Comparison of volatile and non-volatile metabolites in sufu produced with bacillus licheniformis by rapid fermentation

Jingjing Liu, Jingyu Chen, shuangshi Li, weina Tian, Haigang Wu, and Beizhong Han

Department of Food Technology, School of Bioengineering, Beijing Polytechnic, Beijing, China; Beijing Laboratory for Food Quality and Safety, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China; School of Life Sciences, Henan University, Henan, China

ABSTRACT
Sufu is a pleasant-tasting, traditional Chinese fermented soybean food that is rich in nutrients. In this study, the changes of volatile and nonvolatile metabolites in sufu fermented by bacillus licheniformis, were investigated. The results indicated that a total of 55 kinds of nonvolatile compounds were detected, including 2 carbohydrates, 4 alcohols, 17 amino acids, 18 organic acids, 6 biogenic amines, and 8 other substances. Furthermore, a total of 58 volatile compounds identified were composed of 11 esters, 16 alcohols, 10 acids, and 21 miscellaneous compounds. Inoculation of bacillus licheniformis enriched the metabolite profile of sufu and improved its functionality and safety of edibility. It was observed that the pure fermented starter resulted in controlled acceleration of sufu maturation.

Introduction
Sufu or furu is a fermented soybean curd originating in China, which has enriched dishes of Chinese people over thousands of years. Since it possesses a characteristic flavor and has a relatively high-protein content, sufu is used extensively as seasonings in Asian diets. According to the starter culture used, sufu can be classified into bacteria-fermented sufu and mold-fermented sufu. Traditionally, the production of sufu is naturally incubated with various airborne microorganisms, which leads to the control of product quality rather limited and the yield could be affected by various factors such as environment and season. In addition, the long period of fermentation also limited the development of sufu industries.

The maturation of sufu adds to the cost of production, any reduction in maturation time would mean significant savings for the sufu industry, so shortening the ripening time without losing any important characteristics would be very advantageous. Different methods have been employed to shorten sufu maturation times, including the use of adjunct cultures and the addition of exogenous enzyme. However, no pure starter has been reported to accelerate ripening of sufu. In recent years, pure starter represents an effective means to strengthen traditional fermented food. The use of starter culture has been reported to improve the quality of many fermented foods as well as shorten the fermentation process.

Considering these aspects, a proper starter culture bacillus licheniformis from daqu, a traditional fermentation starter in China, was selected in this research. Bacillus species are good secretors of proteins and metabolites. Most species of bacillus have a high capacity to secrete a variety of extracellular enzymes such as amylase, arabinase, cellulase, lipase, and xylanase, and these enzymes play important roles in many fermented food. In addition, the use of bacillus licheniformis starter cultures was reported to improve the aroma characteristics in...
fermented food and condiments, as they produce numerous volatile compounds mainly including pyrazines, aldehydes, ketones and alcohols.\(^8\)

Moreover, knowledge on dynamic changes of metabolites in bacteria-fermented during fermentation is also insufficient. Therefore, the content change of free amino acids, biogenic amines, organic acids, and esters, etc., in whole fermentation process of sufu were tracked, and water content, pH, titratable acidity, and free amino-type nitrogen during the process were measured. The objective of this study was to explore a proper starter culture for safe and rapid sufu fermentation production.

### Materials and methods

#### Materials and strains

Tofu was purchased from Wu Mart (Beijing, China). *Bacillus licheniformis* (B.L-1), which have a high capacity to secrete proteases, was isolated from Chinese fermented starter-*Daqu* collected from Lvliang, Shanxi, China. The culture media (Nutrient broth, NB) were from Beijing Aoboxing Biotech Co., Ltd (Beijing, China), and other chemicals used were reagent grade. B.L-1 was grown in NB agar media at 37 °C for 1 d, then scraped into a sterile bottle homogenized with 200 ml saline solution in a lab-blender. The diluted suspension was ready for production of sufu as a pure starter culture.

#### Preparation of sufu

In this study, sufu samples were prepared essentially according to the procedure described by Tan\(^{[9,10]}\) with slight modifications. A schematic diagram of the sufu production process is shown in figure 1. First, tofu was put in bamboo drawer to have conventional thermal sterilization (115 °C for 15 min) and cooled to room temperature. Then, it was cut into pieces to 3.0 × 3.0 × 1.0 cm for pre-fermentation. About 1 mL diluted suspension containing B.L-1 prepared in Section 2.1 was sprayed.

![Figure 1](image-url) **Figure 1.** Biplot of the PCA based on \(^1\)H-NMR spectral data obtained from the sufu samples during the entire fermentation period. BP: tofu; JP: pehze; SP: salted pehze; HJ-5 d, 10 d, 20 d, 30 d, 45 d, 60 d: ripening time of sufu samples. (A): Amino acids; (B): Biogenic amine; (C): Organic acids; (D): Sugars, alcohols and secondary metabolites.
on the surface of every tofu piece. Samples with B.L-1 were arranged neatly in bamboo drawers and incubated for 48 h at 37 °C. After tofu became pehtze, 12 pehtzes were placed into a 250 mL wide-mouthed glass bottle. About 200 mL of rice wine (Ethanol, 10%, v/v; NaCl, 8%, w/v) was added from the top. Finally, the bottles were sealed with iron screw caps and incubated at 28 °C for 60 days. An aliquot of sample from tofu, pehtze and samples during ripening (5 d, 10 d, 20 d, 30 d, 45 d, 60 d), was taken out for further analysis. All of the samples were stored at −20 °C, and the sample analyses were completed within 2 weeks.

**Physicochemical analysis of sufu**

About 5 g of the samples was homogenized in a stomacher with 45 mL of distilled water, after which the pH of the supernatant of each homogenate was measured using a PB-21 pH meter (Thermo Scientific, USA). Water content was determined using the standard gravimetric method described by AOAC, Association of Official Analytical Chemists.[11] Total acidity was measured by titration of a product homogenate with 0.05 mol L⁻¹ NaOH according to AOAC procedures and was expressed in grams of lactic acid per 100 g of sufu. Primary amino nitrogen was quantified based on the formalin titration method.

**Sensory analysis**

This analysis of sufu during the fermentation process (30 d, 45 d, and 60 d) and three commercial sufu products were performed according to Shen[12] by a panel of 10 trained people. The panel members were asked about the color, aroma, taste and texture. A scale from 1 to 10 was employed for each feature, where 10 was the best and 1 was the worst. For each quality feature, scores given by the 10 panelists were totaled, and the mean was calculated. The overall sensory quality was obtained as the sum of the means of all features.

**Analysis of nonvolatile compounds using ¹H-NMR spectroscopy during sufu fermentation**

The chemical compounds produced during sufu fermentation, including carbohydrates, organic acids, amino acids, biogenic amines, and nitrogen compounds, were analyzed by using ¹H-NMR spectroscopy according to the method described by Lee et al.,[13] with minor modification. Samples of 1 g of sufu were homogenized with 1.5 mL of ultrapure dH₂O in a mini-beadbeater for 60 s and then kept in ice for 10 min. The mixtures were centrifuged at 13,400 × g at 4 °C for 10 min. For every 1 mL of supernatant, 1 mL of 0.2 mM phosphate buffer pH 7.0 containing 20% (w/v) of deuterium oxide (D₂O, 99.9%), 1 mM 3-trimethylsilyl-2,2,3,3-d₄-propionate (TSP, 98%), 10 mM imidazole, and 0.2% (w/v) azide sodium were added and mixed thoroughly. Once again, the mixtures were centrifuged at 13,400 × g at 4 °C for 10 min and then, the supernatants were transferred into NMR tubes. ¹H-NMR spectra were obtained using an Avance 600-MHz NMR spectrometer (Bruker, Germany) operating at a proton NMR frequency of 600.13 MHz and a temperature of 300 K, using a 5 mm PATXI probe. For each sample, 128 scans were recorded, with the following parameters: pulse sequence, NOESYGPR1D (RD–90°–t1–90°–tm–90°-acquisition); relaxation delay, 2.00 s; mixing time (for NOESY), 1.00 s; acquisition time, 2.28 s; number of steady-state transients (dummy scans), 4; gradient pulse times, 1 ms; solvent suppression, presaturation with spoil gradient; spectral width, 7184 Hz; time domain size, 32 k.[14] Metabolites were identified and quantified with Chenomx software (version 7.0; Chenomx Inc., Canada) with the reference of internal standard TSP. Each experiment was repeated three times.

**Profiling of volatiles by HS-SPME-GC-MS during sufu fermentation**

Volatile compounds in sufu samples were analyzed by HS-SPME-GC-MS, as described by Sulejmani et al., with a minor modification.[15] Briefly, sufu samples (5 g) mixed with 10 µL 4-methyl-2-pentanol
(125 mg/L) as an internal standard. The samples were allowed to equilibrate at 45 °C for 30 min before analysis. The volatile compounds were extracted with an SPME fiber (50:30 mm divinylbenzene-carboxen-polydimethylsiloxane, Supelco Co., Bellefonte, PA, USA) at 40 °C for 30 min. Oven temperature gradient started with 40 °C (2 min), increased at 5 °C/min to 70 °C, then at 10 °C/min to 240 °C. Settings included the temperature of the injector at 250 °C, split of 1:5, solvent delay of 10 min, and run time 45 min. The compounds were identified by comparison with the mass spectral data of the NIST 14 mass spectral database.

**Statistical analysis**

The composition of compounds in all sufu samples was analyzed by PCA using the software package SIMCA-P 12.0 (Umetrics, Umea, Sweden) to cluster the samples into different groups. Samples were plotted in two dimensions based on scores for the first two principal components to evaluate relationships among samples. The proportion of variance explained by each principal component was calculated. Significant differences (p < .05) of sensory evaluation score on bacteria-fermented sufu were tested by one-way ANOVA using SPSS software (SPSS v19.0, Inc.).

**Results and discussion**

**Physicochemical and sensory analysis of bacteria-fermented sufu**

Tables 1 and 2 show the average values for the physicochemical parameters and sensory quality, respectively, of bacteria-fermented sufu. Moisture of sufu dropped to 60.56% (w/w) compared to BP and then increased to 67.92% (w/w) during the following stages. The initial pH of sufu at BP stage was 6.56, which significantly increased to 7.65 at pre-fermentation stage (pehtze). From the 5 d in post-fermentation until the end, the pH value showed a gradual decrease (from 6.94 to 6.20), accompanied by an increase in acidity. Amino nitrogen was not detected before pre-fermentation (pehtze). It peaked at pehtze (0.42 g per 100 g sufu) and decreased significantly to 0.35 g per 100 g sufu. Thereafter, the amino nitrogen continued to rise from 0.35 to 0.46 g per 100 g sufu during the ripening fermentation stage.

**Table 1. Physiochemical properties during the production of sufu.**

|                      | BP    | JP    | 5 d   | 10 d  | 20 d  | 30 d  | 45 d  | 60 d  |
|----------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Moisture (%, w/w)    | 74.87 | 60.56 | 67.92 | 65.65 | 68.75 | 65.19 | 65.37 | 65.71 |
| pH                   | 6.56  | 7.65  | 6.94  | 6.82  | 6.34  | 6.15  | 6.13  | 6.20  |
| Acidity (g/100 g)    | 0.05  | 0.18  | 0.32  | 0.47  | 0.48  | 0.48  | 0.46  | 0.47  |
| Amino nitrogen (g/100 g) | –   | 0.42  | 0.35  | 0.40  | 0.42  | 0.44  | 0.46  | 0.46  |

Values represent means ± SD (n = 3).

*BP: tofu; JP: pehtze; SP: salted pehtze; 5 d, 10 d, 20 d, 30 d, 45 d, 60 d: ripening time of sufu samples. “–” means not detected.

**Table 2. Sensory evaluation score of bacteria-fermented sufu.**

|            | 30 d | 45 d | 60 d | C1  | C2  | C3  |
|------------|------|------|------|-----|-----|-----|
| Aroma      | 7.5  | 8.3  | 9.2  | 9.3 | 9.2 | 9.4 |
| Color      | 8.5  | 8.6  | 9.0  | 8.9 | 9.1 | 9.0 |
| Taste      | 7.3  | 8.6  | 9.3  | 9.0 | 8.6 | 8.9 |
| Texture    | 6.5  | 7.6  | 8.5  | 8.0 | 8.3 | 9.0 |
| Score      | 29.8 | 33.1 | 36   | 35.2| 35.2| 36.3|

*30 d, 45 d, 60 d: ripening time of sufu samples; C1, C2 and C3: three brands of commercial bacteria-fermented sufu samples.
The quality of bacteria-fermented sufu is usually determined during the fermentation process, and after its completion. In Table 2, the overall sensory quality scores are not significantly different at a $p$-value $> 0.05$, which is compared with three commercial bacteria-fermented sufu samples. According to the members of the evaluation panel, the quality of sufu at 30 d and 45 d fermented was generally worse than commercial samples. Sufu obtained at 30 d was characterized by too intense odor and hard texture; this result was the main reason for the lower ratings. The quality of sufu at 60 d was superior to commercial samples. The results indicated that the fermentation period affected the sensory features of bacteria-fermented sufu.

**Changes of nonvolatile compounds during the sufu fermentation**

PCA was applied to the variables obtained from $^1$H NMR data to reveal the overall differences of nonvolatile compound profiles during fermentation. Figure 1 A, B, C and D show the 2-dimensional biplots of PCA in amino acids; biogenic amine; organic acids; sugars, alcohols and secondary metabolites, respectively. The amino acids that most significantly characterized the different clusters are glycine, glutamate, valine and leucine. The predominant biogenic amines are cadaverine and serotonin. Among the organic acids, acetate, ascorbate and lactate are responsible for the separation of the clusters. Glucose and mannitol also contribute to grouping.

To better overview the changes of nonvolatile compounds during the sufu fermentation process, a heat map of sufu samples versus metabolites was plotted (figure 2). A total of 55 kinds of nonvolatile compounds were detected in sufu samples, including 2 carbohydrates, 4 alcohols, 17 amino acids, 18 organic acids, 6 biogenic amines, and 8 other substances, which was superior to other reported sufu.[4,16]

Free amino acids (FAAs), released either by the starter culture or through endogenous proteases, are considered to be important in creating the pleasant and palatable taste of fermented foods. They can contribute directly to the taste perception; meanwhile, indirectly devote to the development of their typical aroma since they are precursors of numerous volatile compounds. Generally, the contents of total free amino acids increased at the end of the post-ripening period and reached to the highest amounts after 60 days of maturation (figure 2). Meanwhile, the level of some free amino acids (Gly, Leu, Ile, Lys, and Tyr) in pehtze (JP) was significantly higher than in sufu samples after 60 days of ripening. Apparently, the pure starter is a good source of some essential and flavored amino acids. According to the result, Gly, Glu, Leu, Thr, Val, Lys, and Asp were the major free amino acids, accounting for more than 77% of the total free amino acids at 60 days of ripening. The content of glycine was the highest, which has a pleasant sweet taste, and is widely presented in large quantity in seafoods, such as snow crab, clam and scallop.[17] Glu and its salts are the principal cause of the delicious taste of various fermented soybean foods, such as miso, koji and soy sauce. Leu is bitter, which might be an important precursor of branched-chain volatile flavor. In this study, *bacillus licheniformis* as a pure stater is conducive for speeding up sufu maturation, based on the distribution patterns of the free amino acids and the content of amino nitrogen.

Safety issues have risen in traditional fermented soybean foods in the past, especially those related to the presence of biogenic amines (BAs). It is well known that the excessive consumption of biogenic amines can induce the adverse effects such as nausea, respiratory distress, hot flushes, heart palpitations, headache, and hyper- or hypotension. In this study, the BAs in all samples were shown in figure 2. A sharp change in the types and contents of BAs was observed in pehtze (JP) with starter incubation. During pre-fermentation, BAs (serotonin, glutamine, putrescine, N-Acetylglutamine, histamine, trimethylamine N-oxide, and cadaverine) were detected in pehtze (JP) samples. Cadaverine was the predominant biogenic amine in pehtze (JP) and its precursor is Lysine.[18] Putrescine is an indicator of the degree of freshness or spoilage of food products including fermented soybean. Cadaverine and Putrescine are regarded as two typical BA reported in soybean products including sufu, soy sauce, tempe, miso, and natto.[19] In addition, the European Food Safety Authority (EFSA) considers histamine and tyramine as the most important Bas from a toxicological point of view, since their
consumption in high concentrations can cause hypertension, headaches, palpitations and vomiting in certain individuals. While in our study, these amines decreased or disappeared over time. In the final sufu products, only a small amount of serotonin, cadaverine, N-Acetylglutamine, and trimethylamine N-oxide were detected. Compared with traditional fermented sufu products, [20] contents of biogenic amines in our sufu samples were lower. This result showed that pure starter culture fermentation with bacillus licheniformis was effective in reducing biogenic amine content for sufu products. Similar phenomena had appeared in cheonggukjang (Korean fermented unsalted soybean paste), which bacillus licheniformis showed a high inhibitory ability against bacillus cereus growth and possess a potential for reduction of biogenic amines in cheonggukjang. [21]

The flavor and taste of fermented foods are believed to be produced mainly by organic acids together with free amino acids and carbonyl compounds. Additionally, antimicrobial activity of organic acids may also improve the keeping quality of a food product. In the present study, figure 2 shows changes in the composition of organic acids in sufu during ripening. It was noted that, only small amounts of ascorbate, acetate and guanido acetate were detectable in the non-fermented tofu
samples. However, most organic acids, including ascorbate, lactate, acetate, taurine, 2-hydroxyisobutyrate, methylmalonate, ethylmalonate, succinate, methylmalonate, creatine phosphate, 4-aminobutyrate, propionate, glycolate, creatine, valerate, butyrate, 2-oxoglutarate and guanido acetate, were detected in the sufu samples during pre/post-fermentation. Apparently, they were formed through the catalytic action of enzymes produced by bacillus licheniformis. Interestingly, acetic acid was found to be the predominant organic acid in the pehtze (JP) examined. While, further extending the ripening time sharply decreased the contents of acetic acid significantly. The acetic acid in the sufu samples observed in this study is in agreement with that observed in the enzyme-ripened sufu product.\(^{[22]}\) Meanwhile, with the extension of ripening, lactate content in the sufu samples increased, indicating that b. licheniformis may promote lactic acid formation and thereby the sourness of sufu. Furthermore, b. licheniformis, a more recently developed thermophilic host, has been successfully used for the production of lactic acid with the highest glucose fermentation rate.\(^{[23]}\) Unlike what was observed in other bacteria-fermented sufu,\(^{[4]}\) both acetic acid and lactic acid were the most predominant organic acid detected in the sufu product in the present study. This discrepancy may be attributed to the difference in the microorganisms involved and the fermentation process. Based on the obtained data, glucose was identified as the major sugar present in sufu fermentation and the concentration of which increased gradually during fermentation. Apparently, the increased amounts of glucose in sufu came essentially from metabolism of b. licheniformis.

**Analysis of volatile compounds during the sufu fermentation**

To evaluate the correlations among the important volatile compounds and the contribution of the sufu samples, the concentration of volatile compounds as variables was subjected to principal component analysis (PCA). Figure 3 A, B, C and D shows the 2-dimensional biplots of PCA in alcohols, organic acids, esters and heterocyclic compounds, respectively. The distance between two sufu samples measures their dissimilarity in discriminating the volatile compounds. Further, the similarity (covariance) between two samples is determined by both the length of their vectors and the cosine of the angle between them. Based on alcohols (figure 3 A) and organic acids (figure 3B) detected, samples fell into two apparent groups: pehtze (JP) formed one group, and the remaining samples formed another. Figure 3A and 3B indicate that phenylethyl alcohol, ethanol and 2-methyl-hexanoic acid contributed to this discrimination. The PCA of sufu samples based on ester composition also showed two groups (figure 3C). The esters that most significantly characterized the different cluster were 3-methylbutanoic acid ethyl ester and L-valine ethyl ester. Figure 3D shows the heterocyclic compounds that most significantly characterized the different clusters were styrene and 3-ethyl-2,5-dimethyl-pyrazine.

Sufu contains a variety of volatile compounds that are highly valuable to sensory quality. In this study, a total of 58 compounds tentatively identified were composed of 11 esters, 16 alcohols, 10 acids, and 21 miscellaneous compounds. Although individual volatiles exhibited trends with different magnitudes and timings, the total abundance of some classes of volatiles had obvious trends, as shown in heatmap (figure 4). The abundance of total alcohols reached to peaks at 5 days during post-fermentation, and decreased thereafter, falling to 67% of the amount present at 60 days by the end of fermentation. The abundance of organic acids and esters increased rapidly from 5 to 10 days of post-fermentation, and decreased slowly thereafter. Interestingly, the abundance of heterocyclic compounds increased rapidly from the stage of JP to 5 days of post-fermentation, and reached the highest at 10 days and decreased slowly thereafter. These results show that the most volatile compounds accumulate mainly at the early stage of fermentation, while the heterocyclic compounds mainly at the middle stage. Compared with other soybean fermented food,\(^{[24]}\) heterocyclic fermentation stage of the bacteria-fermented sufu comes to a bit earlier.

Among the detected alcohols, phenylethyl alcohol and ethanol are prominent, which also grouped the samples. Phenylethyl alcohol has a pleasant sweet taste and flowery aroma, and is widely presented in soy sauce\(^{[25]}\) and sufu.\(^{[26]}\) Ethanol can polymerize with organic acids that form the main flavor compounds of fermented food. Among 10 acid compounds identified, 2-methyl-
hexanoic acid was a major volatile, comprising approximately 79% of the total quantified acids in the final sufu samples and widely distributed in reported commercial fermentation starters.\[^{27}\] Organic acids are formed due to bacterial growth and metabolism of large molecular mass compounds in fermented products.\[^{28}\] Organic acids, which have been reported, can directly influence the balance and taste of Chinese liquor, as well as the growth and vitality of microorganisms during the fermentation.\[^{29}\]

Esters are major volatiles in many soybean fermented foods. In this study, ethyl acetate, 3-methylbutanoic acid ethyl ester, and L-valine ethyl ester were the major esters. Ethyl acetate has a fruity, sweet aroma that can contribute to a product’s olfactory complexity; thus, enhancing the bouquet of sufu. The major aroma-forming compounds in Fen-liquor are ethyl acetate and ethyl lactate\[^{30}\], the former is generated by \textit{B. licheniformis}, indicating that this species may contribute to the palatability of the final product. While ethyl esters, in which the acid group is a fatty acid with a medium-length chain and the alcohol group is ethanol,\[^{31}\] are responsible for the fruity flavor in foods. Chen and Xu\[^{32}\] reported that ethyl esters, which have a fruity and flower aroma, were the largest group of volatile compounds in Chinese rice wine. We speculate that ethyl esters detected in sufu samples may be from adjunct rice wine.

**Conclusion**

In this study, the maturation time of bacteria-fermented sufu was shortened to 60 days by adding B. L-1. Meanwhile, a variety of volatile compounds and a small amount of biogenic amines were detected in the final fermented sufu. Using the pure fermented starter resulted in controlled acceleration of sufu maturation, and such a starter could be used to accelerate full-scale industrial production of sufu. The results of our study are very encouraging and will lay the foundation for pilot plant tests and full-scale plant tests. To the best of our knowledge, this is the first report of accelerating bacteria-fermented sufu...
ripening by the use of *Bacillus licheniformis*.

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