Food Chemistry: X 22 (2024) 101280

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x

Effects of soybean cultivar and starter strain on biogenic amines formation during *Cheonggukjang* fermentation

BaoZhu Shi, BoKyung Moon *

Department of Food and Nutrition, Chung-Ang University, 72-1, Nae-ri, Daedeok-myeon, Anseong-si, Gyeonggi 17546, Republic of Korea

ARTICLE INFO

Keywords:
Cheonggukjang
Biogenic amine
Fermentation
Soybean cultivar
Strain

ABSTRACT

Cheonggukjang, a traditional Korean fermented seasoning, contains biogenic amines (BAs). This research aimed to explore the effects of soybean cultivar, strain, and temperature on BAs formation during *Cheonggukjang* fermentation. Moisture, pH, total aerobic plate count, and protease activity were measured. Free amino acids (FAAs) and BAs were determined by high-performance liquid chromatography. Total FAA and BA contents tended to increase with fermentation time. Total BAs in the samples initially ranged from 112.65 to 153.05 mg/kg and ultimately increased to 155.72–614.28 mg/kg at 25 °C and 201.95–738.39 mg/kg at 37 °C, respectively. *Bacillus licheniformis*-inoculated samples had the highest total BA content (over 400 mg/kg) after 96 h of fermentation. Final histamine content in cv. 'Somoktae'-made samples generally exceeded that in samples produced by the other two cultivars. These findings indicate that soybean cultivar, strain, and fermentation temperature all play pivotal roles in controlling the contents of BAs in *Cheonggukjang*.

1. Introduction

Cheonggukjang is a traditional Korean fermented seasoning produced by natural fermentation of soybeans (Jeon et al., 2018). In traditional *Cheonggukjang* fermentation, rice straw is used as the source of microorganisms, but recently, rice straw has been replaced with isolated and screened *Bacillus* strains in order to control the product quality (Tamang et al., 2022). Among soybean cultivars, the large, yellow-colored soybeans like cv. 'Daewon' are generally used for making *Cheonggukjang*. However, *Cheonggukjang* made from other soybean varieties has become a mainstay in the market due to the increased consumer demand for food diversity. 'Somoktae,' a colored soybean cultivar, is especially popular to make *Cheonggukjang*, which may be because of its specific flavor, higher quantities of isoflavones compared to other soybean cultivars, and anti-inflammatory effect (Kim et al., 2012).

Biogenic amines (BAs) are biogenic substances with one or more amine groups. The major BAs commonly found in food products include tryptamine, 2-phenylethylamine, putrescine, cadaverine, spermidine, spermine, histamine, and tyramine (Majcherczyk & Surówka, 2019). Of these eight individual BAs, histamine and tyramine are linked to neurological and gastrointestinal diseases, and their effects can be exacerbated by the presence of putrescine and cadaverine (Bulushi et al., 2009). Additionally, putrescine, cadaverine, spermidine, and spermine have the potential to promote the formation of *N*-nitrosamines, a potent class of carcinogens, through reactions with nitrite (Tasić et al., 2012). The primary mechanism for BAs generation in food involves the microbial decarboxylation of specific amino acids by substrate-specific decarboxylases (EFSA, 2011; Gao, Li, et al., 2023). Therefore, the high protein content in soybeans, combined with the fermentation process used in seasoning production, can promote the formation of BAs in *Cheonggukjang*. In our previous study, the total BA content in 10 brands of commercial *Cheonggukjang* ranged from 104.72 to 698.22 mg/kg, and the tyramine content ranged from 18.15 to 312.89 mg/kg (Shi & Moon, 2023). Park et al. (2020) also reported a high tyramine content in Korean commercial *Cheonggukjang*. Foods with high concentrations of tyramine can have adverse effects on human health, especially for those who are taking monoamine oxidase inhibitor drugs. The high BAs content among *Cheonggukjang* samples underscores the importance of investigating the factors affecting BAs formation in the context of the *Cheonggukjang* industry.

Research has shown that starter strains, additives, and temperature can influence BAs formation during *Cheonggukjang* fermentation (Gao, Li, et al., 2023). Jeon et al. (2018) reported that *Cheonggukjang* fermented by *Enterococcus* could produce more BAs than fermentation with *Bacillus* spp. Tyramine contents in *Cheonggukjang* could be reduced by adding nicotinic acid (Kang et al., 2018), and a lower fermentation

* Corresponding author at: Department of Food and Nutrition, Chung-Ang University, Anseoung 17546, Republic of Korea.
E-mail address: bkmoon@cau.ac.kr (B. Moon).

https://doi.org/10.1016/j.fochx.2024.101280
Received 17 January 2024; Received in revised form 7 March 2024; Accepted 9 March 2024
Available online 15 March 2024

temperature (25 °C compared to generally 37 °C) may decrease tyramine formation (Park et al., 2020). Furthermore, Lim (2022) suggested that *B. subtilis* starter could decrease the accumulation of cadaverine, tryptamine, and tyramine. Although researchers have investigated the effect of the cultivar on biological activities (Kim et al., 2012), volatile compound profiles (Cho et al., 2017), and free amino acids (FAAs) profiles (Kim et al., 2012) of *Cheonggukjang*, there has been no research reported regarding the influence of soybean cultivar on BAs formation in *Cheonggukjang*. Moreover, the FAAs in different soybean cultivars may further influence the BAs formation because the diverse FAAs that serve as precursors for BAs have been shown to impact BA formation (Herbert et al., 2005; Smit et al., 2013). In our previous research, we reported that BAs formation by yeast increased when Arg was added to the media (Yoon et al., 2024). Therefore, it is necessary to investigate the change of BAs accumulation in *Cheonggukjang* depending on the soybean cultivar because of possible differences in their FAA composition.

In that regard, this study set out to explore how soybean cultivars, starter strains, and temperature influence BAs formation during *Cheonggukjang* fermentation. To make *Cheonggukjang*, we collected three soybean cultivars and selected four species of *Bacillus* with diverse BAs productivity from commercial *Cheonggukjang* products. Samples were fermented at 25 and 37 °C for 0, 6, 12, 18, 30, 48, 72, and 96 h and tested for the critical indexes of the BAs formation, including total aerobic plate count (TAPC), BA productivity, protein hydrolysis, FAAs, pH, and BA content.

2. Materials and methods

2.1. Chemicals

BA standards (tyramine hydrochloride, 2-phenylethylamine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, tryptamine hydrochloride, spermidine trihydrochloride) and amino acid standards (L-Asp, L-Glu, L-Ser, L-Gly, L-His, L-Thr, L-Arg, γ-aminobutyric acid [GABA], L-Ala, L-Pro, L-Tyr, L-Val, L-Met, L-Iso, L-Leu, L-Phe, L-Trp, and L-Lys) were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dansyl chloride, perchloric acid, sodium hydrogen carbonate, potassium dichromate, and silver nitrate were purchased from Daejung Chemical Co. (Siheung, Korea). Distilled water, acetone, and acetonitrile (HPLC grade) were purchased from Tedia Co. (Fairfield, OH, USA).

2.2. Sample collection

In this study, three soybean cultivars, including 'Daewon' (the most popular soybean cultivar in Korea), small soybean cv. 'Pungsannamul,' and black soybean cv. 'Somoktae,' were selected and purchased in a local market (Anseong, Korea) to make *Cheonggukjang*. A total of 50 commercial *Cheonggukjang* samples were purchased from local markets in the eight largest cities in Korea and used for the selection and isolation of *Bacillus* strains from commercial *Cheonggukjang* products.

2.3. Isolation and selection of Bacillus strains from commercial Cheonggukjang products

Bacillus spp. collection was conducted following the method of Ju et al. (2019) with a slight modification. Specifically, 5 g of each commercial *Cheonggukjang* sample was separately taken in sterile filter stomacher bags (Difco Laboratories, Detroit, MI, USA) containing 50 mL of 0.2% peptone water and homogenized for 1 min using a stomacher (BagMixer 400, Interscience Laboratory, Inc., St. Nom, France). The resultant sample suspension was kept in a 75 °C water bath for 10 min and cooled to room temperature, followed by dilution into different grades for plating in mannitol-egg yolk-polymyxin agar (MYPA; Difco Laboratories). The yellow and pink colonies were selected and streaked onto tryptic soy agar (TSA; Difco Laboratories) (Kwon et al., 2021).

To select pure strains, observations were conducted, considering morphological features such as color (yellow or pink) on MYPA plates and colony size or shape on TSA plates. Next, a total of 50 single colonies were selected for BA productivity detection.

2.4. BAs productivity of strains

The BAs productivity of strains was determined by the method of Jeon et al. (2018) with modifications. A loop of a strain was inoculated into 5 mL of tryptic soy broth (TSB) and incubated at 37 °C, 120 r/min for 24 h. Afterward, 20 μL of the broth culture was added to a new 5 mL of TSB and incubated under the same conditions for 6 h to obtain a bacterial suspension in the logarithmic phase. Next, 50 μL of this suspension was transferred to 5 mL TSB containing 0.005% pyridoxal phosphate and 0.1% amino acids (L-His, L-Tyr, L-Arg, L-Lys, L-Trp, and L-Phe) and incubated at 37 °C, 120 r/min for 48 h. One milliliter of final broth was mixed with 9 mL of 0.4 M perchloric acid and then filtered through an 11 μm filter paper (Advantech, Tokyo, Japan) for BAs analysis. Four strains with various total BA productivity (highest, second-highest, medium, and lowest) were selected and inoculated into TSB (Difco, Becton Dickinson, Sparks, MD, USA), then incubated at 37 °C for 48 h and stored in glycerol (20%, *v*/v) at −70 °C.

2.5. Identification of strains

The selected *Bacillus* strains were identified (species level) following sequence analysis of the 16S rRNA gene amplified with the universal primers of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGACTTAACCCCAATCGC-3′) (Solgent Co., Daejeon, Korea). The sequences were determined using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6. Sample preparation

Cheonggukjang was prepared following the method of Kim et al. (2012) with slight modifications. Soybeans (4 kg per cultivar) were washed and soaked with water (1:5, *w*/w) at 20 °C for 16 h. The soaked soybeans were separated (100 g per sample) into a tin foil box with a perforated lid and named by cultivar ('Daewon,' 'Pungsannamul,' and 'Somoktae') and strain (*B. velezensis*, *B. amyloliquefaciens*, *B. subtilis*, and *B. licheniformis*). Accordingly, there were 12 samples at each time point and temperature as follows: BAMD (*B. amyloliquefaciens* and cv. 'Daewon'), BAMP (*B. amyloliquefaciens* and cv. 'Pungsannamul'), BAMS (*B. amyloliquefaciens* and cv. 'Somoktae'), BSD (*B. subtilis* and cv. 'Daewon'), BSP (*B. subtilis* and cv. 'Pungsannamul'), BSS (*B. subtilis* and cv. 'Somoktae'), BVD (*B. velezensis* and cv. 'Daewon'), BVP (*B. velezensis* and cv. 'Pungsannamul'), BVS (*B. velezensis* and cv. 'Somoktae'), BLD (*B. licheniformis* and cv. 'Daewon'), BLP (*B. licheniformis* and cv. 'Pungsannamul'), and BLS (*B. licheniformis* and cv. 'Somoktae'). All samples had three replicates.

All the samples were steamed at 121 °C for 30 min using an autoclave, cooled to 40 °C, and subsequently inoculated with the identified strains to the final concentration of approximately 10^6 CFU/g. Inoculated samples were fermented at 25 and 37 °C, with sampling at 0, 6, 12, 18, 30, 48, 72, and 96 h. All samples were first tested for TAPC, protease activity, and BA productivity and then homogenized for storage at −70 °C until further analysis.

2.7. Determination of pH, moisture content, and TAPC

A pH meter (Beckman Coulter, Fullerton, CA, USA) was used to determine the pH of each sample solution (5 g sample mixed with 5 mL of distilled water) following the method of Padilah et al. (2018). Moisture content was determined by the method of the Association of Official Analytical Chemists (AOAC, 2005). Each sample was dried at 105 °C to a

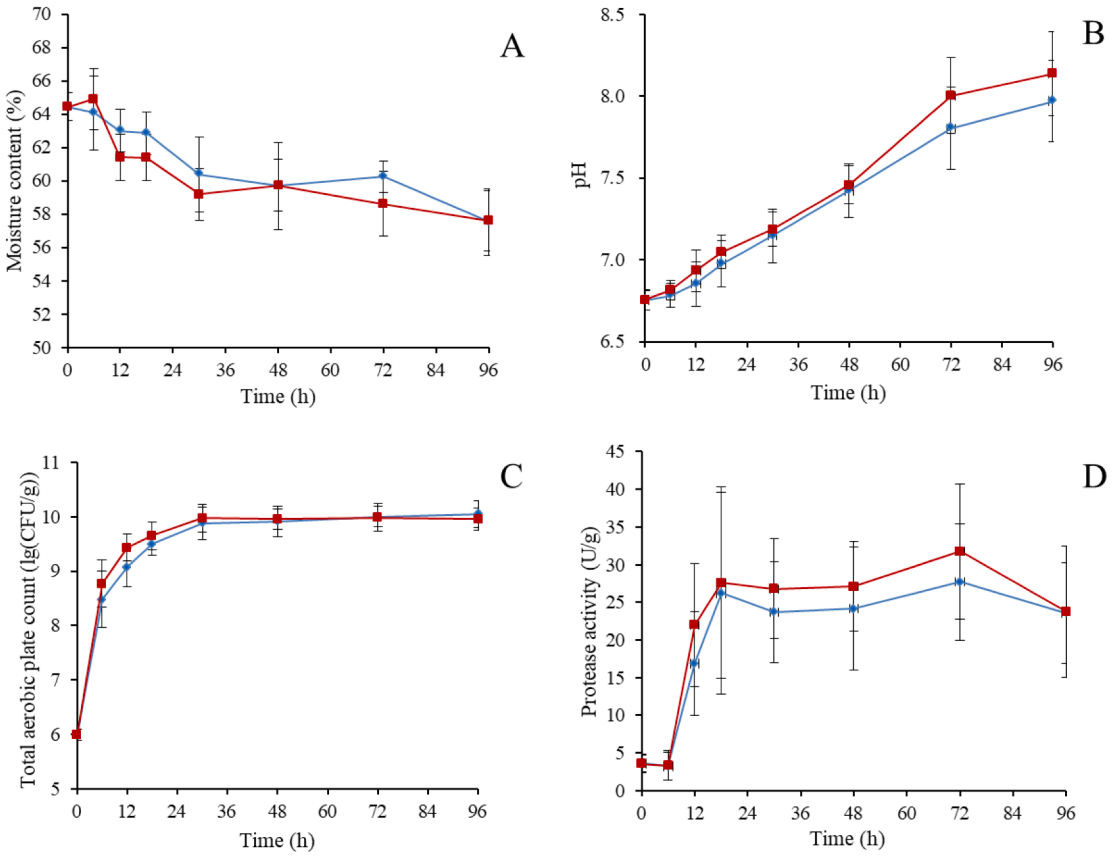

Fig. 1. Mean moisture content (**A**), pH (**B**), total aerobic plate count (**C**), and protease activity (**D**) changes of *Cheonggukjang* samples during fermentation period at 25 °C (—◆—) and 37 °C (—■—).

constant mass, and the ratio of water to solid material was calculated. TAPC was tested using plate count agar according to the method of Jeon et al. (2018). Each sample (5 g) was homogenized with 45 mL of sterilized saline water and then (100 μL) spread on plate count agar. After 48 h of incubation at 37 °C, the colony count was recorded.

2.8. Protease activity and BA productivity of sample

2.8.1. Protease activity assay

The sample (2 g) was mixed with 20 mL of phosphate buffer (pH 7.5), vortexed for 1 min, and centrifuged at 10,000 ×*g* for 5 min at room temperature. Supernatants were used to detect the neutral protease activity in accordance with the method described in a previous study (Liu et al., 2022). Sample solution (1 mL) was mixed with 1 mL of casein solution and 2 mL of trichloroacetic acid at 40 °C, then centrifuged at 10,000 ×*g* for 2 min. The supernatant (1 mL) was combined with 5 mL of Na_2CO_3 (0.5 mol/L) and 1 mL of Folin reagent, followed by reaction for 20 min at 40 °C. Finally, the absorbance value was measured at 680 nm. The calibration curve was constructed by analyzing different concentrations of a casein standard solution.

2.8.2. BAs productivity

The decarboxylase activity was evaluated by determining the corresponding BAs productivity according to the procedure of Kang et al. (2018) and Tan et al. (2019) with modifications. The sample (2 g) was mixed with 10 mL of 0.2 M sodium acetate buffer (pH 5.4) and vortexed for 1 min, followed by centrifugation at 10,000 ×*g* for 5 min. The supernatant (100 μL) was added to 900 μL of 0.2 M ammonium acetate buffer (pH 5.4) containing 0.005% pyridoxal phosphate and 2.5 mM amino acids (L-His L-Tyr, L-Arg, L-Lys, L-Trp, and L-Phe), then incubated at 37 °C for 60 min. Next, 9 mL of 0.4 M perchloric acid was added to the mixture to inactivate the enzyme before BAs analysis. The same solution without 60 min of incubation was set as a control.

2.9. BAs analysis

BAs were determined as described in our previous study (Shi & Moon, 2023). First, 5 g of sample was extracted using 20 mL of 0.4 M perchloric acid solution two times, and the final volume was adjusted to 50 mL with 0.4 M perchloric acid solution. Each sample extract solution was filtered for derivatization, which was conducted according to the method of Frías et al. (2007). Derivatized samples (20 μL) were injected into the high-performance liquid chromatography (HPLC) system equipped with a C18 Supelco column (4.6 mm × 250 mm, i.d., 5 μm; Shiseido, Kyoto, Japan) thermostated at 35 °C, with the flow rate of the mobile phase at 0.5 mL/min and the detection wavelength at 210 nm. The gradient elution procedure was as follows: 0 min, 35% A (0.1 M ammonium acetate), 65% B (acetonitrile); 5 min, 30% A, 70% B; 10 min, 19% A, 81% B; 15 min, 17.5% A, 82.5% B; 20 min, 100% B; 25–35 min, 35% A, 65% B.

2.10. FAAs analysis

FAAs were analyzed using the combined method of the Chinese Industry Standard QB/T 4356–2012 (Ministry of Industry and Information Technology of China, 2012) and Zhu et al. (2016). One gram of sample was extracted using 25 mL of boiling water for 15 min. After the sample was cooled to room temperature and centrifuged at 10,000 ×*g* for 10 min, the final volume of the supernatant was adjusted to 25 mL with distilled water. Then, phenyl isothiocyanate derivatization was performed. Each derivatized sample was filtered through a 0.45 μm syringe filter (Biocomma, Shenzhen, China) for HPLC analysis using an Agilent

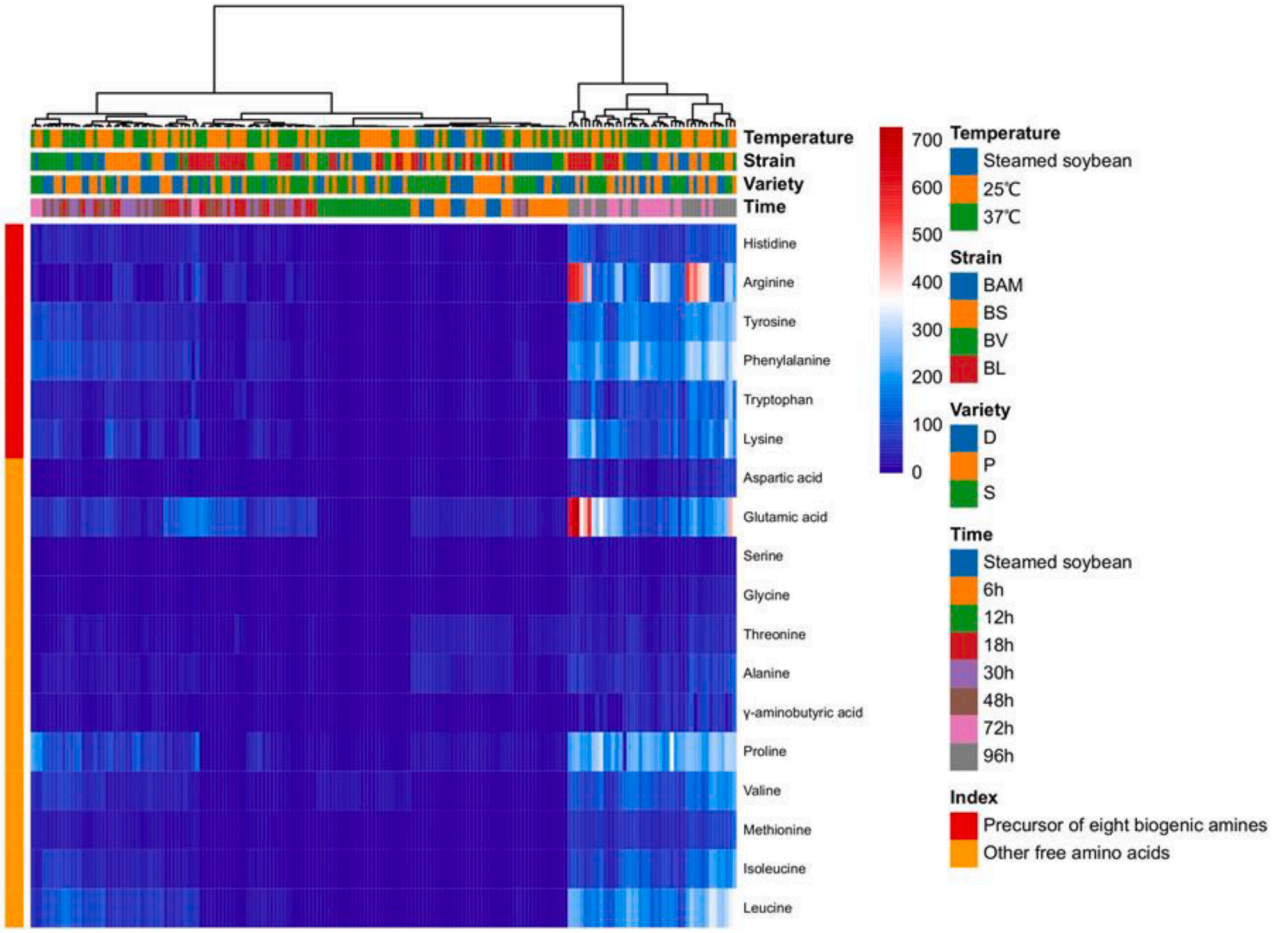

Fig. 2. Heatmap of change in 18 free amino acids during *Cheonggukjang* fermentation.

HPLC system (Alliance 2695 Separations Module, Waters, Milford, MA, USA) equipped with a DAD (1260 Infinity II). Samples (20 μL) were injected into the system and separated on a ZORBAX SB-C18 column (4.6 mm × 250 mm, i.d., 5 μm; Agilent) thermostated at 38 °C, with the flow rate of the mobile phase at 1 mL/min and the detection wavelength at 254 nm. The gradient elution procedure was as follows: 0–2 min, 97.2% A (0.1 M sodium acetate-0.025% triethylamine-3% acetonitrile, *v*/v/v, pH 6.5), 2.8% B (80% acetonitrile, *v*/v); 8 min, 94.9% A, 5.1% B; 13 min, 85.9% A, 14.1% B; 24 min, 76% A, 24% B; 25 min, 73% A, 27% B; 37 min, 62% A, 38% B; 38–42 min, 100% B; 50–55 min, 97.2% A, 2.8% B. For method validation, the method of Yoon et al. (2019) was used.

2.11. Statistical analysis

All analysis data were shown as the mean ± SD ($n = 3$). Statistical analysis was performed using SPSS 24.0 (IBM Corp., Armonk, NY, USA). Paired-samples *t*-test, one-way ANOVA, and Duncan's multiple range tests were used to compare values at $p < 0.05$. Data were processed and drawn using Origin 2021 software (Origin Lab Co., Ltd., Northampton, MA, USA) and online tools (https://www.hiplot.com.cn and https://www.metaboanalyst.ca/).

3. Results and discussion

3.1. Selection and identification of Bacillus strains from commercial Cheonggukjang products

BA productivity (mg/L) of 50 strains selected from retailed *Cheonggukjang* is presented in Table S1. Strains differing in their total BA productivity, namely No. 36 (the highest, 16.29 mg/L), No. 5 (the second-highest, 6.21 mg/L), No. 7 (medium, 3.74 mg/L), and No. 20 (the lowest, 1.93 mg/L), were selected and analyzed by 16S rRNA analysis. From the 16S rRNA analysis result (Table S2), the four strains were identified as *B. velezensis* (No. 5), *B. subtilis* (No. 7), *B. amyloliquefaciens* (No. 20), and *B. licheniformis* (No. 36), respectively.

3.2. Moisture, pH, TAPC, and protease activity

The moisture content, pH, TAPC, and protease activity dynamics in *Cheonggukjang* during fermentation are shown in Fig. 1 and Table S3. The moisture content gradually decreased during fermentation. The average moisture content of 12 samples decreased from 64.45% to 57.54% at 25 °C and to 57.62% at 37 °C, respectively. Similarly, Piao and Eun (2020) also found that the moisture content of *Cheonggukjang* decreased gradually during fermentation. The pH value of samples increased at almost all time points examined during fermentation, which could be explained by the continuous accumulation of ammonia compounds and peptides caused by protein hydrolysis (Yang et al., 2020). Furthermore, most of the pH values of samples fermented at 37 °C were higher than those at 25 °C, which may indicate the relatively faster ammonia compounds and peptides accumulation levels at 37 °C. However, although the TAPC of *Cheonggukjang* fermented at 37 °C was higher than that at 25 °C before 30 h, the values for both temperatures were saturated at that time point and became similar thereafter.

Protease activity, which is responsible for the degradation of proteins in *Cheonggukjang*, is able to affect the protein utilization rate (Gao, Zhao, et al., 2023) as well as the FAAs content (Kim et al., 2012). As Fig. 1D

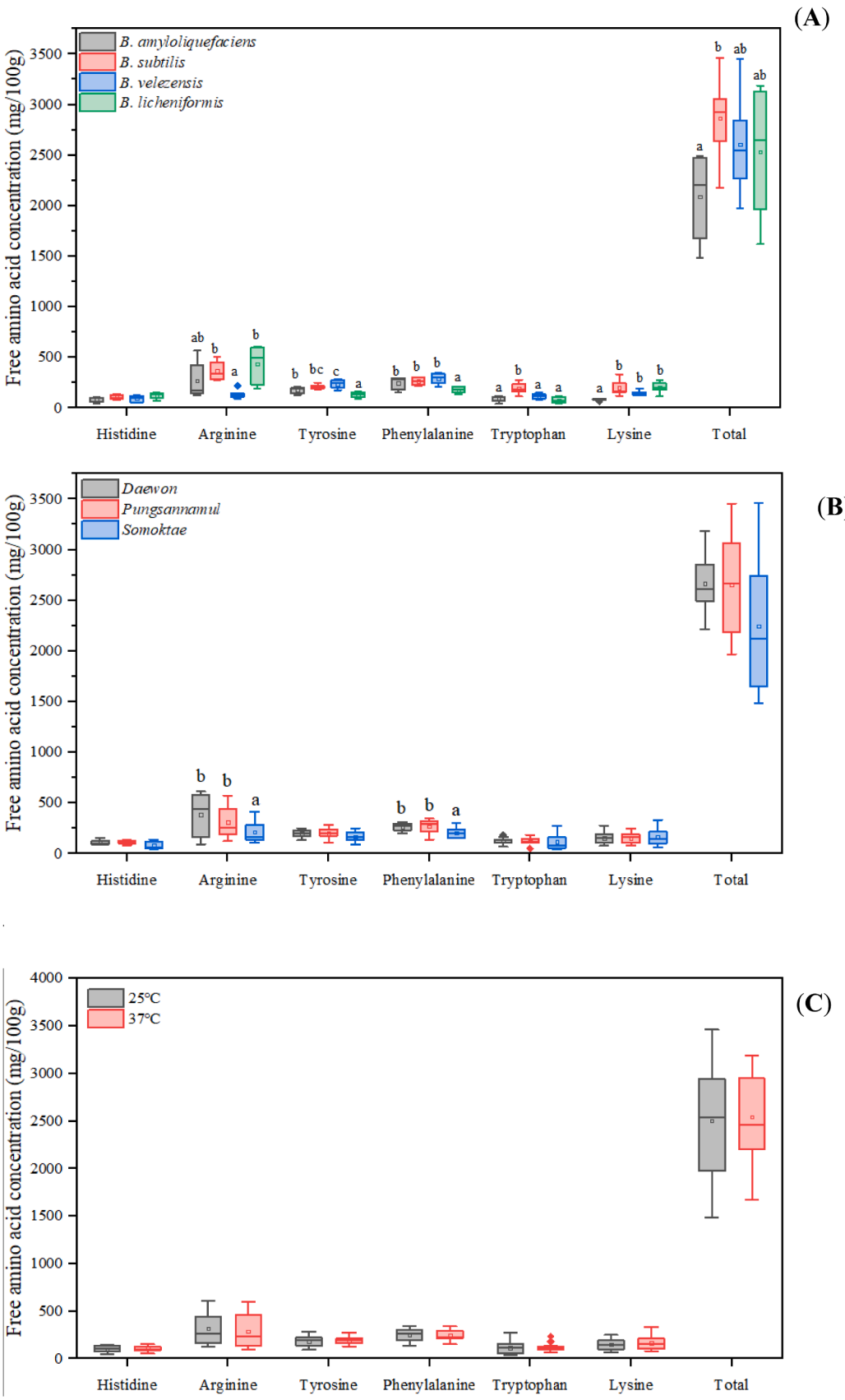

Fig. 3. Concentrations of six free amino acids (precursors of eight biogenic amines) and total 18 free amino acids in *Cheonggukjang* fermented for 96 h grouped by inoculated strain (A), soybean cultivar (B), and temperature (C). a,b: same superscript or no superscript in one group indicates no significant difference ($p > 0.05$).

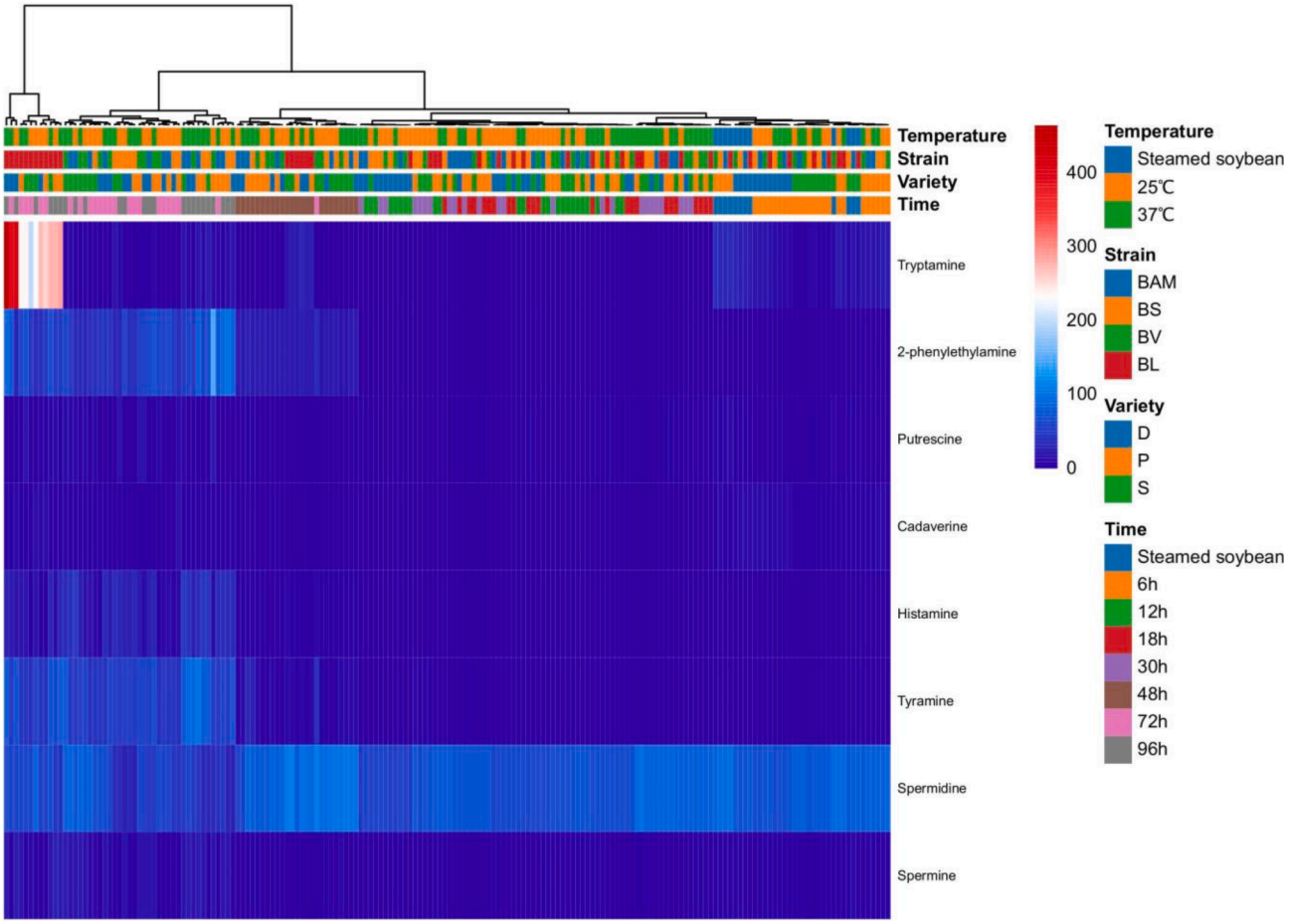

Fig. 4. Heatmap of change in eight biogenic amines during *Cheonggukjang* fermentation.

illustrates, the protease activity of samples is initially low at 0 and 6 h of fermentation but sharply increases after 12 h, then fluctuates until 96 h at both 25 and 37 °C. This tendency was similar to Cho et al. (2014)'s study in which the neutral protease activity of some *Cheonggukjang* samples also initially increased and then fluctuated. Furthermore, the protease activity of samples fermented at 37 °C was generally higher than at 25 °C, which was consistent with the pH tendency and further proved that soybean proteins were more easily degraded into amino acids at 37 °C than at 25 °C.

3.3. FAAs

The HPLC method for the FAAs determination demonstrated good precision, as all relative standard deviation (RSD) values were lower than 3%. The recovery rates, which represent the accuracy, ranged from 81.14% to 118.76% (within the acceptable range from 80% to 120%), indicating the reliability of the HPLC analysis method for 18 FAAs in *Cheonggukjang* according to the SANTE 11312/2021 guidelines (European Commission, 2021).

Heatmap visualization of 18 FAAs during *Cheonggukjang* fermentation (Fig. 2) revealed that the majority of FAAs (His, Arg, Tyr, Phe, Trp, Lys, Glu, GABA, Ala, Pro, Val, Iso, and Leu) showed an overall increasing trend during fermentation because the steamed soybean samples fermented for 6 and 12 h were all shaded in dark blue in the heatmap, whereas those fermented for 72 and 96 h were mostly in the areas shaded in white or red.

The FAAs dynamics during fermentation are shown in Tables S4 and S5. Among the six precursors of eight BAs (His, a precursor of histamine; Arg, the precursor of putrescine, spermidine, and spermine; Tyr, a precursor of tyramine; Phe, a precursor of 2-phenylethylamine; Trp, a precursor of tryptamine; Lys, a precursor of cadaverine) (Afé et al., 2021; EFSA, 2011), Arg exhibited higher levels at 72 and 96 h (white and pink colors in the heatmap). In terms of other amino acids, Glu, highlighted in white and red in the heatmap, emerged as the dominant amino acid in *B. licheniformis*-inoculated samples fermented for 96 h. This result is partly consistent with the report of Tamang et al. (2022), in which Glu was also the major amino acid in *Cheonggukjang*, but Arg was not. This slight discrepancy may be explained by the different soybean cultivars between studies (Kim et al., 2012).

Fig. 3 illustrates the six FAAs (precursor of eight BAs) and total FAA concentrations (18 FAAs) in *Cheonggukjang* fermented for 96 h grouped by inoculated strain (A), soybean variety (B), and temperature (C). Notably, samples prepared with *B. amyloliquefaciens* had the lowest levels of total FAA and Lys, whereas samples fermented with *B. subtilis* exhibited the highest levels of total FAA and Trp. In contrast, samples inoculated with *B. velezensis* had the lowest levels of Arg but the highest levels of Tyr. Additionally, samples made with *B. licheniformis* had the lowest levels of both Tyr and Phe. Baek et al. (2010) also reported a diverse FAA profile in *Cheonggukjang* produced by different strains. In their study, the total FAA and Lys contents in *Cheonggukjang* inoculated with *B. amyloliquefaciens* were lower than those inoculated with *B. licheniformis*, which was consistent with our findings.

Among the soybean cultivars, samples made from 'Somoktae' had significantly ($p < 0.05$) lower Arg and Phe contents after 96 h fermentation. This was in line with a report by Kim et al. (2012) that *Cheonggukjang* made from large and small soybeans showed higher Arg and Phe contents than colored soybean products. However, their study also displayed a difference in the FAAs profile between samples made from cv.

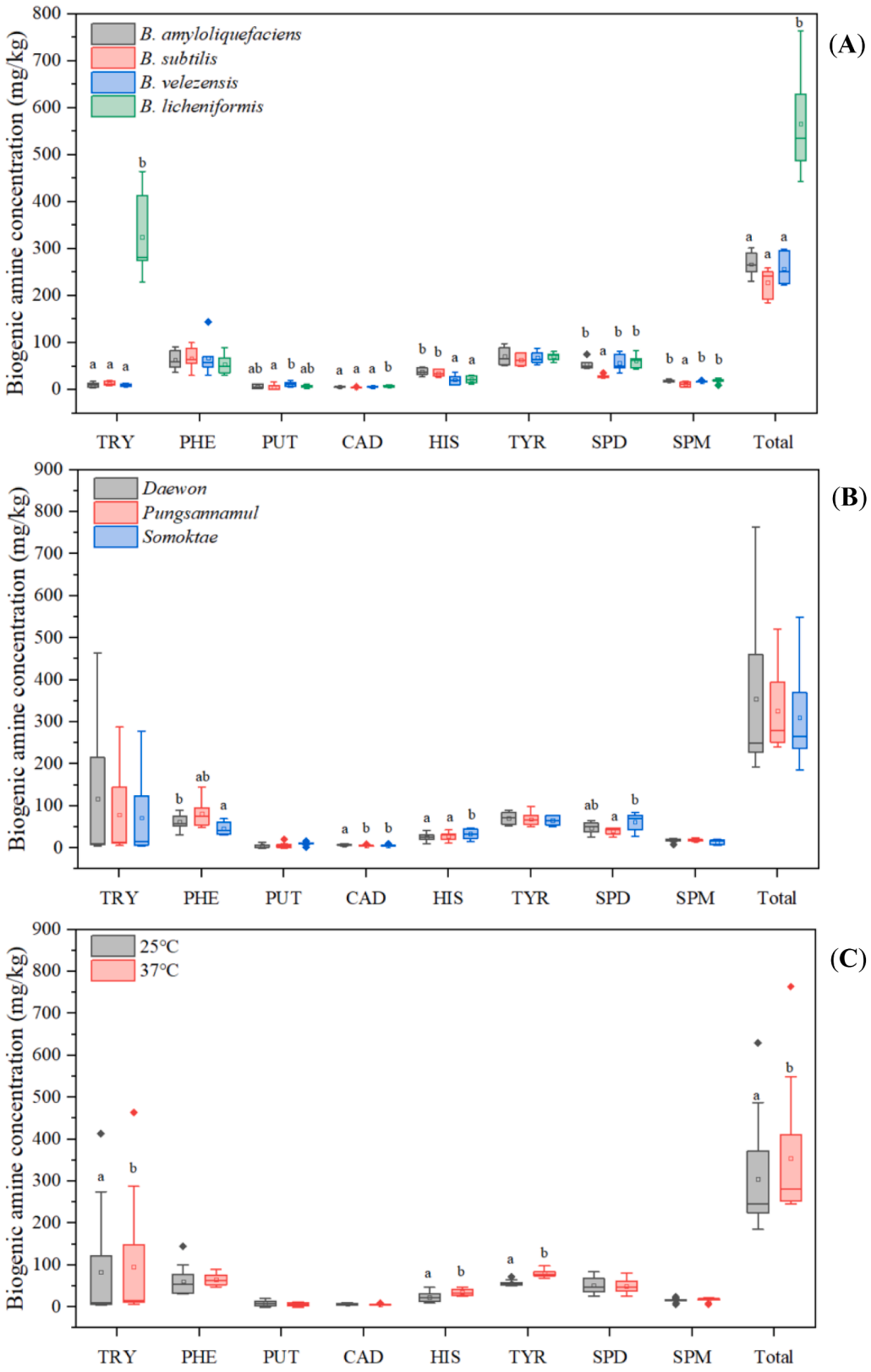

Fig. 5. Biogenic amine concentration of *Cheonggukjang* fermented for 96 h grouped by inoculated strain (**A**), soybean cultivar (**B**), and temperature (**C**). a,b: same superscript or no superscript in one group indicates no significant difference ($p > 0.05$). TRY: tryptamine. PHE: 2-phenylethylamine. PUT: putrescine. CAD: cadaverine. HIS: histamine. TYR: tyramine. SPM: spermine. SPD: spermidine.

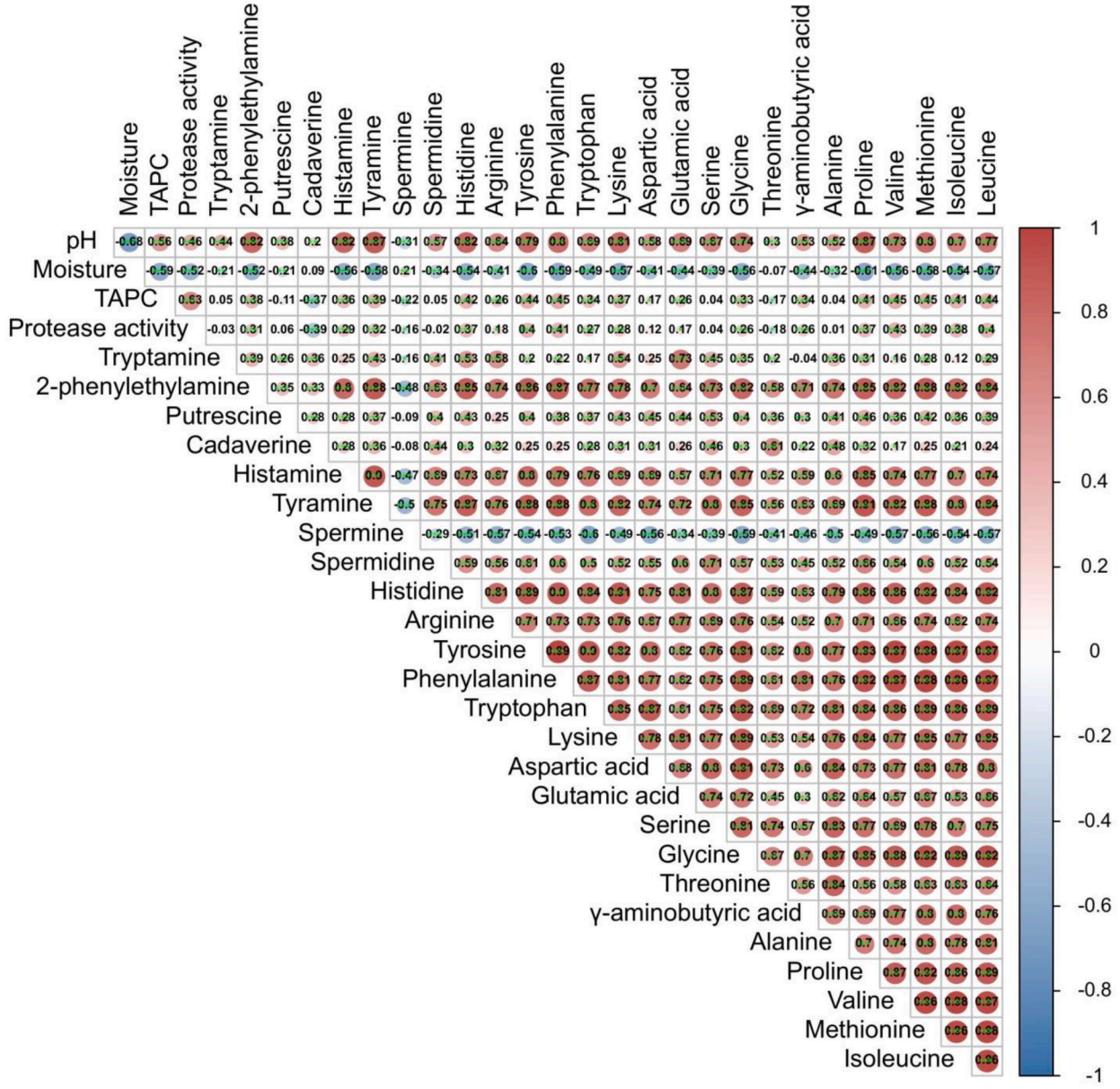

Fig. 6. Pearson correlation heatmap between pH, moisture content, protease activity, total aerobic plate count, eight biogenic amines, and 18 free amino acids.

'Daewon' and cv. 'Pungsannamul,' whereas no significant difference was detected in our study. This may be attributed to the use of different inoculated strains. Additionally, no significant difference was observed in the FAAs content when comparing two fermentation temperatures, even though the results of pH and protease activity measurements illustrated that the higher temperature can facilitate protein degradation. This may be linked to the faster BAs accumulation at the end of the fermentation carried out at the higher temperature (Table S6). In turn, these results indicated that FAAs were extensively transformed into BAs or other compounds during fermentation, influencing the accumulation levels of FAAs.

3.4. Changes of BAs and correlation with other indexes

3.4.1. Changes of BAs in Cheonggukjang during fermentation with different starter strains

Heatmap visualization of eight BAs dynamics during *Cheonggukjang* fermentation (Fig. 4) showed that the majority of BAs (except for spermidine) increased with fermentation time. Furthermore, the total BA content in the samples initially ranged from 112.65 to 153.05 mg/kg and ultimately increased to 155.72–614.28 mg/kg at 25 °C and to 201.95–738.39 mg/kg at 37 °C, respectively. The highest total BA content was observed in BLD fermented for 96 h at both 25 °C (629.86 mg/kg) and 37 °C (764.44 mg/kg), while the lowest values were found in BSS at 25 °C (185.17 mg/kg) and in BSP at 37 °C (244.96 mg/kg) (Table S6).

Moreover, it can be seen in the heatmap that samples inoculated with *B. licheniformis* showed higher tryptamine contents (shaded in white or red), over 200 mg/kg at 72 and 96 h, than other individual BAs contents (shaded in blue), which had <100 mg/kg. This was consistent with the high tryptamine productivity of *B. licheniformis* (12.21 mg/L), which was several times that of the productivity of other individual BAs by this strain. Su-Yeon et al. (2017) also reported that the tryptamine content in *Cheonggukjang* fermented by four different *B. licheniformis* strains was several times that of other individual BAs.

It is also notable that there was a 10-fold increase in tryptamine content in *B. licheniformis*-inoculated samples from 48 to 72 h. This change may be linked to the highest BA productivity in samples at 48 h.

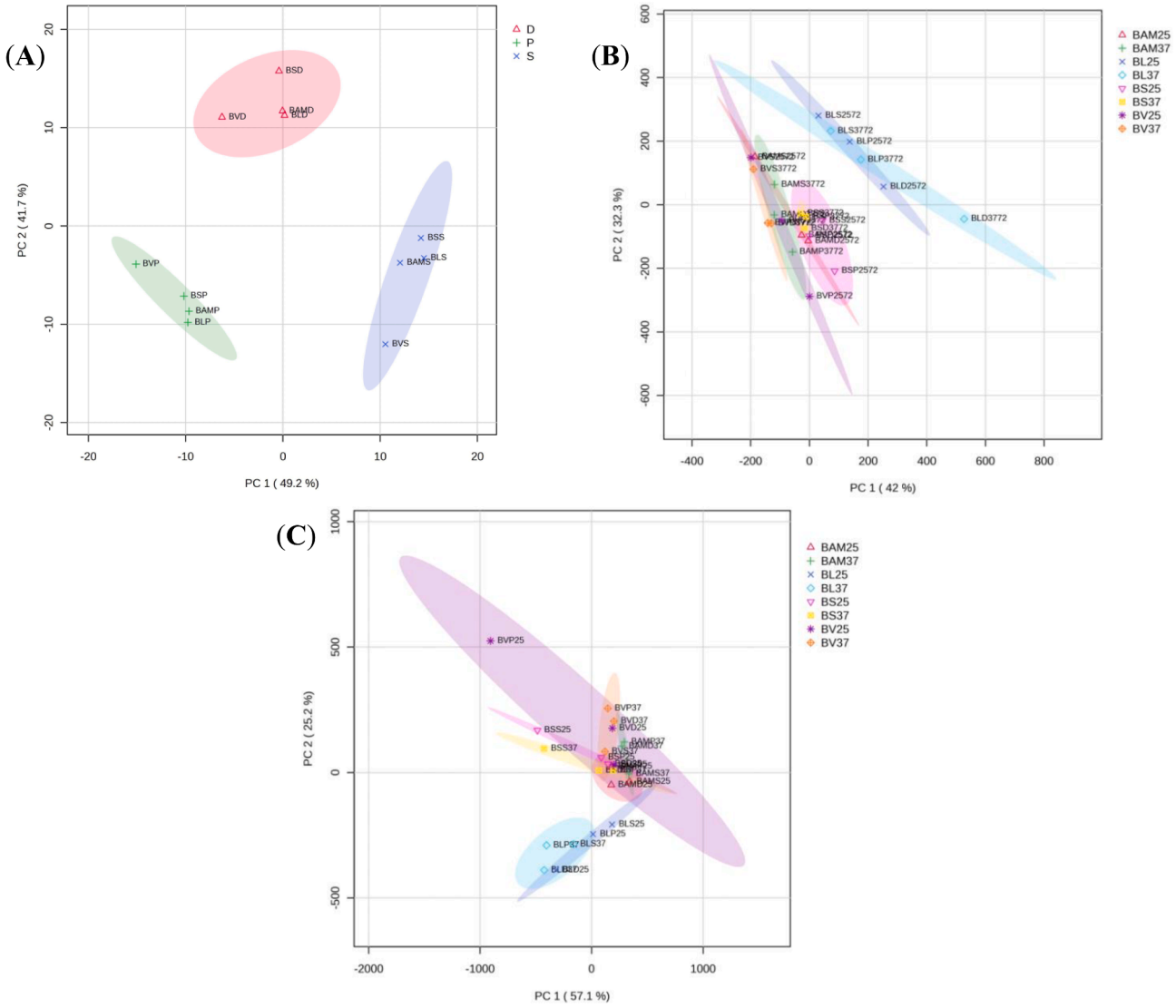

Fig. 7. Principal component analysis plot of moisture content, pH, total aerobic plate count, protease activity, biogenic amines, and free amino acids in *Cheonggukjang* at various fermentation times. (**A**) 0 h, samples were grouped by soybean variety; (**B**) 72 h, samples were grouped by inoculated strain; (**C**) 96 h, samples were grouped by inoculated strain.

Specifically, the tryptamine productivity, representing tryptophan decarboxylase (TDC) activity, gradually increased from around 15 mg/L in the first stage to over 30 mg/L at 48 h, then dramatically decreased to <20 mg/L at 72 h (Table S7) and further reduced to <10 mg/L at 96 h. The tendency may be partially attributed to the rise in pH, as the optimal pH for TDC activity is reported to be between 6.5 and 7.5 (Molchan et al., 2012; Sherwin, 1970), but the pH values for samples made with *B. licheniformis* exceeded 7.5 after 72 h.

In order to further demonstrate the influence of the inoculated strain, the BA concentration in *Cheonggukjang* grouped by inoculated strain at 96 h is illustrated in Fig. 5A. The samples inoculated with *B. licheniformis* exhibited a significantly ($p < 0.05$) higher total BA content compared to those inoculated with other strains. This observation aligns with the ranking of total BA productivity among the strains, as presented in section 3.1 (Table S1). Additionally, the histamine content among the samples was highest in those inoculated with *B. amyloliquefaciens*. This can be explained by the difference in histamine productivity, as *B. amyloliquefaciens* was the only strain detected to produce histamine. The results proved that the productivity of BAs of the inoculated strain could be a crucial index to assess the ability of the strain to influence BA accumulation in *Cheonggukjang*.

3.4.2. Changes of BAs in Cheonggukjang during fermentation by different soybean cultivars and effect of temperature

The BAs concentration in *Cheonggukjang* fermented over 96 h grouped by inoculated strain is displayed in Fig. 5B. There was no significant effect of soybean cultivar on the total BA content in samples after 96 h fermentation, which was consistent with the results of the total FAA content. This may be because the content of FAA precursors of BAs was not sufficiently different between the three cultivars to affect the total BA amount in *Cheonggukjang*. In addition, the formation of putrescine, spermidine, and spermine is complex, as they influence each other's synthesis, and thus, it is difficult to link the content of these three individual BAs with their amino acid precursor, Arg (Makletsova et al., 2022).

Nevertheless, concerning histamine (the most toxic BA), the final content in samples made with cv. 'Somoktae' was generally found to be higher than in samples produced by the other two cultivars ($p < 0.05$). Specifically, after 96 h fermentation, the histamine content of the sample made with cv. 'Somoktae' ranged from 15.68 to 47.89 mg/kg, while the corresponding ranges for cv. 'Daewon' and cv. 'Pungsannamul' were 9.93–41.29 and 10.93–43.02 mg/kg, respectively. The influence of soybean cultivar may be linked to the significantly higher

initial His content (over 14 mg/100 g) of cv. 'Somoktae,' which was more than twice that of the other two cultivars (<7 mg/100 g). Furthermore, the average final His content of the sample made with cv. 'Somoktae' was 77.72 mg/100 g, which was relatively lower than those made with cv. 'Daewon' (116.93 mg/100 g) and cv. 'Pungsannamul' (117.47 mg/100 g). Likewise, a previous study found that wine prepared from a grape variety with a relatively higher initial FAA concentration produced more BAs during the fermentation period (Hernández-Orte et al., 2008).

Samples fermented at the higher temperature also appeared to contain a higher total BA content (Fig. 5C), potentially due to differences in decarboxylase activity because the value of total BA productivity at 37 °C was generally higher than at 25 °C. As reported by Morii and Kasama (2004), temperature is one of the factors affecting the activity of amino acid decarboxylases. This indicates that soybean cultivar, strain, fermentation time, and temperature are all crucial control factors for reducing the BA content in *Cheonggukjang*.

3.4.3. Correlation analysis and principal component analysis (PCA)

Pearson's correlation heatmap between pH, moisture content, TAPC, protease activity, eight BAs, and 18 FAAs is shown in Fig. 6. It can be found that the pH, all FAAs, and the majority of BAs, except for spermine, had positive associations ($p < 0.01$) with each other, indicating that these indexes had a similar trend during *Cheonggukjang* fermentation time. This can be explained by the enhanced availability of FAAs and the activity of proteases and decarboxylases with the increase in pH, efficiently promoting BAs formation (Lan et al., 2020; Liu et al., 2022).

The PCA plots of moisture content, pH, TAPC, protease activity, BAs, and FAAs in *Cheonggukjang* at different time points, which included separated 95% confidence regions, are presented in Fig. 7. At 0 h (Fig. 7A), all samples were separated by soybean cultivar. However, after 72 and 96 h of fermentation, only samples inoculated with *B. licheniformis* could be differentiated from the others in the PCA plot (Fig. 7B and C). At the other time points, there was no separated 95% confidence region whether samples were grouped by soybean cultivar or inoculated strain. This illustrates that the differentiation between samples prepared with *B. licheniformis* and other samples becomes more pronounced as fermentation time increases, whereas the others gradually become less uniform in terms of both soybean cultivar and inoculated strain. The results demonstrated that the microorganism used for fermentation is a more important factor affecting BA content than temperature and soybean cultivar, which aligns with the viewpoint of a previous report (Gao, Li, et al., 2023). The results further showed that *B. licheniformis* has a major influence on the formation of BAs in *Cheonggukjang* and that strains with high BA productivity should be avoided when making *Cheonggukjang*.

4. Conclusion

This study investigated the effects of soybean cultivar, inoculated strain, and temperature on BAs formation during *Cheonggukjang* fermentation. Throughout the fermentation period, the moisture content of *Cheonggukjang* samples decreased, whereas the pH value increased. TAPC of the samples reached saturation at 30 h, and protease activity initially increased before fluctuating. FAAs exhibited a general increasing trend with fermentation time, and differences in FAAs profiles were observed among samples grouped by both inoculated strains and soybean varieties. Furthermore, the BAs content was influenced by the strain. *B. licheniformis* showed the highest total BA content at 72 and 96 h due to having the highest BA productivity. Histamine formation varied depending on the soybean cultivar, which may be associated with the significantly ($p < 0.05$) higher initial but comparably lower final His content of cv. 'Somoktae.' Additionally, the fermentation temperature was also an effective factor influencing BAs accumulation. These results revealed the dynamics of BAs content and the related indexes during the *Cheonggukjang* fermentation period. The findings offer an opportunity for further research into reducing BAs in *Cheonggukjang* by modifying the environmental conditions and selecting specific strains or soybean cultivars.

CRediT authorship contribution statement

BaoZhu Shi: Writing – original draft, Formal analysis, Data curation. **BoKyung Moon:** Writing – original draft, Methodology, Conceptualization.

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.

Acknowledgment

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101280.

References

Afé, O. H. I., Saegerman, C., Kpoclou, Y. E., Douny, C., Igout, A., Mahillon, J., … Scippo, M.-C. (2021). Contamination of smoked fish and smoked-dried fish with polycyclic aromatic hydrocarbons and biogenic amines and risk assessment for the Beninese consumers. *Food Control, 126*. https://doi.org/10.1016/j.foodcont.2021.108089. Article 108089.

AOAC. (2005). *Official methods of analysis* (18th ed.). Association of Official Analytical Chemists.

Baek, J. G., Shim, S.-M., Kwon, D. Y., Choi, H.-K., Lee, C. H., & Kim, Y.-S. (2010). Metabolite profiling of *Cheonggukjang*, a fermented soybean paste, inoculated with various *Bacillus* strains during fermentation. *Bioscience, Biotechnology, and Biochemistry, 74*(9), 1860–1868. https://doi.org/10.1271/bbb.100269

Bulushi, A. I., Poole, S., Deeth, H. C., & Dykes, G. A. (2009). Biogenic amines in fish: Roles in intoxication, spoilage, and nitrosamine formation—A review. *Critical Reviews in Food Science and Nutrition, 49*(4), 369–377. https://doi.org/10.1080/10408390802067514

Cho, K. M., Lim, H.-J., Kim, M.-S., Kim, D. S., Hwang, C. E., Nam, S. H., … Shin, E.-C. (2017). Time course effects of fermentation on fatty acid and volatile compound profiles of *Cheonggukjang* using new soybean cultivars. *Journal of Food and Drug Analysis, 25*(3), 637–653. https://doi.org/10.1016/j.jfda.2016.07.006

Cho, M. J., Lee, J. Y., & Kim, J. H. (2014). Microbial and physiochemical properties of *Cheonggukjang* fermented using *Bacillus* strains with antibacterial or antifungal activities. *Food Science and Biotechnology, 23*(5), 1525–1532. https://doi.org/10.1007/s10068-014-0208-z

EFSA. (2011). Scientific opinion on risk based control of biogenic amine formation in fermented foods. *EFSA Journal, 9*(10). https://doi.org/10.2903/j.efsa.2011.2393. Article 2393.

European Commission. (2021). Analytical quality control and method validation procedures for pesticide residues analysis in food and feed (SANTE 11312/2021). Retrieved from https://www.eurl-pesticides.eu/userfiles/file/EurlALL/SANTE_11312_2021.pdf Accessed September 26, 2023.

Frías, J., Martínez-Villaluenga, C., Gulewicz, P., Perez-Romero, A., Pilarski, R., Gulewicz, K., & Vidal-Valverde, C. (2007). Biogenic amines and HL60 citotoxicity [sic] of alfalfa and fenugreek sprouts. *Food Chemistry, 105*(3), 959–967. https://doi.org/10.1016/j.foodchem.2007.04.043

Gao, X., Li, C., He, R., Zhang, Y., Wang, B., Zhang, Z.-H., & Ho, C.-T. (2023). Research advances on biogenic amines in traditional fermented foods: Emphasis on formation mechanism, detection and control methods. *Food Chemistry, 405*. https://doi.org/10.1016/j.foodchem.2022.134911. Article 134911.

Gao, X., Zhao, X., Hu, F., Fu, J., Zhang, Z., Liu, Z., … Ho, C.-T. (2023). The latest advances on soy sauce research in the past decade: Emphasis on the advances in China. *Food Research International, 173*. https://doi.org/10.1016/j.foodres.2023.113407. Article 113407.

Herbert, P., Cabrita, M. J., Ratola, N., Laureano, O., & Alves, A. (2005). Free amino acids and biogenic amines in wines and musts from the Alentejo region. Evolution of

amines during alcoholic fermentation and relationship with variety, sub-region and vintage. *Journal of Food Engineering, 66*(3), 315–322. https://doi.org/10.1016/j.jfoodeng.2004.03.024

Hernández-Orte, P., Lapeña, A. C., Peña-Gallego, A., Astrain, J., Baron, C., Pardo, I., … Ferreira, V. (2008). Biogenic amine determination in wine fermented in oak barrels: Factors affecting formation. *Food Research International, 41*(7), 697–706. https://doi.org/10.1016/j.foodres.2008.05.002

Jeon, A. R., Lee, J. H., & Mah, J.-H. (2018). Biogenic amine formation and bacterial contribution in *Cheonggukjang*, a Korean traditional fermented soybean food. *LWT, 92*, 282–289. https://doi.org/10.1016/j.lwt.2018.02.047

Ju, S., Cao, Z., Wong, C., Liu, Y., Foda, M. F., Zhang, Z., & Li, J. (2019). Isolation and optimal fermentation condition of the *Bacillus* subtilis subsp. *natto* strain WTC016 for nattokinase production. *Fermentation, 5*(4). https://doi.org/10.3390/fermentation5040092. Article 92.

Kang, H.-R., Kim, H.-S., Mah, J.-H., Kim, Y.-W., & Hwang, H.-J. (2018). Tyramine reduction by tyrosine decarboxylase inhibitor in *Enterococcus faecium* for tyramine controlled *cheonggukjang*. *Food Science and Biotechnology, 27*(1), 87–93. https://doi.org/10.1007/s10068-017-0205-0

Kim, M.-H., Han, S.-Y., Ko, J.-M., & Kim, Y.-S. (2012). Degradation characteristics of proteins in *cheonggukjang* (fermented unsalted soybean paste) prepared with various soybean cultivars. *Food Science and Biotechnology, 21*(1), 9–18. https://doi.org/10.1007/s10068-012-0002-8

Kwon, E.-A., Lee, J.-I., Park, J. W., & Kim, S.-S. (2021). Application of comparative genomics in the development of DNA probes to detect *Bacillus cereus* and *Bacillus subtilis*. *LWT, 142*. https://doi.org/10.1016/j.lwt.2021.110996. Article 110996.

Lan, G., Li, C., He, L., Zeng, X., & Zhu, Q. (2020). Effects of different strains and fermentation method on nattokinase activity, biogenic amines, and sensory characteristics of natto. *Journal of Food Science and Technology, 57*(12), 4414–4423. https://doi.org/10.1007/s13197-020-04478-3

Lim, E.-S. (2022). Influence of bacteriocin-producing *Bacillus* strains on quality characteristics of fermented soybean product with biogenic amine-forming lactic acid bacteria. *Applied Biological Chemistry, 65*. https://doi.org/10.1186/s13765-021-00664-9. Article 5.

Liu, X., Liang, J., Ma, Y., Sun, J., Liu, Y., Gu, X., & Wang, Y. (2022). The impact of protein hydrolysis on biogenic amines production during sufu fermentation. *Food Control, 140*. https://doi.org/10.1016/j.foodcont.2022.109105. Article 109105.

Majcherczyk, J., & Surówka, K. (2019). Effects of onion or caraway on the formation of biogenic amines during sauerkraut fermentation and refrigerated storage. *Food Chemistry, 298*. https://doi.org/10.1016/j.foodchem.2019.125083. Article 125083.

Makletsova, M. G., Rikhireva, G. T., Kirichenko, E. Y., Trinitatsky, I. Y., Vakulenko, M. Y., & Ermakov, A. M. (2022). The role of polyamines in the mechanisms of cognitive impairment. *Neurochemical Journal, 16*(3), 283–294. https://doi.org/10.1134/S1819712422030059

Ministry of Industry and Information Technology of China. (2012). *Determination of free amino acids in Chinese rice wine: High performance liquid chromatography (QB/T 4356–2012)*.

Molchan, O., Romashko, S., & Yurin, V. (2012). L-tryptophan decarboxylase activity and tryptamine accumulation in callus cultures of *Vinca minor* L. *Plant Cell, Tissue and Organ Culture, 108*(3), 535–539. https://doi.org/10.1007/s11240-011-0060-2

Morii, H., & Kasama, K. (2004). Activity of two histidine decarboxylases from *Photobacterium phosphoreum* at different temperatures, pHs, and NaCl concentrations. *Journal of Food Protection, 67*(8), 1736–1742. https://doi.org/10.4315/0362-028x-67.8.1736

Padilah, B., Bahruddin, S., Fazilah, A., Ahmad, M., & Gulam, R. R. A. (2018). Biogenic amines analysis in shrimp pastes belacan obtained from the northern states of peninsular Malaysia. *International Food Research Journal, 25*(5), 1893–1899.

Park, Y. K., Jin, Y. H., Lee, J.-H., Byun, B. Y., Lee, J., Jeong, K. C., & Mah, J.-H. (2020). The role of *Enterococcus faecium* as a key producer and fermentation condition as an influencing factor in tyramine accumulation in *Cheonggukjang*. *Foods, 9*(7). https://doi.org/10.3390/foods9070915. Article 915.

Piao, Y.-Z., & Eun, J.-B. (2020). Physicochemical characteristics and isoflavones content during manufacture of short-time fermented soybean product (*cheonggukjang*). *Journal of Food Science and Technology, 57*(6), 2190–2197. https://doi.org/10.1007/s13197-020-04255-2

Sherwin, J. E. (1970). A tryptophan decarboxylase from cucumber seedlings. *Plant and Cell Physiology, 11*(6), 865–872. https://doi.org/10.1093/oxfordjournals.pcp.a074577

Shi, B., & Moon, B. (2023). Monitoring and risk assessment of biogenic amines in Korean commercial fermented seasonings. *Heliyon, 9*(8). https://doi.org/10.1016/j.heliyon.2023.e18906. Article e18906.

Smit, A. Y., du Toit, W. J., Stander, M., & du Toit, M. (2013). Evaluating the influence of maceration practices on biogenic amine formation in wine. *LWT - Food Science and Technology, 53*(1), 297–307. https://doi.org/10.1016/j.lwt.2013.01.006

Su-Yeon, K., Hyeong-Eun, K., & Yong-Suk, K. (2017). The potentials of *Bacillus licheniformis* strains for inhibition of *B. Cereus* growth and reduction of biogenic amines in *cheonggukjang* (Korean fermented unsalted soybean paste). *Food Control, 79*, 87–93. https://doi.org/10.1016/j.foodcont.2017.03.028

Tamang, J. P., Das, S., Kharnaior, P., Pariyar, P., Thapa, N., Jo, S.-W., … Shin, D.-H. (2022). Shotgun metagenomics of *Cheonggukjang*, a fermented soybean food of Korea: Community structure, predictive functionalities and amino acids profile. *Food Research International, 151*. https://doi.org/10.1016/j.foodres.2021.110904. Article 110904.

Tan, Y., Zhang, R., Chen, G., Wang, S., Li, C., Xu, Y., & Kan, J. (2019). Effect of different starter cultures on the control of biogenic amines and quality change of douchi by rapid fermentation. *LWT, 109*, 395–405. https://doi.org/10.1016/j.lwt.2019.04.041

Tasić, T., Ikonić, P., Mandić, A., Jokanović, M., Tomović, V., Savatić, S., & Petrović, L. (2012). Biogenic amines content in traditional dry fermented sausage *Petrovská klobása* as possible indicator of good manufacturing practice. *Food Control, 23*(1), 107–112. https://doi.org/10.1016/j.foodcont.2011.06.019

Yang, B., Tan, Y., & Kan, J. (2020). Regulation of quality and biogenic amine production during sufu fermentation by pure *Mucor* strains. *LWT, 117*. https://doi.org/10.1016/j.lwt.2019.108637. Article 108637.

Yoon, S. H., Koh, E., Choi, B., & Moon, B. (2019). Effects of soaking and fermentation time on biogenic amines content of *maesil* (*Prunus mume*) extract. *Foods, 8*. https://doi.org/10.3390/foods8110592. Article 592.

Yoon, S. H., Lee, S., Lee, S.-Y., & Moon, B. (2024). Effect of precursors and stress factors on yeast isolated from fermented *maesil* extract and their biogenic amine formation. *Food Science and Biotechnology, 33*, 211–218. https://doi.org/10.1007/s10068-023-01328-8

Zhu, Y., Luo, Y., Wang, P., Zhao, M., Li, L., Hu, X., & Chen, F. (2016). Simultaneous determination of free amino acids in Pu-erh tea and their changes during fermentation. *Food Chemistry, 194*, 643–649. https://doi.org/10.1016/j.foodchem.2015.08.054