HS-SPME-GC × GC/MS combined with multivariate statistics analysis to investigate the flavor formation mechanism of tank-fermented broad bean paste

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ABSTRACT

With the advancement of industrialization, tank fermentation technology is promising for Pixian broad bean paste. This study identified and analyzed the general physicochemical factors and volatile metabolites of fermented broad beans in a thermostatic fermenter. Headspace solid-phase microextraction (HS-SPME)-two-dimensional gas chromatography-mass spectrometry (GC × GC–MS) was applied to detect the volatile compounds in fermented broad beans, while metabolomics was used to explore their physicochemical characteristics and analyze the possible metabolic mechanism. A total of 184 different metabolites were detected, including 36 alcohols, 29 aldehydes, 26 esters, 21 ketones, 14 acids, 14 aromatic compounds, ten heterocycles, nine phenols, nine organonitrogen compounds, seven hydrocarbons, two ethers, and seven other types, which were annotated to various branch metabolic pathways of carbohydrate and amino acid metabolism. This study provides references for subsequent functional microorganism mining to improve the quality of the tank-fermented broad beans and upgrade the Pixian broad bean paste industry.

1. Introduction

Pixian broad bean paste is a famous fermented condiment from the Pidu district in Chengdu City, Sichuan Province, China, and is considered the soul of Sichuan cuisine. The production process mainly consists of koji-making, broad bean fermentation, pepper fermentation, and mixed fermentation. Although open fermentation has traditionally been used for production, the subsequent products are easily contaminated during the fermentation process, such as air pollution, insects, undesired microbes, dust, etc., while product quality is difficult to control. Therefore, tank fermentation equipment was developed to facilitate the standardized and industrial production of Pixian broad-bean paste. Our previous research presented the equipment details (Ding et al., 2022). However, the studies on tank-fermented broad beans are not mature enough, requiring significant research to popularize mechanical production.

For thermostatic fermentation, many studies have focused on characterizing flavor substances, determining key aroma compounds, and identifying microbial diversity (Ding, Zhao, Xie, Liu, Zhang, Che, et al.,

2021; Ding et al., 2022; Ding et al., 2021). The volatile substances in fermented condiments have attracted considerable research attention, while the related generation mechanism remains unclear. This study uses metabolomics, an established research method for mass metabolite data processing, information mining, and the sensory and nutritional quality prediction of final fermented food products. It is essential to establish the relationship between the components and functions of food products and observe the changes in their metabolic profiles during fermentation (Utpott et al., 2022). GC–MS, liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) are commonly used to detect metabolites. This study applies two-dimensional gas chromatography-mass spectrometry (GC × GC–MS) for volatile metabolite analysis, which relies on combining two chromatographic dimensions using a modulator. This combination significantly increases the separation capacity of the analytical system, while the modulator allows a comprehensive transfer from the first (1D) to the second (2D) GC dimension (Giddings, 1995). Compared to one-dimensional gas chromatography, comprehensive GC × GC provides a more significant peak capacity and highly structured chromatograms

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(Gruber, Weggler, Jaramillo, Murrell, Piotrowski, & Dorman, 2018). Therefore, it is more effective in separating substances and can detect more compounds. GC × GC–MS generates quantitative data, continuously measuring quantitative variables (on an interval or ratio scale) (Szymanska et al., 2015). Several studies have investigated GC × GC–MS data using the integrated peak area for every compound detected. The quantitative data are processed using qualitative goals, including compound identification, exploratory data analysis, feature selection, and clustering analysis (Stefanuto et al., 2021). With the development of user-friendly GC × GC systems, the technology has evolved from "single-sample analysis" to large-scale "omics" screening (Cordero, Kiefl, Reichenbach, & Bicchi, 2019; Keppler, Jenkins, Davis, & Bean, 2018).

This study utilizes headspace solid-phase microextraction (HS-SPME) for volatile component collection, while GC × GC–MS is adopted for the untargeted detection of volatile metabolites. Multivariate statistical analysis is used to screen and compare the differential metabolites and analyze the related annotated metabolic pathways to predict the possible mechanism behind metabolite production. This promotes an understanding of the volatile substance characteristics during constant temperature tank fermentation at the molecular level, provides a reference for optimizing broad bean tank fermentation, and offers theoretical knowledge for industrial upgrading.

2. Materials and methods

2.1. The broad bean fermentation process

The broad bean fermentation process principle is shown in Fig. 1. The broad beans were fermented in a closed system device mainly composed of a 50 L tank fermenter, an air supply system, and a thermostat. The temperature remained constant at 40 °, while a fermentation tank with a filling coefficient of 0.8 was used for fermentation. The fermenter was stirred every 12 h and ventilated for 5 min every 6 h. The broad bean koji inoculated with Aspergillus oryzae3.024 was obtained from Sichuan PXDB Co., Ltd., and then mixed with water at a ratio of 1:1, after which 18 % (w/w) salt was added. Finally, the mixtures were transferred to the fermentation tank for formal fermentation. The experiment lasted 39 d.
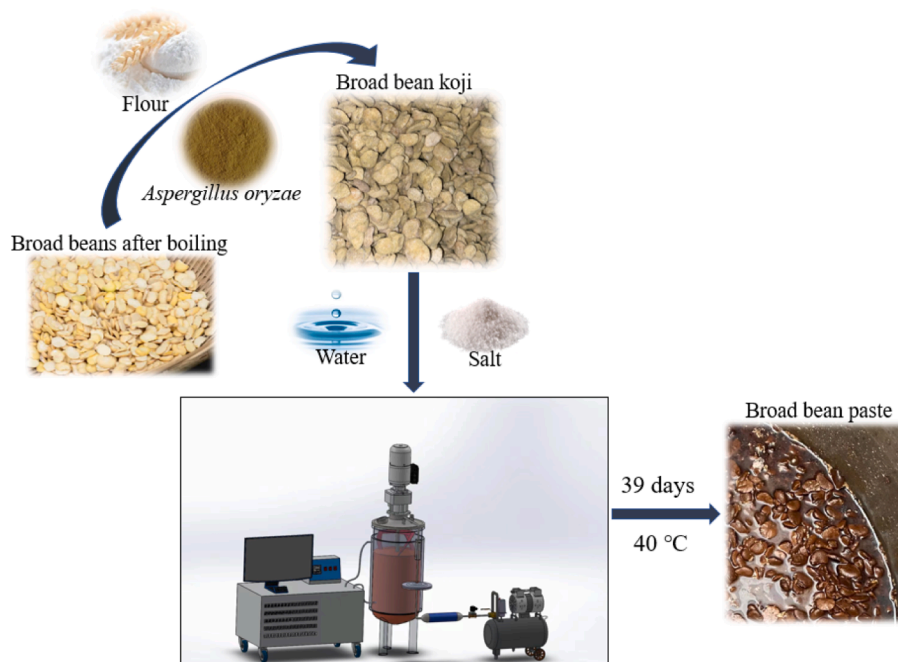
2.2. Sample collection

Sterile sampling bags were used to collect the samples at 0 d, 6 d, 13 d, 20 d, 28 d, and 39 d of the fermentation period and were labeled A, B, C, D, E, and F, respectively. Before each sampling, the materials in the fermenters were mixed well, and then were divided into three layers at equal distances according to their heights, each layer was sampled according to the five-point sampling method, specifically, is to take the midpoint of the plane as the central sampling point, and select four points on the diagonal with equal distance from the midpoint for sampling and then mixed evenly. The experiments were repeated five times according to metabonomic methodology.

2.3. Determination of the basic physicochemical indexes

Total titratable acidity was measured using 0.05 mol/L NaOH, yielding a titration endpoint at pH 8.2 (Zhao, Mu, & Sun, 2019). The amino acid nitrogen was quantified according to the acidity meter method (Xia, Li, Zheng, Ran, & Kan, 2014), while the reducing sugars were determined using the 3,5-Dinitrosalicylic acid technique (Khatri & Chhetri, 2020), and the pH value of the sample was measured using a pH meter. In addition, direct drying was used to determine the water content.

2.4. Detection of the volatile compounds using HS-SPME-GC × GC–MS

HS-SPME combined with comprehensive GC × GC–MS were applied to detect the changes in the volatile components during the fermentation of the broad bean paste. The SPME and instrument parameter settings were determined according to previously described methods (Liu et al., 2022) with some modifications.

The sample was mashed into a homogenized paste, after which 5.000 g was placed in a 15 mL SPME vial. For full volatilization of compounds, the samples were equilibrated in a water bath at 60 °C for 25 min. Next insert a 75 μm CAR/PDMS-coated SPME fiber probe (Supelco, Bellefonte, PA) into the SPME vial to absorb volatile components in the vial, this process lasts 25 min. Then the fiber was inserted into the GC injection port for desorption for 1 min. All the extractions

Fig. 1. The broad bean fermentation process.

were conducted in quintuplicate.

The 1D column consisted of a DB wax quartz capillary column (30 m × 0.25 mm × 0.25 μm), while the 2D column comprised a DB-17MS capillary column (1.2 m × 0.18 mm × 0.18 μm) and applied a solid-state thermal modulator HV (c720-21005), Helium gas (purity ≥99.9999 %) at a flow rate of 1.0 mL/min was used as a carrier for the splitless injection. The temperature was maintained at 40 °C for 2 min, after which it was increased to 230 °C at 5 °C/min, where it was maintained for 4 min, while the oven temperature was 230 °C, two-dimensional analysis time was synchronized with the one-dimensional column with a modulation period of 4 s.

2.5. Data processing and analysis

The two-dimensional chromatographic data processing software, Canvas Panel, was applied to detect and match the original data, eliminate noise peaks and merge duplicate peaks, identify chromatographic peaks with signal-to-noise ratios exceeding 100, and eliminate compounds with positive and negative matching degrees less than 750 via the similarity matching of the mass spectrum information using the NIST 20 (NIST, Gaithersburg, MD, USA) library. The data were then processed for peak alignment. After processing, the data was exported into an

EXCEL format and contained information regarding the metabolite name, CAS number, peak area, molecular formula, and 1D and 2D retention times. The data were imputed and standardized using IBM SPSS statistics 25 and imported into SIMCA 14.1 after normalization for multivariate statistical analysis via Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). The variable importance in projection (VIP) value obtained via OPLS-DA analysis is a crucial indicator for evaluating the screening of metabolic markers. Therefore, a VIP value over 1.0 was adopted to select the effective compounds. The compounds with VIP values exceeding 1.0 were subjected to ANOVA analysis using SPSS software (Addinsoft, Paris, France). A P-value less than 0.01 represented a significant difference. Therefore, compounds with P-values less than 0.01 were filtered out after removing a subset of compounds with no chemical information and consequently not suitable for analysis, resulting in a list of differential metabolites.

2.6. Prediction of the metabolic pathways

The differential metabolites were imported into the online bioinformatic analysis website, MetaboAnalyst (https://www.metaboanalyst.ca/), while the KEGG database was used for the metabolite set
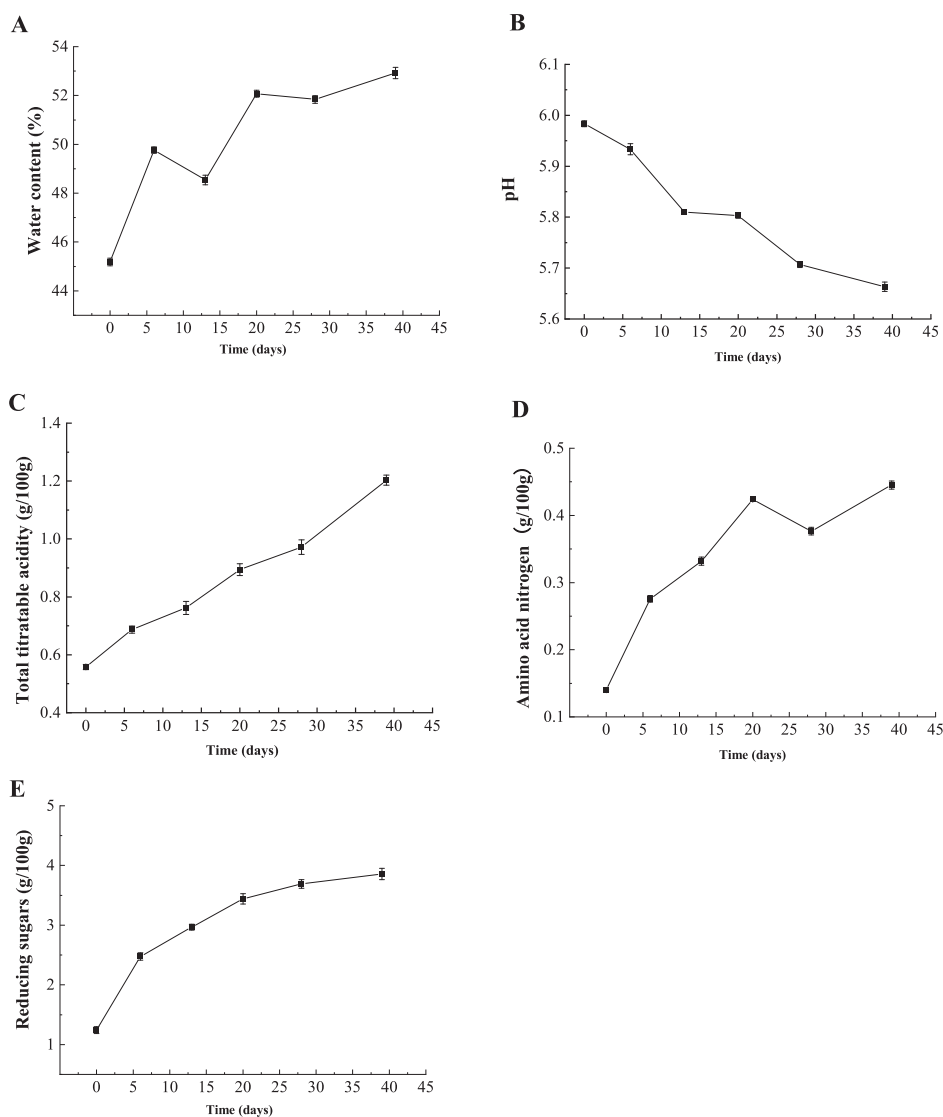
Fig. 2. The physicochemical parameters for the constant temperature fermentation of broad beans. (A) Water content. (B) pH. (C) Total titratable acid. (D) Amino acid nitrogen. (E) Reducing sugars. The experiment for each parameter was performed in triplicate, while the standard error was visualized via an error bar.

enrichment analysis to resolve the possible metabolic pathways.

3. Results and discussion

3.1. Analysis of the basic physicochemical factors

The basic physical and chemical indicators are shown in Fig. 2. As shown in Fig. 2A, the water content of the broad beans ranged between 45 % and 52 % during the fermentation stage. Water content was an important indicator during broad bean fermentation, affecting the biological activities of the microorganisms in the fermentation environment and the progress of the Maillard reaction. However, the changes in the moisture content were not the main factor, it was indicated that these conditions met the requirements of the fermentation microorganisms and Maillard reaction.

The pH value was used to measure the acidity of the product. As shown in Fig. 2B, the pH values were between 5.98 and 5.66, showing a gradual decrease with the extension of the fermentation time. Various microorganisms were present in the fermentation environment of the broad beans, which continuously utilized the raw materials to produce organic acids via metabolism (Wang, Zhang, Liu, Jin, & Xia, 2022), such as acetic acid and lactic acid, decreasing the pH in the fermentation environment.

The total acid content increased stepwise during fermentation (Fig. 2C), from 0.558 g/100 g to 1.203 g/100 g, meeting the national standard regulations that the total acid (measured as lactic acid) be no more than 2.0 g/100 g in sauce products. The total acid value was used as an important criterion representing the quality of the Pixian broad bean paste, as well as a hygienic indicator. The elevation of total acid content may be due to the abundant *Aspergillus oryzae* in the koji, which can secrete amylase, combined with a series of biochemical reactions to generate acid substances such as organic acids (Park, Seo, & Kim, 2019). Studies (Ito & Matsuyama, 2021) have shown the presence of lactic acid bacteria and yeasts in koji, while glucose is broken down into small molecules such as lactate and acetic acid due to metabolic action. These organic acids ultimately increase the total acid content, which is also consistent with the pH results.

As an important index in traditional Chinese fermented foods, the amino acid nitrogen content reflected the degree of broad bean protein hydrolysis during fermentation and free amino acid levels. As shown in Fig. 2D, the amino acid nitrogen content increased gradually throughout the fermentation process, reaching a level of 0.445 g/100 g at the end of fermentation, which indicated that the broad bean paste approached maturity.

Reducing sugars are an important source of the carbon elements required for microbial growth and metabolism. They were derived from the hydrolysis of starch by the amylase mainly secreted by *Aspergillus oryzae* and several other microorganisms. The reducing sugar content increased to ultimately reach 3.858 g/100 g (Fig. 2E). Examination of broad bean physicochemical indexes during constant temperature tank fermentation indicated that the ammonia nitrogen and reducing sugar levels were higher than those obtained during traditional fermentation. However, the total acid content was lower than during traditional fermentation at the same time as in previous studies. Therefore, constant temperature tank fermentation significantly reduced the ripening period and improved production efficiency, which was conducive to product quality control. This may be because a constant temperature of 40 °C in the fermenter provides suitable conditions for microorganism and enzyme activity (Hoang et al., 2018).

3.2. Multivariate data analysis

A total of 771 volatile components were detected at six fermentation time points using GC × GC–MS, followed by multivariate statistical analysis to screen the differential metabolites. Principal component analysis (PCA) was employed to discriminate between the fermented broad bean samples in the six groups (0–39 d), indicating separation to varying degrees. As shown in Fig. 3A, the two principal components accounted for 60.4 % of the total variance, with the first principal component (PC1) and second principal component (PC2) accounting for 25.1 % and 35.3 %, respectively. Group A was clearly distinguishable from the others, while the E and F groups were also clearly discernable from the BCD cluster. However, a slight overlap was evident between groups E and F, as well as groups C and D in the BCD cluster. In general, discrepancies were apparent between the broad bean samples exposed to different fermentation times and could be differentiated by identifying the volatile substances. The PCA results illustrated that the samples could be separated at varying fermentation periods. The next step involved establishing a discriminant model, adopting OPLS-DA to explore the differential substances. OPLS-DA combines orthogonal signal correction (OSC) to filter out signals that do not correlate with the model classification, improving the sample separation between the groups and enhancing the explanation. As indicated by the OPLS-DA score plot (Fig. 3B), the separation between the groups was obvious with a high degree of clustering, highlighting the reproducibility of the OPLS-DA method. The OPLS-DA results showed that PC1 accounted for 19.9 % and PC2 for 39.6 %, respectively, producing a total variance of 59.5 %, with an R2Y of 0.956 and a Q2 of 0.809, indicating that the superior reliability of the current model. To further determine the reliability of the OPLS-DA model, it was evaluated using a permutation test. As shown in Fig. 3C, the intercept of Q2 on the Y-axis was less than 0.5. When the transverse coordinate was 1, Q2 was less than R2, while R2 was very close to Q2, confirming the reliability of the model without overfitting. Therefore, the original model sufficiently explained the differences between the samples.

3.3. Identification of the characteristic metabolites

The VIP values were generated using the OPLS-DA model. The compounds with VIP values more than 1 and P-values less than 0.01 were screened out as differential metabolites. A total of 184 differential metabolites were screened and classified as alcohols, aldehydes, esters, ketones, acids, aromatics, heterocyclics, phenols, organonitrogens, hydrocarbons, ethers, and other substances, while the numbers of each compound type were 36, 29, 26, 21, 14, 14, 10, 9, 7, 9, 2, and 7, respectively. The proportion of each compound type is shown in Fig. 4. The alcohols, aldehydes, esters, and ketones accounted for 60.9 % of all the compounds, representing the primary volatile compounds, the details of which are shown in Supplementary Table 1. A heat map was created according to the normalized metabolite peak area data to observe the substance changes and clustering more intuitively while helping to distinguish between the different samples.

3.4. Analysis of the characteristic volatile compounds

The heat map (Fig. 5) showed good aggregation within the groups and significant variability between the groups. The clustering results further indicated that the samples obtained at different fermentation time points were clustered into three groups. The samples collected at 0 d of fermentation were clearly distinguished from those at 20 d and 39 d, while these clustering results were consistent with those obtained via previous multivariate statistical analysis. These findings indicated that the screened differential metabolites effectively distinguished the fermented broad beans at specific fermentation periods. Therefore, the thermostatic tank fermented broad beans were divided into three stages: pre-, mid-, and post-fermentation. Most of the differential metabolites accumulated during the post-fermentation stage, while the unique flavor also formed during this phase.

Alcohols originate from the microbial fermentation and metabolism of amino acids and sugar compounds. The heat map showed significant metabolite accumulation with the extension of fermentation time, while the increasing reducing sugar and amino acid nitrogen levels provided a
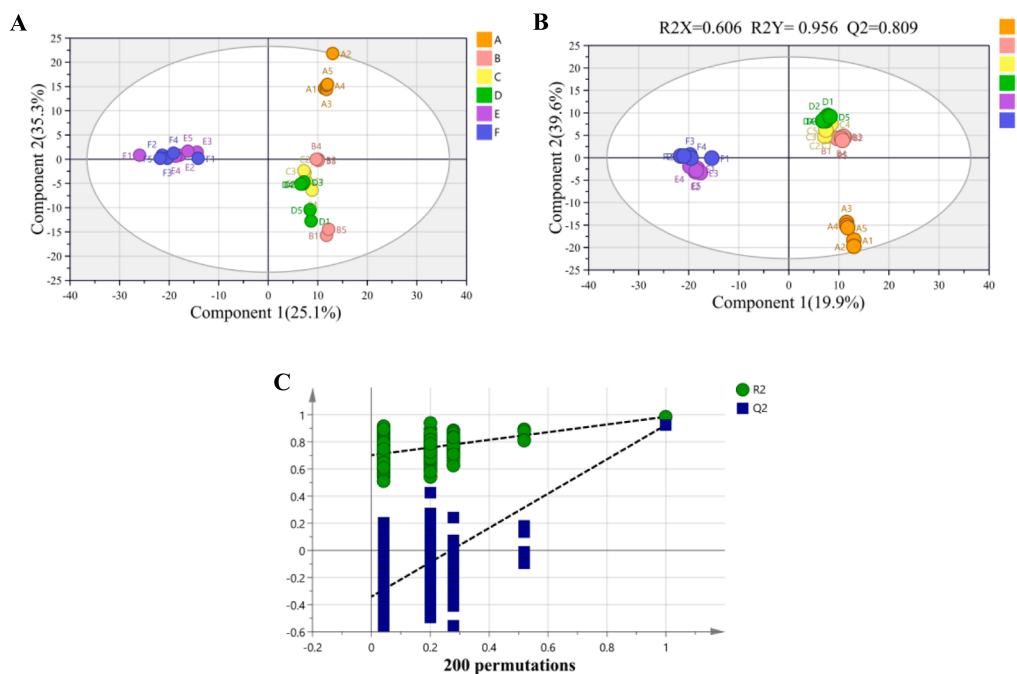
Fig. 3. The results of the multivariate statistical analysis of the fermented broad bean samples. (A) The PCA analysis of the volatile compounds identified in the broad beans at different fermentation periods. (B) The OPLS-DA score plot. (C) The result of 200 times permutation testing for OPLS-DA. Five parallel experiments were performed for each sample.
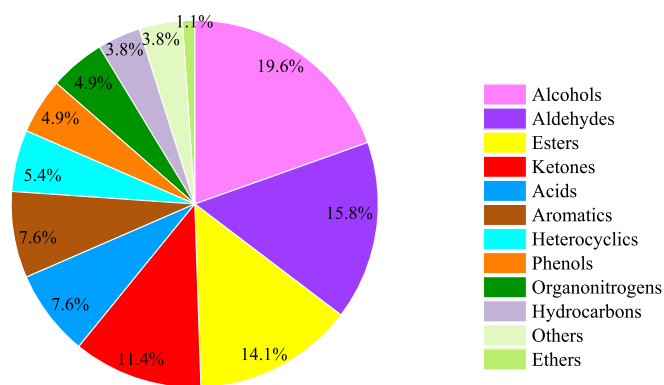
Fig. 4. The proportion of volatile differential compounds.

sufficient material basis for microbial activity, leading to the enrichment of alcohols (Cordente, Schmidt, Beltran, Torija, & Curtin, 2019; Du, Yang, Yang, & Yang, 2022), which was consistent with the result shown in Figs. 2D and E.

Aldehydes display low thresholds and generally present pleasant nutty, floral, and fruity aromas (Lee, Seo, & Kim, 2006). The heat map showed abundant aldehyde aggregation during the beginning and end of the fermentation process. Although aldehyde accumulation was evident during the initial fermentation stage, the types of aldehydes varied significantly during the later stage, indicating that the raw materials contained a more substantial number of aldehydes. Some aldehydes may be originally metabolized to other substances, while new aldehydes are synthesized during the post-fermentation period when exposed to certain biochemical reactions.

Esters are important flavor substances in fermented condiments, playing an important role in the coordination of overall flavor. Alcohol acyl transferases (AATs) allowed some microorganisms to synthesize esters by condensing acyl CoAs and alcohols from biomass-derived fermentable sugars and organic acids (Layton & Trinh, 2016).

The production of acid compounds was closely related to the microorganisms in the fermentation system. During fermentation, proteins and carbohydrates were degraded by microorganisms to produce small peptides, amino acids, and sugars that contributed significantly to flavor and were further metabolized to form pyruvate, a key intermediate in the organic acid production pathway, resulting in the formation of a large number of volatile organic acids (Liu, Li, Shin, Liu, Du, & Chen, 2017). Pyruvic acid accumulated in the fermentation medium and diminished considerably during the late fermentation stage, indicating that it might have been transformed during fermentation. Another key acid annotated in the differential metabolites, namely isovaleric acid, was enriched during the mid-fermentation stage, while the other acid compounds were all enriched during the post-phase. In terms of flavor analysis, not all 184 volatile substances had an important impact on the flavor of the Pixian broad bean paste. Previous research results confirmed that the key aroma compounds in the Pixian broad bean paste included 3-(methylthio)propionaldehyde, 2,5-dimethyl pyrazine, benzene ethanol, benzaldehyde, 2,3,5,6-tetramethyl pyrazine, benzene acetaldehyde, 2-ethyl phenol, 2-acetyl pyrrole, phenol, 2–4-ethyl acetyl pyrrole, furfural, 4-ethyl guaiacol, and isovaleraldehyde (Lin, Liu, He, Liu, Che, Wang, et al., 2019).

The compounds representing both differential metabolites and key aroma substances were marked on the heat map after the compound name (dotted in blue) and included 3-Methyl-1-butanol, phenethyl alcohol, furfuryl alcohol, 3-Methylthiopropanol (alcohols), benzaldehyde, phenylacetaldehyde, isovaleraldehyde, furfural, 3-(methylthio) propionaldehyde, 1-Nonanal (aldehydes), isovaleric acid (acids), 2,5-Dimethyl pyrazine (heterocyclics), styrene (aromatics), and 4-Ethyl-2-methoxyphenol (phenols). As shown in the heat map, 3-methyl-1-butanol displayed the highest content in group E, which decreased significantly at the end of fermentation (group F). The variation trend of 2,5-dimethylpyrazine was consistent with 3-methyl-1-butanol, which were enriched during the late stage of fermentation and exhibited a more substantial decrease at the end of fermentation (group F). Furthermore, 1-nonanal, 4-Ethyl-2-methoxyphenol, furfuryl alcohol, phenylethanol, 3-(methylthio)propionaldehyde, furfural, benzaldehyde,
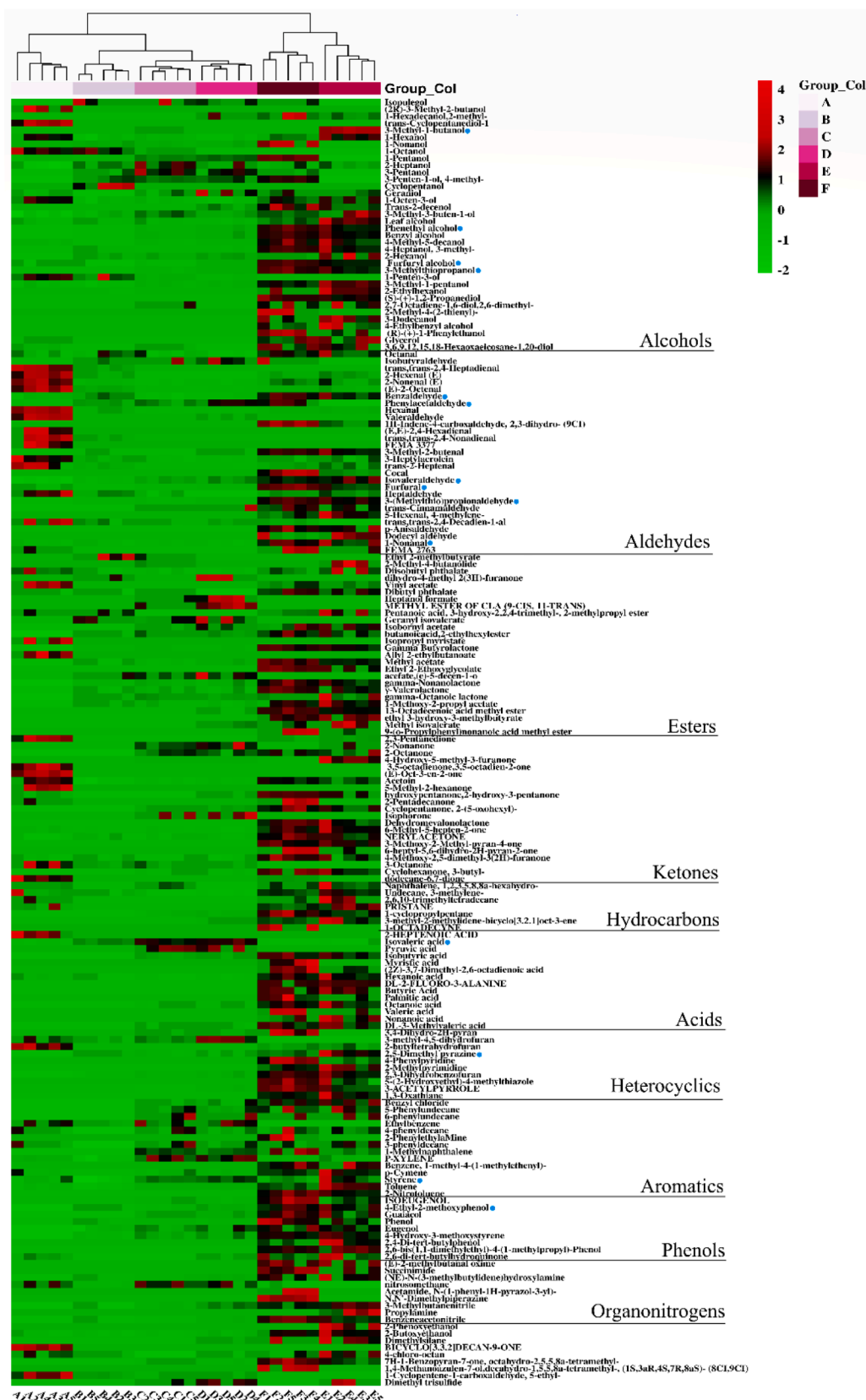
Fig. 5. The heat map of the differential metabolite peak areas. A, B, C, D, E, and F represent the samples collected at 0 d, 6 d, 13 d, 20 d, 28 d, and 39 d of fermentation, respectively, while the numbers 1, 2, 3, 4, and 5 represent parallel experiments. The blue dots indicate the key flavor substances identified in previous studies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and isovaleraldehyde, were all present at an exceedingly low level during pre-fermentation but displayed considerable accumulation during the post-fermentation stage. Phenylacetaldehyde was more abundant during the mid-fermentation stage, showing an initial decline, followed by a significant increase during the late fermentation stage. Isovaleric acid was enriched during the mid-fermentation stage but was inconsequential during the early and late stages. The 3-Methylthiopropanol was almost exclusively present in group E, while styrene appeared almost throughout the fermentation process, but it was more abundant during the post-fermentation period. The changes in the content of various compounds could be ascribed to the complex biochemical reactions that occurred during the constant temperature fermentation. A complex system was present, consisting of various bacterial and fungal communities that underwent active metabolic activities during fermentation, providing the broad beans with a unique flavor. Since fermented foods are closely associated with microorganisms, the differential metabolites are subjected to function and pathway enrichment analysis to determine the possible metabolic pathways.

3.5. Analysis of the metabolic pathways

By KEGG database analysis, the differential metabolites were annotated to 91 pathways, and the top ranked ones were metabolic pathways (21), microbial metabolism in diverse environments (13), degradation of aromatic compounds (10), biosynthesis of secondary metabolites (7). The regulation of metabolic responses in living organisms is often complex, mediated by distinct genes and functional protein networks. Their mutual influence and regulation ultimately lead to systemic changes in the metabolome (Topfer, Kleessen, & Nikoloski, 2015).

The broad bean paste fermentation occurred in a semi-solid high-
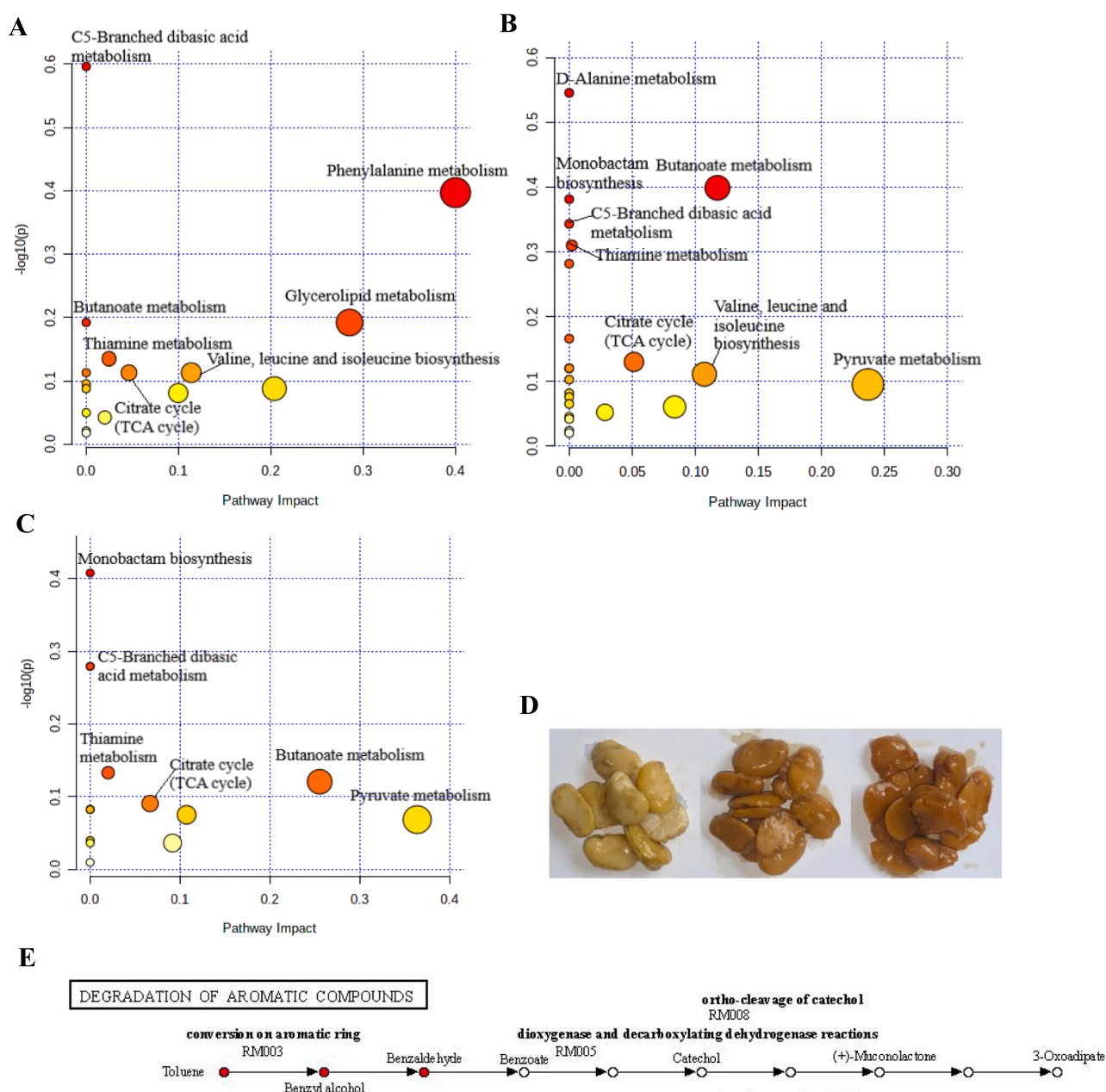
Fig. 6. KEGG pathway-based differential metabolite analysis. (A) The KEGG pathway annotated in the yeast database. (B) The KEGG pathway annotated in the *Bacillus* database. (C) The KEGG pathway annotated in the *Staphylococcus* database. The importance of pathways was visualized by the size and color shade of the bubbles. (D) A comparison between the color of the fermented broad beans. Images of the pre-, mid-, and post-fermentation stages are shown from left to right. (E) The degradation of toluene.

temperature environment. Broad beans are rich in protein, starch, fat, cellulose, and other macromolecular substances. Since the fermentation involved a considerable number of microbial activities that included bacteria and fungi, as well as various metabolic pathways, individual examination was tough. According to previous studies, the relative abundance of yeasts, such as *Candida, Zygosaccharomyces*, and *Pichia,* increased during broad bean fermentation. Furthermore, the yeasts contained abundant hydrolytic enzyme systems, including protease, amylase, cellulase, and saccharolytic enzymes, which were considered important functional bacterial groups during fermentation (Lin, Bi, Zhou, Fang, Liu, Ding, et al. 2021). Bacillus and *Staphylococcus* represented the predominant bacteria in the broad bean paste (Jia et al., 2020), while Aspergillus, Bacillus, and Lactobacillus were closely correlated with unique flavor metabolites (Yang et al., 2021). Additional studies showed that Bacillus was the dominant bacteria during broad bean fermentation (Lu, Tan, Lv, Yang, Chi, & He, 2020).

Combining the above analysis, all the differential metabolic pathways based on the differential metabolites were searched via comparison with *Staphylococcus, Bacillus,* and *Saccharomyces*. The pathway results were obtained, as shown in Figs. 6A, B and C.

C5-Branched dibasic acid metabolism represents one of the organic acid production pathways. As the fermentation time increased, higher total acid levels were evident in the fermentation system. The total acid contains free acids, bound acid, and organic acid produced via the metabolism of the food itself or bacteria and fungi. Therefore, the system must contain an active organic acid metabolic reaction.

The broad beans darkened in color as fermentation continued, as shown in Fig. 6D. In addition, an important metabolic pathway, namely phenylalanine metabolism (phenylacetaldehyde and phenethylamine), was observed, which could be responsible for the production of aromatic compounds and the deepening of color. Distinct differences were evident between the color of the broad beans during the pre-, mid-, and post-fermentation periods. Combined with the analysis of the previous physicochemical indexes, the gradual increase in the reducing sugar and amino acid nitrogen levels provided a sufficient material basis for browning. Fermented condiments, such as douchi, soy sauce, doenjang, and miso, undergo browning during the fermentation process. The forms of browning are usually divided into non-enzymatic browning, like the Maillard reaction, and enzymatic browning. Many cereal proteins contain significant levels of amide-containing amino acids, glutamine, and asparagine. The amide bond readily hydrolyzes these side chains, releasing ammonia during the Maillard browning reaction to form aromatic compounds and brown pigments (Riha, Izzo, Zhang, & Ho, 1996). On the other hand, the proteins in the board beans were decomposed to amino acids by microorganisms. Tyrosine was catalyzed by phenolic hydroxyl groups and polyphenol oxidase in aerobic conditions, undergoing a series of reactions, such as oxidation, cyclization, rearrangement, and polymerization to produce brown and black pigment, increasing the brown color of the final product. In addition, microbial phenylalanine metabolism is promising for producing aromatic substances and has been applied in metabolic engineering. Previous studies have applied metabolic engineering approaches to derive heterologous aromatic compounds from the tyrosine and phenylalanine biosynthesis pathway in *Saccharomyces cerevisiae*, such as styrene (Gottardi, Reifenrath, Boles, & Tripp, 2017).

Pyruvate metabolism represents another essential metabolic pathway. Pyruvate was enriched during the mid-stage of fermentation and decreased during the post-stage, while butanoic acid was higher during the post-stage. The lactic acid, acetic acid, ethanol, and acetoin produced during fermentation represented crucial flavor compounds, of which acetoin presented a creamy, buttery aroma. Pyruvate was an important intermediate in the formation of these substances.

D-Alanine metabolism is an essential cellular metabolic process. D-amino acids such as D-alanine (D-Ala) and D-glutamate are present in all eubacterial cell walls (Yoshimura & Esaki, 2003). Part of the D-Ala metabolic pathway in bacteria involves the conversion of L-alanine to D-Ala via alanine racemase and the formation of D-alanyl–D-alanine by D-alanine–D-alanine ligase, the product of which was involved in cell wall peptide glycan synthesis (Qiu et al., 2016). For thiamine metabolism, thiamine is a crucial cofactor involved in maintaining carbohydrate metabolism and participates in multiple cellular metabolic processes (Pacei et al., 2020).

Previous studies (Lin, Yu, Fang, Lu, Liu, Xing, et al., 2018) have revealed the presence of many kinds of organic acids in Pixian broad bean paste, representing important flavor substances and providing energy for cellular activities via tricarboxylic acid cycle, glycolytic, and gluconeogenetic pathways. A significant number of organic acids and considerable intermediate pyruvate were synthesized during fermentation. Most of the enriched pathways were affiliated with carbon hydrate and amino acid metabolism, which were consistent with the abundance of starch and protein in the raw materials of the broad bean paste. Moreover, carbohydrate and amino acid metabolism represented the main metabolic category of tank fermentation (Ding et al., 2022).

In addition, ten metabolites were annotated in the degradation of aromatic compounds according to KEGG. A possible pathway was observed for the production of benzaldehyde as a key aroma substance (Fig. 6E) by consulting the relevant information about the autoxidation mechanism of toluene (Hermans, Peeters, Vereecken, & Jacobs, 2007). These three substances were enriched during post-fermentation, confirming a close relationship. During the oxidation reaction, toluene was converted to the benzyl alcohol, benzaldehyde, and then oxidized to benzoic acid or benzoate. Toluene mainly participates in radical substitution, electrophilic substitution, and radical addition reactions. In light and irradiation conditions, it can undergo radical substitution reactions with certain reactants on methyl groups.

4. Conclusion

This study investigated the general quality of tank-fermented broad beans by monitoring the basic physicochemical indicators. HS-SPME-GC × GC–MS was applied to detect the volatile metabolites during different fermentation periods. In total, more than 700 compounds were identified during the fermentation process, while 184 volatile differential metabolites were screened by combining multivariate statistical analysis. The KEGG database was used to annotate these differential metabolites to several metabolic pathways related to carbohydrates and amino acids, indicating that the metabolism of raw materials by microorganisms was closely related to the production of volatiles. Furthermore, the continuous transformation and accumulation of volatile substances revealed the flavor and color differences of fermented broad beans at different fermentation stages. The real concentration of flavor contribution in tank-fermented broad bean paste needs to be studied in the future and It's necessary to further investigate the key flavor substances and strains in combination with multi-omics to improve the effect of tank fermentation and provide a theoretical basis for the industrial production of Pixian broad bean paste.

CRediT authorship contribution statement

Shiqi Liao: Investigation, Methodology, Formal analysis, Software, Writing – original draft. **Jinlin Han:** Formal analysis, Data curation. **Chunyan Jiang:** Investigation, Data curation. **Binbin Zhou:** Investigation, Data curation. **Zhenju Jiang:** Methodology. **Jie Tang:** Methodology. **Wenwu Ding:** Methodology, Conceptualization. **Zhenming Che:** Conceptualization, Resources. **Hongbin Lin:** Supervision, Writing – review & editing, Project administration, Resources.

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.

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100556.

References

Cordente, A. G., Schmidt, S., Beltran, G., Torija, M. J., & Curtin, C. D. (2019). Harnessing yeast metabolism of aromatic amino acids for fermented beverage bioflavouring and bioproduction. *Applied Microbiology and Biotechnology, 103*(11), 4325–4336. https://doi.org/10.1007/s00253-019-09840-w

Cordero, C., Kiefl, J., Reichenbach, S. E., & Bicchi, C. (2019). Characterization of odorant patterns by comprehensive two-dimensional gas chromatography: A challenge in omic studies. *TrAC-Trends in Analytical Chemistry, 113*, 364–378. https://doi.org/10.1016/j.trac.2018.06.005

Ding, Zhao, X. Y., Xie, S., Liu, Y., Zhang, M. N., Che, Z. M., & Lin, H. B. (2021). Dynamics and correlation of microbial community and flavor in Pixian Douban fermented with closed process of constant temperature. *Journal of the Science of Food and Agriculture, 101*(10), 4142–4153. https://doi.org/10.1002/jsfa.11050

Ding, W., Ye, X., Zhao, X., Liu, Y., Zhang, M., Luo, Y., … Tang, X. (2022). Fermentation characteristics of Pixian broad bean paste in closed system of gradient steady-state temperature field. *Food Chemistry, 374*, Article 131560. https://doi.org/10.1016/j.foodchem.2021.131560

Ding, W. W., Liu, Y., Peng, C. B., Ye, X. Q., Zhang, M. N., Che, Z. M., … Xu, M. (2021). Characterization of volatile compounds between industrial closed fermentation and traditional open fermentation doubanjiang-meju. *European Food Research and Technology, 247*(8), 2067–2077. https://doi.org/10.1007/s00217-021-03772-4

Du, Y., Yang, W., Yang, C., & Yang, X. (2022). A comprehensive review on microbiome, aromas and flavors, chemical composition, nutrition and future prospects of Fuzhuan brick tea. *Trends in Food Science & Technology, 119*, 452–466. https://doi.org/10.1016/j.tifs.2021.12.024

Giddings, J. C. (1995). Sample dimensionality - A predictor of order-disorder in component peak distribution in multidimensional separation. *Journal of Chromatography A, 703*(1–2), 3–15. https://doi.org/10.1016/0021-9673(95)00249-m

Gottardi, M., Reifenrath, M., Boles, E., & Tripp, J. (2017). Pathway engineering for the production of heterologous aromatic chemicals and their derivatives in Saccharomyces cerevisiae: Bioconversion from glucose. *FEMS Yeast Research, 17*(4). https://doi.org/10.1093/femsyr/fox035

Gruber, B., Weggler, B. A., Jaramillo, R., Murrell, K. A., Piotrowski, P. K., & Dorman, F. L. (2018). Comprehensive two-dimensional gas chromatography in forensic science: A critical review of recent trends. *TrAC-Trends in Analytical Chemistry, 105*, 292–301. https://doi.org/10.1016/j.trac.2018.05.017

Hermans, I., Peeters, J., Vereecken, L., & Jacobs, P. A. (2007). Mechanism of thermal toluene autoxidation. *ChemPhysChem, 8*(18), 2678–2688. https://doi.org/10.1002/cphc.200700563

Hoang, N. X., Ferng, S., Ting, C.-H., Lu, Y.-C., Yeh, Y.-F., Lai, Y.-R., … Hsu, C.-K. (2018). Effect of initial 5 days fermentation under low salt condition on the quality of soy sauce. *LWT, 92*, 234–241. https://doi.org/10.1016/j.lwt.2018.02.043

Ito, K., & Matsuyama, A. (2021). Koji molds for Japanese soy sauce brewing: Characteristics and key enzymes. *Journal of Fungi, 7*(8), 658. https://www.mdpi.com/2309-608X/7/8/658.

Jia, Y., Niu, C. T., Lu, Z. M., Zhang, X. J., Chai, L. J., Shi, J. S., … Li, Q. (2020). A bottom-up approach to develop a synthetic microbial community model: Application for efficient reduced-salt broad bean paste fermentation. *Applied and Environmental Microbiology, 86*(12). https://doi.org/10.1128/aem.00306-20

Keppler, E. A. H., Jenkins, C. L., Davis, T. J., & Bean, H. D. (2018). Advances in the application of comprehensive two-dimensional gas chromatography in metabolomics. *TrAC-Trends in Analytical Chemistry, 109*, 275–286. https://doi.org/10.1016/j.trac.2018.10.015

Khatri, D., & Chhetri, S. B. B. (2020). Reducing sugar, total phenolic content, and antioxidant potential of nepalese plants. *Biomed Research International, 2020*. https://doi.org/10.1155/2020/7296859

Layton, D. S., & Trinh, C. T. (2016). Expanding the modular ester fermentative pathways for combinatorial biosynthesis of esters from volatile organic acids. *Biotechnology and Bioengineering, 113*(8), 1764–1776. https://doi.org/10.1002/bit.25947

Lee, S. M., Seo, B. C., & Kim, Y. S. (2006). Volatile compounds in fermented and acid-hydrolyzed soy sauces. *Journal of Food Science, 71*(3), C146-C156. https://doi.org/10.1111/j.1365-2621.2006.tb15610.x.

Lin, H., Yu, X., Fang, J., Lu, Y., Liu, P., Xing, Y., … He, Q. (2018). Flavor compounds in pixian broad-bean paste: Non-volatile organic acids and amino acids. *Molecules, 23*(6). https://doi.org/10.3390/molecules23061299

Lin, H. B., Bi, X. P., Zhou, B. B., Fang, J. X., Liu, P., Ding, W. W., … He, Q. (2021). Microbial communities succession and flavor substances changes during Pixian broad-bean paste fermentation. *Food Bioscience, 42*. https://doi.org/10.1016/j.fbio.2021.101053

Lin, H. B., Liu, Y., He, Q., Liu, P., Che, Z. M., Wang, X. M., & Huang, J. Q. (2019). Characterization of odor components of Pixian Douban (broad bean paste) by aroma extract dilute analysis and odor activity values. *International Journal of Food Properties, 22*(1), 1223–1234. https://doi.org/10.1080/10942912.2019.1636816

Liu, J. J., Li, J. H., Shin, H. D., Liu, L., Du, G. C., & Chen, J. (2017). Protein and metabolic engineering for the production of organic acids. *Bioresource Technology, 239*, 412–421. https://doi.org/10.1016/j.biortech.2017.04.052

Liu, Y., Zhang, Y. M., Shi, Y. J., Zhang, M. N., Liu, Y., Che, Z. M., … Ding, W. W. (2022). Flavor quality evaluation of Pixian Douban fermented in the closed system of multi-scale temperature and flow fields. *LWT-Food Science and Technology, 163*. https://doi.org/10.1016/j.lwt.2022.113598

Lu, Y., Tan, X., Lv, Y., Yang, G., Chi, Y., & He, Q. (2020). Physicochemical properties and microbial community dynamics during Chinese horse bean-chili-paste fermentation, revealed by culture-dependent and culture-independent approaches. *Food Microbiology, 85*. https://doi.org/10.1016/j.fm.2019.103309

Pacei, F., Tesone, A., Laudi, N., Laudi, E., Cretti, A., Pnini, S., … Colombo, C. (2020). The relevance of thiamine evaluation in a practical setting. *Nutrients, 12*(9). https://doi.org/10.3390/nu12092810

Park, M. K., Seo, J.-A., & Kim, Y.-S. (2019). Comparative study on metabolic changes of Aspergillus oryzae isolated from fermented foods according to culture conditions. *International Journal of Food Microbiology, 307*, Article 108270. https://doi.org/10.1016/j.ijfoodmicro.2019.108270

Qiu, W., Zheng, X., Wei, Y., Zhou, X., Zhang, K., Wang, S., … Li, M. (2016). d-Alanine metabolism is essential for growth and biofilm formation of Streptococcus mutans. *Molecular Oral Microbiology, 31*(5), 435–444. https://doi.org/10.1111/omi.12146

Riha, W. E., 3rd, Izzo, H. V., Zhang, J., & Ho, C. T. (1996). Nonenzymatic deamidation of food proteins. *Critical Reviews in Food Science and Nutrition, 36*(3), 225–255. https://doi.org/10.1080/10408399609527724

Stefanuto, P. H., Smolinska, A., & Focant, J. F. (2021). Advanced chemometric and data handling tools for GCxGC-TOF-MS Application of chemometrics and related advanced data handling in chemical separations. *TrAC-Trends in Analytical Chemistry, 139*. https://doi.org/10.1016/j.trac.2021.116251

Szymanska, E., Gerretzen, J., Engel, J., Geurts, B., Blanchet, L., & Buydens, L. M. C. (2015). Chemometrics and qualitative analysis have a vibrant relationship. *TrAC-Trends in Analytical Chemistry, 69*, 34–51. https://doi.org/10.1016/j.trac.2015.02.015

Topfer, N., Kleessen, S., & Nikoloski, Z. (2015). Integration of rnetabolomics data into metabolic networks. *Frontiers in Plant Science, 6*. https://doi.org/10.3389/fpls.2015.00049

Utpott, M., Rodrigues, E., Rios, A. D., Mercali, G. D., & Flo, S. H. (2022). Metabolomics: An analytical technique for food processing evaluation. *Food Chemistry, 366*. https://doi.org/10.1016/j.foodchem.2021.130685

Wang, Y., Zhang, C., Liu, F., Jin, Z., & Xia, X. (2022). Ecological succession and functional characteristics of lactic acid bacteria in traditional fermented foods. *Critical Reviews in Food Science and Nutrition, 1–15*. https://doi.org/10.1080/10408398.2021.2025035

Xia, X. J., Li, G. N., Zheng, J., Ran, C. X., & Kan, J. Q. (2014). Biochemical, textural and microstructural changes in whole-soya bean cotyledon sufu during fermentation. *International Journal of Food Science and Technology, 49*(8), 1834–1841. https://doi.org/10.1111/ijfs.12492

Yang, M. L., Huang, J., Zhou, R. Q., Qi, Q., Peng, C., Zhang, L., … Wu, C. D. (2021). Characterizing the microbial community of Pixian Doubanjiang and analysing the metabolic pathway of major flavour metabolites. *LWT-Food Science and Technology, 143*. https://doi.org/10.1016/j.lwt.2021.111170

Yoshimura, T., & Esaki, N. (2003). Amino acid racemases: Functions and mechanisms. *Journal of Bioscience and Bioengineering, 96*(2), 103–109. https://doi.org/10.1016/s1389-1723(03)90111-3

Zhao, Z., Mu, T. H., & Sun, H. N. (2019). Microbial characterization of five Chinese traditional sourdoughs by high-throughput sequencing and their impact on the quality of potato steamed bread. *Food Chemistry, 274*, 710–717. https://doi.org/10.1016/j.foodchem.2018.08.143