities (e.g., carbohydrate metabolism) throughout the sourdough fermentation process. The CAZy functional contributions

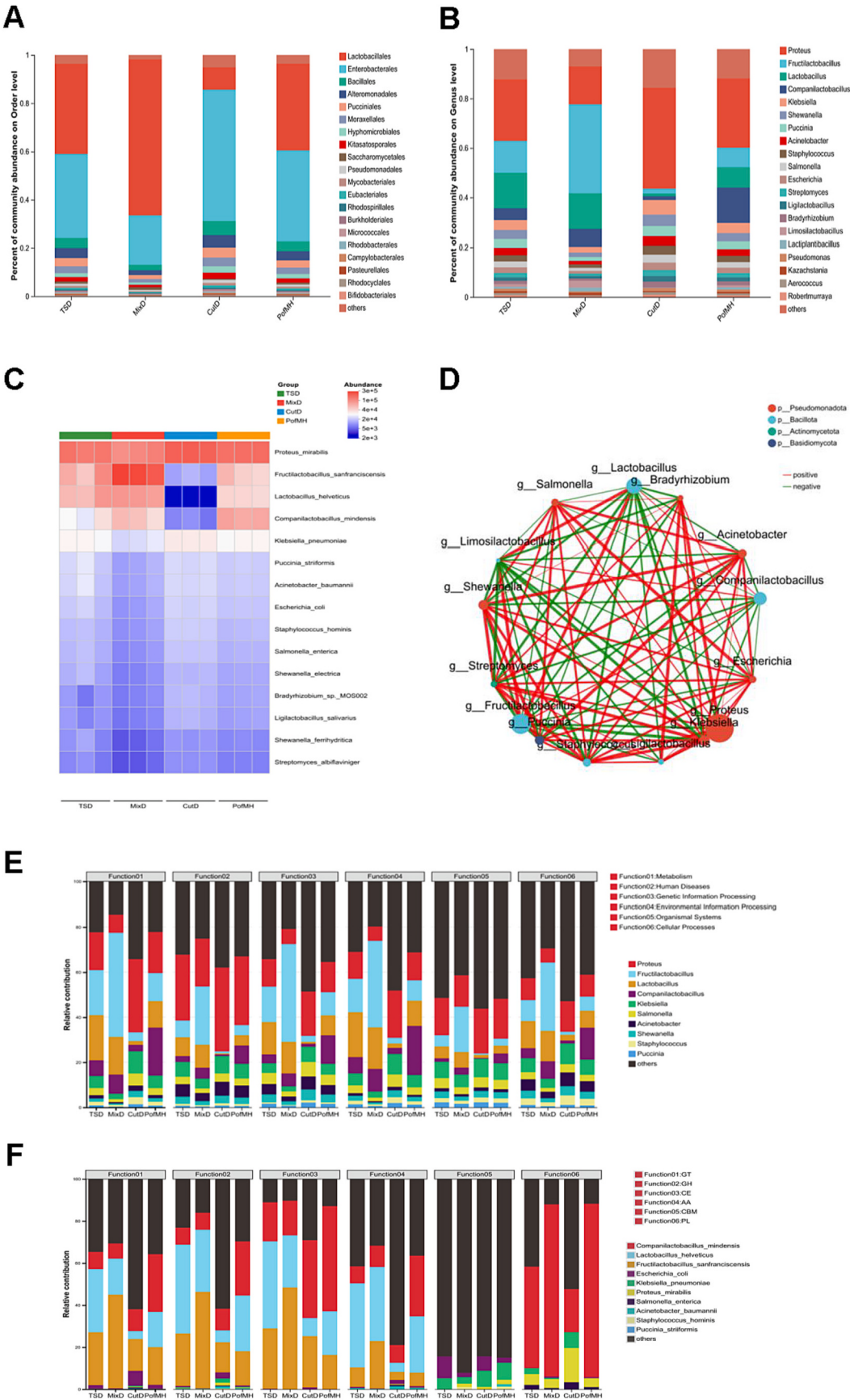


Fig. 7. Microbial community dynamics in Mianhua dough processing before steaming. Barplot analysis of top 20 microbiota at (A) order level and (B) genus level; (C) Heatmap of species composition; (D) Co-occurrence networks of top 15 genus based on spearman correlation; (E) KEGG functional contributions of microbial genera; (F) CAZy functional contributions of microbial species.

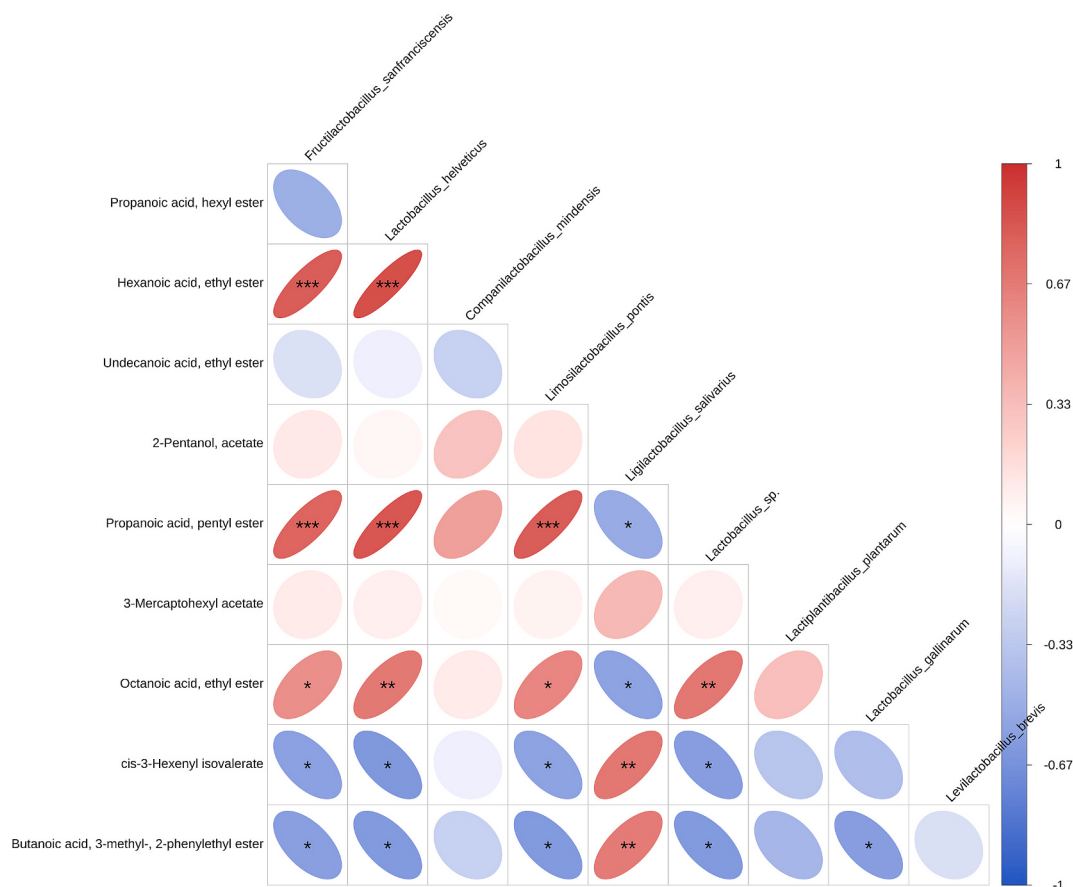


Fig. 8. Correlation analysis between top 9 LAB species and abundant fruity flavor esters based on statistically significant spearman correlation coefficient ($p < 0.05$).

of microbial species in Mianhua processing were visualized in Fig. 7F. *F. sanfranciscensis*, *L. helveticus* and *C. mindensis* collectively accounted for the majority of predicted glycoside hydrolase (GH), glycosyl-transferase (GT) and carbohydrate esterases (CE) in all samples. *C. mindensis* dominated polysaccharide lyase (PL) contributions, especially with >80 % relative abundance in both MixD and PofMH. The contributions from these species indicate their potential cooperative hydrolysis of starch, xyloglucan and arabinoxylan, ensuring efficient carbon flux for flavor compound synthesis.

3.5. Correlation analysis of ester compounds and microbiota

The spearman correlation analysis was performed between core esters (roAV >1) and dominant *Lactobacillaceae* taxa based on the identification of metabolites and microorganisms. Strong positive correlations were observed between propanoic acid hexyl ester, hexanoic acid ethyl ester and most *Lactobacilli* (Fig. S2), suggesting their biosynthesis in Mianhua is tightly linked to fermentation of LAB in traditional sourdough ecosystems. Fig. 8 visualized the correlation matrix of the top 9 abundant fruity flavor esters with dominate LAB species, where R-values are colour-coded and significance were marked. *F. sanfranciscensis* and *L. helveticus* showed strong positive correlations with propanoic acid hexyl ester and hexanoic acid ethyl ester, *Limosilactobacillus pontis* exhibited positive correlation with propanoic acid hexyl ester ($p < 0.001$). Octanoic acid ethyl ester correlated positively with above LAB species ($p < 0.05$). These esters characterized by their fruity aromas implicated the above LAB species as key contributors to fruity sensory in Mianhua processing through ester formation.

Sun et al. demonstrated that volatile flavor compounds are more closely related to the genera level of bacteria and fungi than species level, they noted the impact of flora dynamics and metabolic pathways

on the flavor profile of sourdough at the species level requires further exploration (Sun, Ye, et al., 2025). In the production of type I sourdough steamed bread using retarded sponge-dough fermentation, *F. sanfranciscensis*, *Lactobacillus pontis*, *Kazachstania humilis*, and *Saccharomyces cerevisiae* appeared to generate a stable consortium, which probably contributed to the production of numerous flavor compounds. (X. Wang, Zhao, & Yuan, 2020). This study demonstrated that *F. sanfranciscensis*, *L. helveticus* and *C. mindensis* were the most abundant LAB with the relative abundance significantly higher than other sub-dominant species throughout the production of Mianhua. Generally, *F. sanfranciscensis* is frequently predominant in both European and Chinese sourdoughs (Boyaci Gunduz et al., 2022; Wang, Zhu, et al., 2020). It is a key bacterium in traditional type I sourdoughs perfectly adapted to the sourdough surroundings. The metabolic versatility toward diverse substrates positions *L. helveticus* as a promising candidate for starter cultures, with potential applications in dietary fiber-enriched food development. *L. helveticus* is classically employed as both starter and adjunct cultures in yoghurt and cheese production due to its powerful proteolytic system and generation of flavor-related compounds (Nguyen et al., 2023). The presence of *L. helveticus* as a dominant species in Mianhua suggests a functional convergence between dairy and cereal fermentation, where its proteolytic activity may enhance amino-acid-derived aroma precursors.

3.6. Microbial metabolic networks related to the formation of characteristic esters in Mianhua processing

A thorough analysis was performed by integrating the volatile characteristic aroma compounds and metagenomic data during Mianhua fermentation. Microorganisms directly metabolize proteins, carbohydrates and lipids (Hu et al., 2022), generating intermediates such as

amino acids, fatty acids and pyruvic acid which are subsequently transformed into flavor substances. Metabolic pathways associated with substrate degradation and the formation of ester compounds were constructed via KEGG annotations, as represented in Fig. 9. Fermented flour products are rich in flavorful higher alcohols, primarily produced through the breakdown of amino acids via the Ehrlich pathway or the decarboxylation of corresponding α -keto acids (e.g., conversion of leucine in the flour to 3-methyl-1-butanol, a fruity flavor compound frequently detected in steamed bread). During steaming, most fatty acid-derived odorants such as decanal, nonanal and heptanal, showed increasing trends. Steaming emerged as a critical step affecting its aroma, likely driving thermal-induced non-enzymatic degradation of unsaturated fatty acids promoting the accumulation of key fresh odorants. The microbial enzymes facilitated the production of aldehydes, alcohols, acetyl-CoA and organic acids via processes like transamination, deamination and decarboxylation (Bermudez et al., 2014). Microorganisms are essential for facilitating the formation of precursors through the secretion of enzymes or by modulating enzyme activity, thereby contributing to the highest enrichment of most alcohols and esters in TSD.

Central to this process is the synergistic interaction between LAB and *Kazachstania* in traditional starters fermentation of Mianhua. Esters typically have low threshold and are crucial to the aroma of wheat products, evoking floral and fruity scents. The co-occurrence networks of top 20 genus through spearman correlation shown in Fig. S3 revealed that *Kazachstania* demonstrated strong positive correlations with *Fructilactobacillus* and *Lactobacillus* (spearman's $r > 0.75$, $p < 0.05$), indicating potential synergistic interactions. Subsequent co-culture experiments are warranted to validate the metabolic complementarity

between these LAB species and *Kazachstania*. Enrichment of esters in TSD is due to the esterification of alcohol and acids in dough fermentation. During dough fermentation, the formation of ester involves yeast and bacterial activity, where enzymatic reactions directly convert precursors into volatile esters. Yeasts play a pivotal role in this process by regulating alcohol acetyltransferase (AAT) activity, which catalyzes the esterification of alcohols with acetyl-CoA. KEGG pathway and CAZy analysis show that dominant LAB species (*F. sanfranciscensis*, *L. helveticus*, *C. mindensis*) contribute extensively to carbohydrate-active enzymes and organic acid production, whereas yeasts such as *Kazachstania* are expected to supply higher alcohols and AAT activity, providing precursors and enzymes for ester formation. LAB produce organic acids, which along with alcohols, generate ester flavor compounds through the action of yeast. Overall, LAB-yeast mutualism is essential in substrate degradation and flavor development of this traditional fermented wheat products. Subsequent isolation, cultivation, and functional validation of core strains will be essential. Future research will translate these insights into tangible applications by developing defined LAB-yeast consortia based on correlation strengths between key species and desired flavor compounds. This will be followed by comparing the ability of these synthetic communities to reconstruct target fruity ester profiles against traditional methods, with the goal of implementation for consistent product quality and standardization.

4. Conclusion

This study systematically investigated the correlation between microbiota, functional characteristics and volatiles transformation

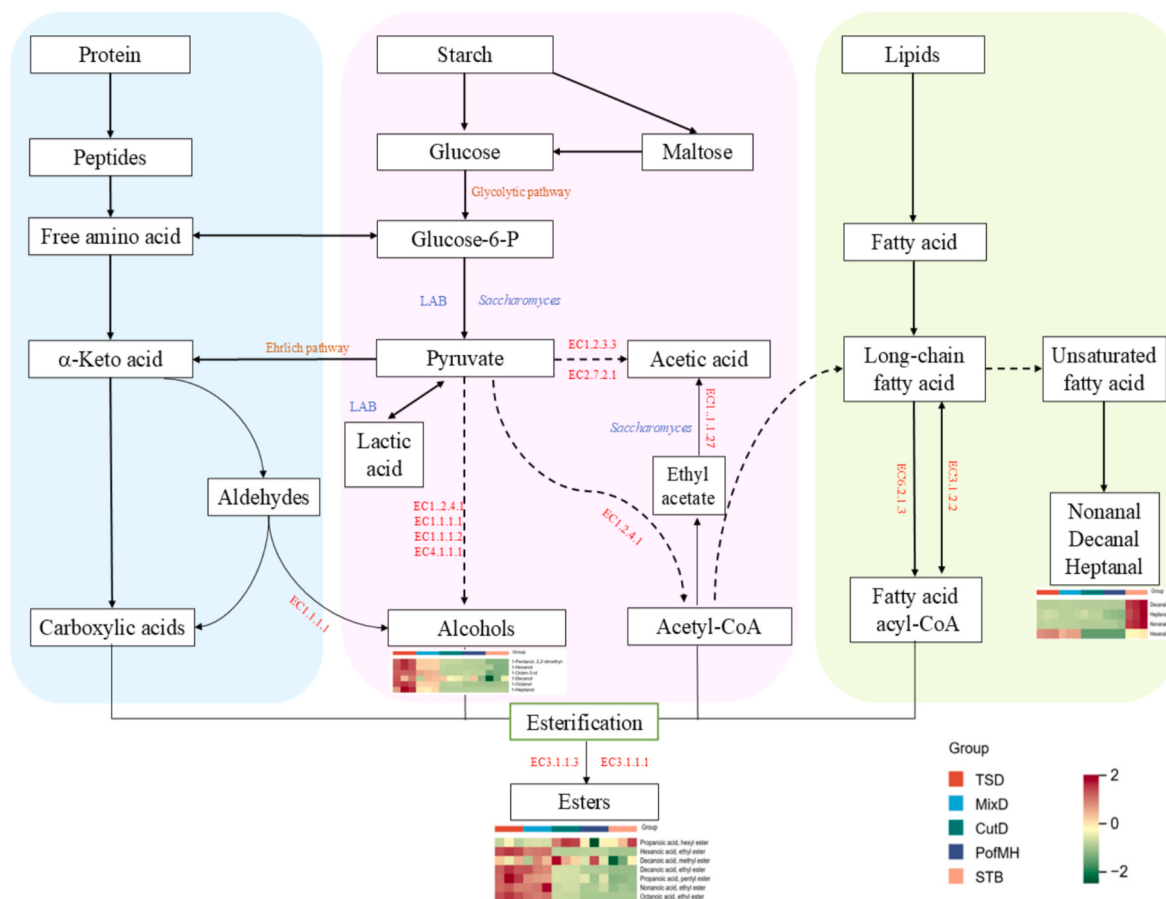


Fig. 9. Construction of the metabolic pathways for substrate degradation and characteristic ester formation in Mianhua processing based on the KEGG database. Arrows indicated the direction of reactions, while different colored regions depicted corresponding functions. The numbers above the arrows indicated the EC numbers of the enzymes in the reactions.

throughout the industrial manufacture of Mianhua. The results identified 848 volatile compounds, in which the most volatiles were ester compounds (18.51 %), which endowed the Mianhua dough with fruity and sweet flavor. Hexanoic acid ethyl ester, 3-mercaptopentyl acetate, octanoic acid ethyl ester, propanoic acid pentyl ester, and decanoic acid ethyl ester were recognized as the key esters characterized with fruity aromas imparted by traditional starters to Mianhua. *Fructilactobacillus*, *Lactobacillus*, *Companilactobacillus* and *Limosilactobacillus* were the dominant LAB during Mianhua processing, while *Kazachstania* was the dominant yeast genus. *F. sanfranciscensis* and *L. helveticus*, the dominant bacterial species in Mianhua processing, showed significant positive correlations with propanoic acid hexyl ester, hexanoic acid ethyl ester, and octanoic acid ethyl ester. Similarly, *Lim. pontis* exhibited positive correlation with the accumulation of propanoic acid hexyl ester, proposing a potential role in enhancing fruity flavor. Microorganisms involved in the metabolism of proteins, lipids and carbohydrates help form precursors that can enhance flavor development. The results provide a fresh outlook on how microbial communities influence flavor compounds during Mianhua processing, thereby might permit to keep quality stability and refine the processing technology of Mianhua. Building upon the established microbiota-flavor correlations, future research will develop and validate defined microbial consortia in fermented dough comprising key lactic acid bacteria (e.g., *F. sanfranciscensis*, *C. mindensis*, *L. helveticus*) and yeasts. Precisely optimizing strain inoculation ratios of specific species and fermentation parameters enables standardized product quality and enhanced process efficiency in industrial applications. This work provides the scientific basis and specific targets for future targeted strain selection, which can guide studies on the mechanistic regulation of flavor formation and the microbial basis for fruity characteristics enhancement in fermented flour products.

CRedit authorship contribution statement

Qiaozhi Song: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Jiixin Li:** Resources, Investigation. **Yu Liu:** Visualization, Software. **Wen Li:** Validation, Data curation. **Ming Li:** Validation, Conceptualization. **Bo Zhang:** Writing – review & editing, Project administration. **Boli Guo:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgements

This work was financially supported by Agricultural Science and Technology Innovation Program of Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences (CAAS-ASTIP-Q2024-IFST-09); Project of Drought-Alkali Wheat Processing Technology Innovation Center of Hebei Province (SJ2024032); Agriculture Research System of China of MOF and MARA (CARS-03).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2025.103446>.

Data availability

No data was used for the research described in the article.

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