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Research Paper

Impact of salt concentration on bacterial diversity and changes in biogenic amines during fermentation of farmhouse soybean paste in Northeast China

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ABSTRACT

Farmhouse soybean paste in Northeast China is a traditional fermented product made from soybean, and more than 11% (w/w) salt is usually added during production to control the fermentation process. In this study, the variations in bacterial diversity, biogenic amines(BAs) and physicochemical properties during the natural fermentation of soybean paste with different salt concentrations (8%, 9%, 10%, 11%, and 12%) were studied. The results show that at 0 days (0 d) of fermentation in soybean paste, the dominant genera included *Staphylococcus*, unidentified *Clostridiales*, and *Sporolactobacillus*. During fermentation from 30 d to 90 d, the dominant genera were *Tetragenococcus* and *Staphylococcus*. However, the proportions of the dominant genera were different depending on the salt concentration. Putrescine(Put), tryptamine(Try), β-phenethylamine(Phe), cadaverine (Cad), histamine(His), and tyramine(Tyr) showed negative correlations with salt concentration. The amino type nitrogen(ANN), titratable acidity(TTA) and total number of colonies were also negatively correlated with salt concentration. Analysis of the correlation between genera and BAs showed that 12 genera were positively correlated with BAs, and 4 genera were negatively correlated with BAs. The results of this study indicated that salt has a significant impact on bacterial diversity during the fermentation of soybean paste, which in turn affects the changes in bacterial metabolites. From the perspective of food safety, the amount of salt added in the soybean paste can be reduced to 10% under the existing fermentation conditions.

1. Introduction

Farmhouse soybean paste is a traditional fermented soybean product in Northeast China, that has been popular with consumers for a long time due to its unique flavor and high nutritional value (Sun et al., 2018; Xie et al., 2019). Its production method involves fermenting steamed soybeans to make koji (a soybean cube with penicillium or white mold growing on the surface after natural fermentation, traditionally called koji). Then the ripe koji is washed, crushed and mixed with a certain concentration of salt brine for natural fermentation, after which it is stirred once a day and is ready to eat after three months. Since soybean paste is fermented in an open natural environment, many beneficial microbes are involved in the fermentation process from the surrounding environment. Many previous studies have shown that in soybean paste, the *Tetragenococcus* and *Staphylococcus* are usually the dominant microbiotas, and many other microbiotas such as *Leuconostoc* and *Weissella* also participate in the fermentation process. These microbiotas usually occupy a small proportion and vary in different soybean pastes as the fermentation environment changes (An et al., 2020, 2021). These microbiotas decompose the nutrient matrix of soybeans to metabolize and transform it into various substances, which mainly contribute to the unique taste and nutritional composition of their products (Zhang et al., 2019). However, there are also some harmful microbiotas involved in the fermentation process, which may compete with the beneficial ones and consume soybean nutrients, producing undesirable metabolites and resulting in declines in the quality and safety of soybean paste. Therefore, it can be seen that the quality and safety of soybean paste is largely determined by the microbiotas contained in it. These microbiotas have different salt tolerances, and their activities are affected by not only the environment but also by the salt concentration. Salt in soybean paste determines the flavor by controlling the growth and metabolism of the microbiotas (Kim et al., 2020a; Kim et al., 2020b). The concentration of salt will also affect the total phenolic content (TPC), the oxygen radical absorbance capacity (ORAC), the Trolox equivalent antioxidant capacity (TEAC), and the phenolic, flavonoid, and isoflavone profiles in the soybean paste (Kim et al., 2018; Yang et al., 2021). Therefore, in the

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traditional method of farmhouse soybean paste, salt with a concentration of more than 11% (w/w) is usually added during the fermentation process.

However, in recent years, many studies have shown that reducing sodium intake is very important and necessary for health(Wolkers--Rooijackers et al., 2013; Bistola et al., 2020; Celik and Bektas, 2020; Jin et al., 2020). Excessive intake of salt is considered harmful to human health, causing cardiovascular and cerebrovascular diseases (Yang et al., 2021). Therefore, many countries have proposed plans to reduce daily salt intake (Zandstra et al., 2016; China, 2017; Cappuccio, Beer, Strazzullo, & the European Salt Action Network, 2018). However, for farmhouse soybean paste, when the salt concentration is too low, the inhibitory effect of salt on some harmful microbiotas will be weakened (Jia et al., 2020) and will lead to unpleasant odors such as sourness and bitterness in the soybean paste (Chun et al., 2020); it will also produce some toxic and harmful substances, such as biogenic amines(BAs) (Li et al., 2020). BAs are metabolites of microbiota (Lorenzo et al., 2017) and are very common in fermented products. There are inevitably high levels of BAs in fermented soybean products such as stinky tofu (Gu et al., 2018), natto (Kim et al., 2012), sufu (Liang et al., 2019), and soy sauces (Guo et al., 2020). In our previous research, the content of BAs in soybean paste ranged from 25.41 to 444.93 mg/kg (Hao et al., 2018; Hao and Sun, 2020). Most BAs are toxic to humans (Ladero et al., 2010; Rio et al., 2017). For example, histamine can cause severe allergic-like symptoms. Tyramine, Spermine, Spermidine, and Tryptamine can all cause migraine headaches and high blood pressure to varying degrees. Other substances in some foods can also synergize with BAs, increasing the toxicity of BAs such as nitrite and ethanol. BAs are an important factor that affects the edible safety of soybean paste, and it is particularly important to monitor their accumulation during the fermentation process. Therefore, for farmhouse soybean paste, the reduction of salt should be controlled within a certain limit, and the appropriate amount of salt should be added under the premise of ensuring product quality and safety. However, there is almost no research on the influence of salt concentration on the quality of farmhouse soybean paste during fermentation.

This study aims to reveal the influence of salt concentration and fermentation time on the bacteria diversity, BAs and physicochemical properties of soybean paste. Meanwhile, the correlation between salt concentration with the changes of bacteria diversity, and the correlation between bacteria diversity with BAs were studied. These results will deepen our understanding the role of salt on the fermentation of soybean paste, which will also provide some support for other products like soybean paste that need to reduce the added amount of salt within an adequate range.

2. Materials and methods

2.1. Methodology used for sample preparation and collection

The preparation of all samples followed these steps. Briefly, (i) soybeans were selected, and stones, grass seeds and other impurities were removed. (ii) The selected soybeans were washed two or three times and then soaked in water that covered the soybeans until they were soft without any hard cores. (iii) The soybeans were then steamed in a high-pressure steamer until they could be easily crushed by hand. The steaming conditions were maintain 15 min under 0.15 MPa steam pressure. (iv) The cooked soybeans were all crushed, and a mold (a cuboid closed on five sides, usually of wood or stainless steel) was used to make the soybean into cubes approximately 30 cm × 20 cm × 10 cm in size with a weight of approximately 6 kg. (v) The soybean cubes were wrapped in kraft paper and placed in a low temperature, humid and dark environment for fermentation. The temperature was maintained between 15 and 20 °C, and the relative humidity was maintained between 60 and 70% rh. (vi) After two months of fermentation, white mold and penicillium were overgrown on the soybean cubes, and the weight was reduced to approximately 3–3.5 kg. The soybean cubes with white mold and penicillium obtained in this step are traditionally called koji. (vii) The kraft paper on the surface of the koji was removed, cleaned with water, and cut into small cubes. (viii) Then, 30 kg of koji cut into small pieces was mixed with 70 kg of water, and the mixture was placed in a pottery vessel, and certain amounts of salt were added to make 8%, 9%, 10%, 11%, and 12% (w/w) salt concentrations of soybean paste. Then a breathable white cotton cloth and waterproof cover were attached to each pottery vessel. The above five groups were fermented under the same conditions (with plenty of sunlight, and maintaining the temperature above 20 °C). When the day was sunny, the white cotton cloth and waterproof cover were opened to allow the soybean paste to be fully exposed to the sunlight, and the white cotton cloth and waterproof cover were closed on cloudy days and at night to prevent rainwater and other impurities from mixing into the soybean paste. The mixtures were fully raked and mixed evenly, and fermentation was completed after 90 days. Three batches of farmhouse soybean paste fermentation were prepared under different brine concentrations (8%, 9%, 10%, 11%, and 12%, w/w). The above processes were conducted from May to September in the soybean paste production plant at Shuncai Food Co., Ltd. Shuangcheng District, Harbin City, Heilongjiang Province, China.

Five pieces of ripened koji were randomly selected, crushed and mixed them evenly, and 200 g was removed as a fermentation 0 d sample and marked as IM. Then, the koji was mixed with salt brine, and sampling was performed every 30 days thereafter for a total of 3 times. The sampling process was performed as follows: after thoroughly mixing the soybean paste in each pottery vessel, a 200 g sample was removed from each vessel and stored at −18 °C for the determination of bacterial diversity, BAs, and physicochemical properties. The above treatment process was repeated 3 times in each sample.

2.2. Determination of bacterial diversity

2.2.1. Sample processing

Ten grams of soybean paste were mixed with 20 mL of a 0.1 mol/L phosphate buffer solution (pH = 7.0) and then shaken thoroughly for 5 min and centrifuged at 3000 rpm/min for 10 min. The supernatant was collected, and the sediment was washed again and centrifuged twice. Then, all of the above supernatants were combined and centrifuged at 12,000 rpm/min for 10 min, and the separated pellets containing bacteria were collected. The collected bacterial cells were washed with 5 mL of phosphate buffer solution (pH = 7.0) and centrifuged at 12,000 rpm/min for 10 min. This wash cycle was repeated 3 times. Finally, the samples were stored in 5 mL of phosphate buffer solution (pH = 7.0) at −20 °C for subsequent analyses.

2.2.2. Genomic DNA extraction

The genomic DNA of the microbial community was extracted with a TIANamp Bacteria DNA Kit bacterial genomic DNA extraction kit (centrifugal column type) (catalog number: DP302 Tiangen Biochemical Technology Co., Ltd, China). One percent agarose gel electrophoresis (DDY-6C Electrophoresis Apparatus, Beijing Liuyi Instrument Factory, China) was performed to check the integrity of the DNA samples. The voltage was 100 V, and the electrophoresis time was 40 min.

2.2.3. PCR amplification and sequencing

The sample DNA was placed in a centrifuge tube, and the sample was diluted to 1 ng/μl with sterile water. Using the diluted genomic DNA as a template, the universal primers 515F (5′-GTG CCA GCM GCC GCGG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Hao and Sun, 2020) were used to amplify the 16S rDNA V4 region from approximately 10 ng of template DNA (Bio-Rad T100 Gradient PCR Amplifier, BIO-RAD, USA). Thermal cycling included (i) initial denaturation at 95 °C for 5 min; (ii) denaturation at 94 °C for 1 min; (iii) annealing at 56 °C for 1 min; (iv) extension at 72 °C for 1 min; steps (ii), (iii), and (iv) were repeated for 30 cycles, and the samples were finally maintained at 72 °C

for 4 min. The PCR products of duplicate samples were combined and subjected to 2% agarose gel electrophoresis (voltage: 80 V, electrophoresis time: 40 min). After verification, they were used for subsequent high-throughput sequencing. According to the characteristics of the amplified region, based on the Illumina NovaSeq 6000 (Beijing Novogene Co., Ltd, Beijing, China) sequencing platform, paired-end sequencing was performed to generate a small-fragment library for paired-end sequencing.

2.2.4. Analysis of sequencing data

Using Uparse software (Uparse v7.0.1001), sequences with a similarity of more than 97% were classified as operating units or operational taxonomic units (OTUs), and the taxonomic information of each OTU was obtained. The OTU data were organized to obtain corresponding species information and species richness. QIIME software (Version 1.9.1) was used to calculate alpha diversity indexes (observed species, Chao1, Shannon, Simpson, ACE) and beta diversity. Alpha diversity index represents the diversity and richness of the sample itself. The observed species, Chao1, and ACE represent the bacterial richness; the larger the value is, the higher the bacterial richness. Shannon and Simpson indices represent the bacterial diversity (Gu et al., 2018); the larger the value is, the higher the bacterial diversity. Beta diversity represents the difference between samples. The OTU sequences were annotated using the Mothur method and the SSUrRNA database of SILVA132 to perform species annotation analysis (the threshold was set to 0.8–1.0), and taxonomic information was obtained at each classification level to define the community composition of the sample.

2.3. Determination of BA contents

The content of BAs in soybean paste was determined by high-performance liquid chromatography (HPLC) (LC-20AT, SHIMADZU, Japan). Eight BAs or amine hydrochloride and an internal standard (1,7-diaminoheptane) were purchased from Shanghai Shifeng Biotechnology Co., Ltd., China, and a derivative (dansyl chloride) was purchased from Sigma–Aldrich (USA). The eight BAs are tryptamine (Try), β-phenethylamine (Phe), putrescine (Put), cadaverine (Cad), histamine (His), tyramine (Tyr), spermine (Spm), and spermidine (Spd).

To determine the content of BAs, 500 μl of 1.0 mg/mL 1,7-diaminoheptane internal standard was added to a 10 g sample of soybean paste, 20 mL of 5% trichloroacetic acid (TCA) was added to the sample, and the mixture was homogenized for 30 min. The homogenate was centrifuged at 5000 rpm/min for 10 min. The supernatant was collected, and the residue was extracted once with the same volume of 5% TCA. All supernatants were combined, and the final volume was made up to 50 mL with 5% TCA.

One milliliter of extracted sample or standard BAs solution was mixed with 200 μL of 2 M NaOH, 300 μL of saturated $NaHCO_3$ and 2 mL of dansyl chloride solution (10 mg/mL, using acetone as the solvent) and was then heated in a water bath at 40 °C for 45 min. After that, 100 μL of ammonia water (25%) was added and then reacted for 30 min in the dark. When the above reaction was finished, the sample was filtered with a 0.22 μm membrane for subsequent determination (Gao et al., 2018).

The BAs content were analyzed using a HPLC detector equipped with a UV–vis detector (LC-20AT, SHIMADZU, Japan). Separation was performed using a 5 μm (250 mm × 4.6 mm) C18 column (SHIMADZU, Japan). Mobile phase A was 90% acetonitrile and 10% 0.01 M ammonium acetate (containing 0.1% acetic acid). Mobile phase B was 10% acetonitrile and 90% 0.01 M ammonium acetate (mixed with 0.1% acetic acid). The column temperature was 35 °C. The injection volume was 20 μl, and the detection wavelength was 254 nm. The above experimental process was repeated 3 times independently for each sample determination.

2.4. Determination of physicochemical properties

The amino-type nitrogen(ANN) and titratable acidity(TTA) contents were measured according to the method described by Kim et al. (2020b) and Liang et al. (2019). The total number of colonies was determined according to GB 4789.2–2016. The above experimental process was repeated 3 times independently for each sample determination.

2.5. Data analysis

The process of bacterial diversity data processing and correlation analysis was carried out on the official website of Novogene Co., Ltd (https://magic.novogene.com/customer/). Statistical analysis was performed using by SPSS software (version 26.0, SPSS Inc. Chicago, USA). Data were analyzed with two-way analysis of variance (ANOVA) and Duncan's test to determine the differences between different fermentation times and between different treatments. A P value of <0.05 or <0.01 was considered significant or extremely significant. All measurements were conducted in triplicate and the results are expressed as the mean ± standard deviation (n = 3). Other statistical and PCA analyses and graphic construction were performed in Origin software (version 2021, OriginLab, USA).

3. Results

3.1. Bacterial diversity analysis of farmhouse soybean paste in Northeast China

3.1.1. Alpha diversity indices of farmhouse soybean paste in Northeast China

As shown in Fig. 1, the bacterial diversity (Shannon and Simpson) in soybean paste with salt concentrations of 8% and 9% increased to the highest point at 30 d and decreased at 30–90 d of fermentation. The bacterial diversity (Shannon and Simpson) in the soybean paste with salt concentrations of 10%, 11% and 12% decreased to the lowest point at 30 d and increased from 30 to 90 d. The bacterial richness (Chao1 and ACE) in the soybean paste with salt concentrations of 8%, 9%, 10%, and 11% increased from 0 to 30 d, decreased from 30 to 60 d and then increased until 90 d of fermentation. The bacterial richness (Chao1 and ACE) of 12% decreased from 0 to 60 d of fermentation and then increased until 90 d. At 30 and 60 d of fermentation, the bacterial diversity (Shannon and Simpson) and bacterial richness (Shannon and Simpson) of soybean paste showed overall decreasing trends with increasing salt concentration. At 90 d of fermentation, the bacterial diversity (Shannon and Simpson) did not change obviously with the increasing of salt concentration, while the bacterial richness (Chao1 and ACE) showed a decreasing trend.

3.1.2. Changes in bacterial populations of farmhouse soybean paste in Northeast China

At 0 d of fermentation in soybean paste, the dominant genera were *Staphylococcus* (35.4%), unidentified *Clostridiales* (26.0%), and *Sporosarcina* (22.6%) (Fig. 2). These three genera accounted for 84% of the overall proportion. In addition, there were some special genera at 0 d that were only present in the samples at 0 d and were not detected in the subsequent fermentation process, including *Chujaibacter*, *Acidibacter, Bordetella, Rhodanobacter,* and *Acidipila*. However, other genera, such as *Salinimicrobium, Jeotgalicoccus, Pontibacter, Enterococcus,* and *Weissella* were not detected at the beginning of fermentation, but persisted during 30–90 d of fermentation. At 30 d of fermentation, the dominant genera in the soybean paste became *Tetragenococcus*, *Staphylococcus* and unidentified *Corynebacteriaceae*. When the salt concentration increased from 8% to 12%, the proportion of *Tetragenococcus* gradually increased from 20.3% to 51.4%. The proportion of *Staphylococcus* in soybean paste with a salt concentration of 8%–10% was between 16.8% and 20.8%. When it increased from 10% to 12%, the

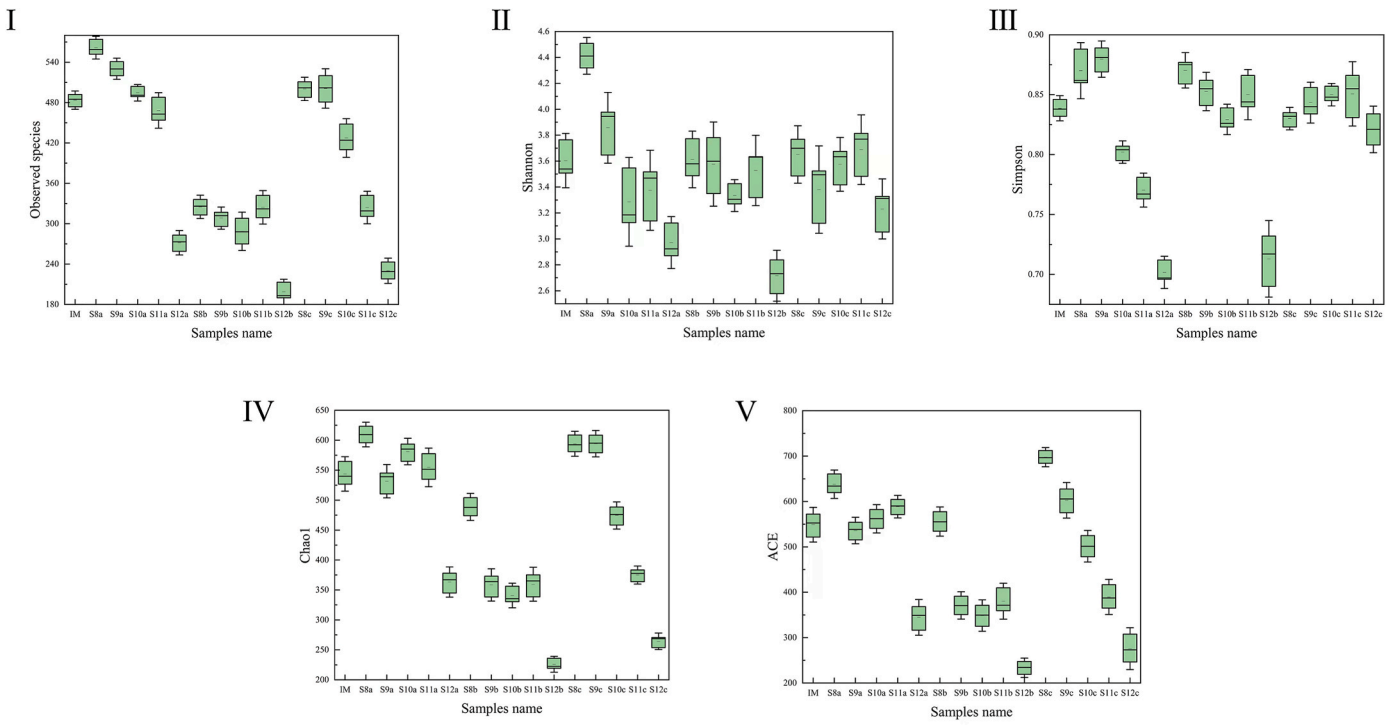

Fig. 1. The α-diversity indexes of farmhouse soybean paste in Northeast China
I-V represent the Observed species, Chao1, ACE, Shannon, and Simpson, respectively. Observed species, Chao1, and ACE represent the bacterial richness; the larger the value is, the higher the bacterial richness. Shannon and Simpson indices represent the bacterial diversity; the larger the value is, the higher the bacterial diversity. S8–S12 represent the salt concentrations of 8%, 9%, 10%, 11% and 12%, respectively. IM represents the sample fermented for 0 d, **a** represents the sample fermented for 30 d, **b** represents the sample fermented for 60 d, and **c** represents the sample fermented for 90 d.

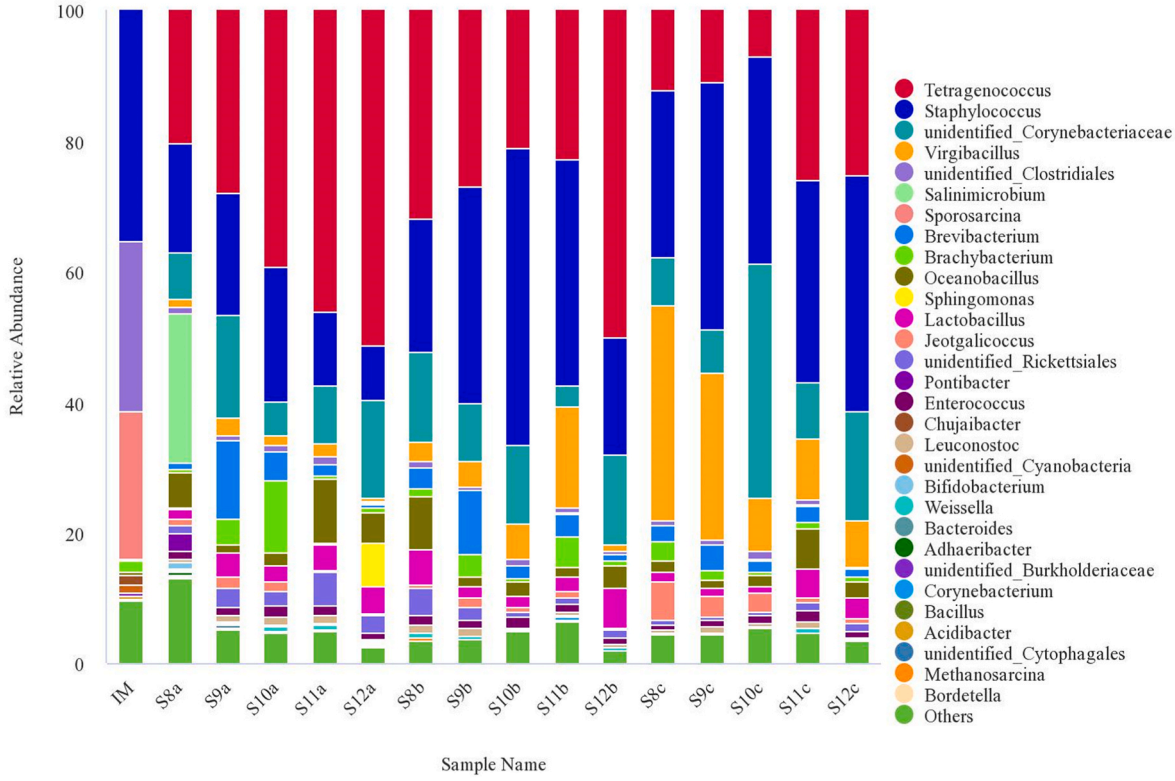

Fig. 2. Species distribution at the genus level of farmhouse soybean paste in Northeast China
S8–S12 represent the salt concentrations of 8%, 9%, 10%, 11% and 12%, respectively. IM represents the sample fermented for 0 d, **a** represents the sample fermented for 30 d, **b** represents the sample fermented for 60 d, and **c** represents the sample fermented for 90 d.

proportion of *Staphylococcus* decreased from 20.8% to 8.3%. At 60 d of fermentation, the proportion of *Tetragenococcus* was lower than that at 30d, and the proportion of *Staphylococcus* was higher than that at 30 d in samples with the same salt concentration. At 90 d of fermentation, the overall proportion of *Tetragenococcus* continued to decrease, but the proportion of *Staphylococcus* did not change significantly compared with that at 60 d in samples with the same salt concentration. At the same time, a high proportion of *Virgibacillus* appeared. When the salt concentration increased from 8% to 12%, the proportion of *Tetragenococcus* increased from 12.6% to 25.4%, and the proportion of *Virgibacillus* decreased from 33.1% to 7.0%.

3.1.3. Beta diversity index of farmhouse soybean paste in Northeast China

The species differences between samples of soybean paste are shown in Fig. 3, where the numbers in the grid are the difference coefficients between the two samples. The difference coefficient represents the difference in species diversity between the two samples. As shown in Fig. 3, the two samples with the smallest differences in species diversity were the 90 d samples with salt concentrations of 8% and 9%, and the difference coefficient was 0.043. The two samples with the largest differences were the samples with a salt concentration of 8% at 0 d and 30 d, of which the difference coefficient was 0.440. During the fermentation process of soybean paste, the differences in species diversity between the samples at 0 d and 30 d were the largest, and the average value of the difference coefficient was 0.370. The differences between the samples at 60 d and 90 d were the smallest, and the average value of the difference coefficient was 0.128. These results further illustrated that there was a species succession process from 0 to 30 d of fermentation, while the bacterial microbiotas gradually stabilized after 30 d of fermentation during the fermentation process of soybean paste. Among the different salt concentrations of soybean paste, the differences between the samples with salt concentrations of 10% and 8% were the largest, the average value of the difference coefficient was 0.207, and the differences between the samples with salt concentrations of 12% and 11% were the smallest, with an average value of the difference coefficient of 0.103. At the same time, the difference in species was negatively related to the salt concentration, meaning that when the salt concentration was lower, the species differences between the two adjacent salt concentrations were larger.

According to the PCA of the top 30 genera (Fig. 4), there was an obvious difference between the sample at 0 d of fermentation and other samples, while the difference between the samples fermented from 30 to 90 d was relatively small. The 30 d samples were mainly concentrated on the left side of the PC1 axis, the 60 d samples were mainly concentrated

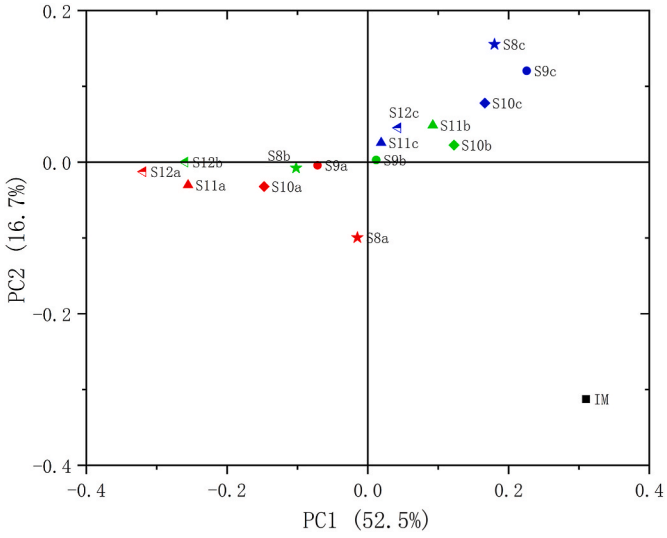

Fig. 4. The PCA of farmhouse soybean paste in Northeast China
S8–S12 represent the salt concentrations of 8%, 9%, 10%, 11% and 12%, respectively. IM represents the sample fermented for 0 d, **a** represents the sample fermented for 30 d, **b** represents the sample fermented for 60 d, and **c** represents the sample fermented for 90 d.

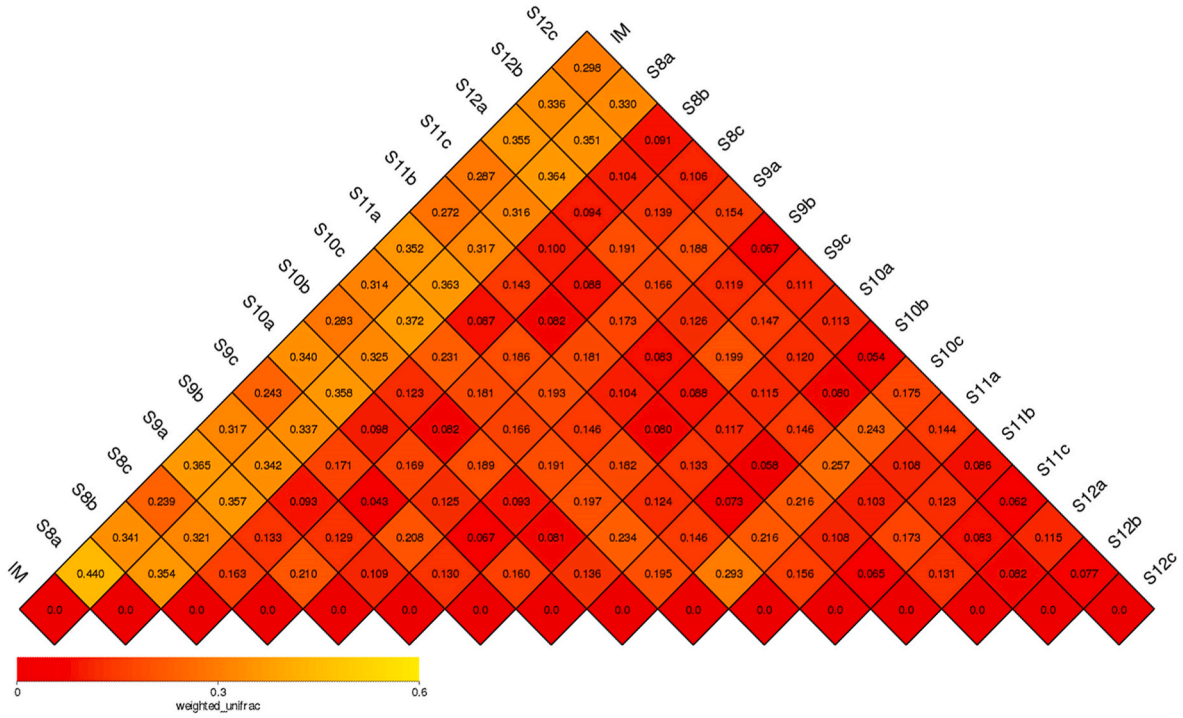

Fig. 3. The β-diversity indexes of farmhouse soybean paste in Northeast China
S8-S12 represent the salt concentrations of 8%, 9%, 10%, 11% and 12%, respectively. IM represents the sample fermented for 0 d, **a** represents the sample fermented for 30 d, **b** represents the sample fermented for 60 d, and **c** represents the sample fermented for 90 d.

on the middle of the PC1 axis, and the 90 d samples were concentrated on the right side of the PC1 axis. Comparing the samples with the same fermentation time, the samples gradually moved from right toleft on the PC1 axis with increasing salt concentration at 30 d and 90 d. At 60 d, when the salt concentration is increased from 8% to 11%, the samples gradually moved from left to right on the PC1 axis. Fig. 3 shows that the difference between samples with the same salt concentration at different fermentation times is larger than the samples with different salt concentrations at the same fermentation time.

3.2. BA content in farmhouse soybean paste in Northeast China

Fig. 5 shows that there were certain amounts of BAs in the soybean paste at 0 d of fermentation, and these BAs were produced during the preparation of koji. At this time, there was a large amount of Spm (131.25 mg/kg) in the sample, the contents of the other BAs were all below 10 mg/kg, and no Cad was detected. At 30 d of fermentation, Spm and Spd had no significant changes compared with 0 d, while the contents of other BAs significantly increased. In the samples with different salt concentrations, the contents of Spd, Try, Tyr, Cad, Put, Phe, and His showed significant differences. Compared with the 8% samples, Spd, Try, Tyr, Cad, Put and His decreased by 2.15 mg/kg (38%), 2.33 mg/kg (26%), 13.25 mg/kg (67%), 30.62 mg/kg (93%), 8.84 mg/kg (69%), 5.6 mg/kg (35%), and 100.98 mg/kg (86%), respectively, in the 12% samples. At 60 d of fermentation, compared with 30 d, Spm still had no significant change, Spd began to decrease significantly, and other BAs continued to increase significantly. When the salt concentration increased from 8% to 12%, Spd, Try, Cad, Phe, Put, and His decreased by 2.39 mg/kg (55%), 2.63 mg/kg (25%), 31.69 mg/kg (69%), 17.54 mg/kg (62%), 6.47 mg/kg (32%), and 105.58 mg/kg (71%), respectively. At 90 d of fermentation, compared with 60 d, the contents of Spd, Spm, Put and His significantly decreased. Try, Cad and Phe continued to increase significantly while Tyr had no significant change. When the salt concentration increased from 8% to 12%, Try, Cad, Phe, Put and His decreased by 3.1 mg/kg (26%), 74.67 mg/kg (67%), 9.42 mg/kg (33%), 8.64 mg/kg (55%), and 59.72 mg/kg (56%), respectively. During the whole fermentation process, there were no significant differences between Spm in samples with different salt concentrations ($P < 0.05$).

3.3. Physicochemical properties of farmhouse soybean paste in Northeast China

From 0 d to 60 d of fermentation, the amino acid nitrogen (ANN) contents in all samples increased significantly with the extension of fermentation time (Fig. 6). From 60 d to 90 d, there was no significant change. At all sampling times, ANN decreased significantly with increasing salt concentration. The ANN contents of soybean paste with the 8% and 9% salt concentrations were higher than those in the salt concentrations of 10%, 11%, and 12%. From 0 d to 90 d of fermentation, the content of total acid (TTA) in all samples increased significantly with the extension of fermentation time until the end of fermentation. At all sampling times, the TTA decreased significantly with increasing salt concentration. For the total number of colonies in the soybean paste (Fig. 6) increased significantly with the extension of fermentation time from 0 d to 60 d in all samples. From 60 d to 90 d, there was no significant change. When the salt concentration ranged from 8% to 11%, the total number of colonies decreased with increasing salt concentration, and there was no obvious change when the salt concentration was higher than 11%.

3.4. Correlations between bacterial genera and BAs in farmhouse soybean paste in Northeast China

In this study, the analysis of the correlations between the main 35 genera and BAs in the soybean paste is shown in Fig. 7, among which 15 genera showed correlations with BAs. Twelve genera showed significant positive correlations with BAs ($P < 0.05$), of which 6 genera showed extremely significant positive correlations with BAs ($P < 0.01$). *Virgibacillus, Jeotgalicoccus*, and *Yaniella* had extremely significant positive correlations ($P < 0.01$) with Cad. *Sporosarcina* and *Rhodanobater* had extremely significant positive correlations ($P < 0.01$) with Spd. Unidentified *Cyanobacteria* had extremely significant positive correlations ($P < 0.01$) with Spd and Spm. Four genera showed significant negative correlations with BAs ($P < 0.05$), of which 1 genus showed an extremely significant negative correlation with BAs ($P < 0.01$). *Virgibacillus* had extremely significant negative correlations ($P < 0.01$) with Spd and Spm.

4. Discussion

The dominant genera in farmhouse soybean paste at the beginning of fermentation were mainly brought into the fermentation system from koji. The proportions of these dominant genera gradually decreased with increasing fermentation time from 30 to 90 d. As a result, they were gradually replaced by some new dominant genera from the surrounding environment. That is, a process of gradual alternation of the dominant genera appeared. From the results described above, it was found that the dominant genera gradually changed to salt-tolerant *Tetragenococcus* and *Staphylococcus*, as similarly discussed in previous studies (An et al., 2020, 2021). Although in such studies the dominant genera at the beginning of the fermentation were not exactly the same, after the addition of salt, most of the dominant genera became *Tetragenococcus*. It has been reported that this genus can grow under salt concentrations of 0%–25% (w/v) thus considered a super salt-tolerant genus (Ogasawara et al., 2006; Yongsawatdigul et al., 2010). During this study, the proportion of *Tetragenococcus* in soybean paste increased with increasing salt concentration, which may account for the effect of the increasing salt concentration resulting in the number of genera with weak salt tolerance gradually decreasing while the proportion of the more salt-tolerant *Tetragenococcus* increased. However, aside from *Tetragenococcus*, the other genera involved in the fermentation process of soybean paste certainly varied from previous studies (Nam et al., 2012; Kim et al., 2016; Sun et al., 2018). This difference may be because soybean paste is produced by natural fermentation in an open environment, in which the microbiota is greatly affected by the specific environmental conditions of different production areas.

BAs, ANN and TTA are all important metabolites of microbiota in soybean paste. One important way of producing BAs is synthesis via microorganism activities through decarboxylases of precursor amino acids (Houicher et al., 2021; Zhao et al., 2020). At the same time, some microbiota can produce decarboxylases to degrade BAs (Li and Lu, 2020; Pištěková et al., 2020). The content of BAs in fermented products is the result of the joint action of these two different metabolic pathways. In this study, Cad was not detected in the samples at 0 d of fermentation, and a large amount of Cad began to accumulate as the fermentation progressed. Therefore, it is possible that Cad may be produced by genera that do not exist in the koji but enter the soybean paste from the environment (Fig. 2), such as *Jeotgalicoccus*, as mentioned in Sections 3.1.2 (Fig. 2) and 3.4 (Fig. 7). In the results of this article, special attention should be paid to the changes in Spm and Spd. As polyamines, the production processes of Spm and Spd are more complicated than those of other BAs. Spm and Spd take Put as the precursor substance (del Rio et al., 2018). Put is transformed from ornithine decarboxylation into Spd under the action of Spd synthase and finally into Spm under the action of Spm synthase (Shantz and Pegg, 1999). The BA with the highest content at 0 d was Spm, and during the subsequent fermentation process, the content of Spm and Spd continued to decrease. At the same time, although the content of Put decreased with increasing salt concentration, Spm and Spd did not change with the change in salt concentration. From this it can be speculated that Spd synthase may be produced by the genera mentioned above that only exist in koji, which has weak salt tolerance (Fig. 2). When these genera gradually disappear as

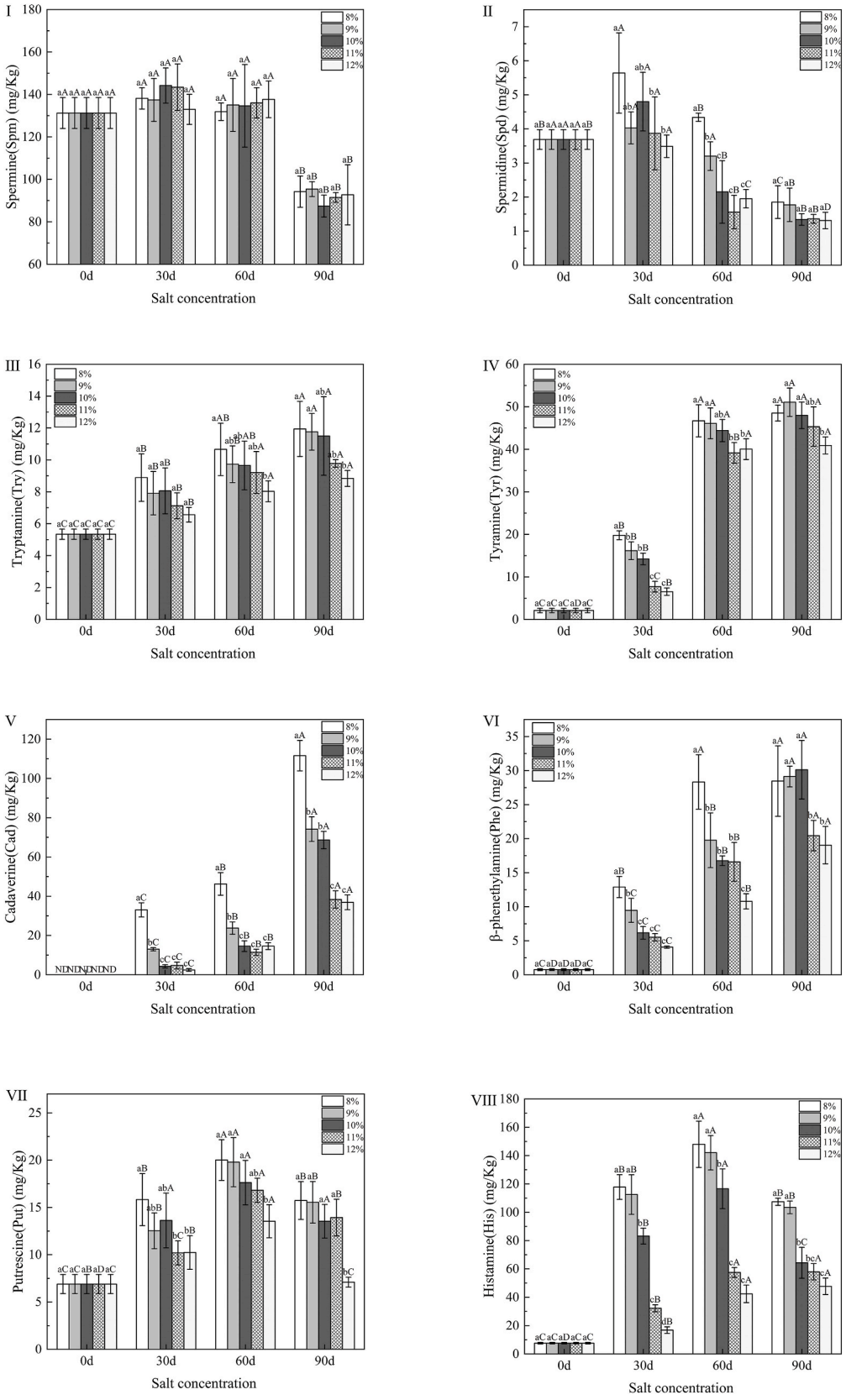

Fig. 5. Contents of 8 kinds of BAs in farmhouse soybean paste in Northeast China.
I-Ⅷ represent the content of Spermine (Spm), Spermidine (Spd), Tryptamine (Try), Tyramine (Tyr), Cadaverine (Cad), β-phenethylamine (Phe), Putrescine (Put) and Histamine (His), respectively. The different uppercases(A-D) show significantly different between samples with same salt concentration during the different fermentation time($P < 0.05$), and the different lowercases(a-d) show significantly different between samples with the different salt concentrations during the same fermentation time($P < 0.05$). ND represent not detected.

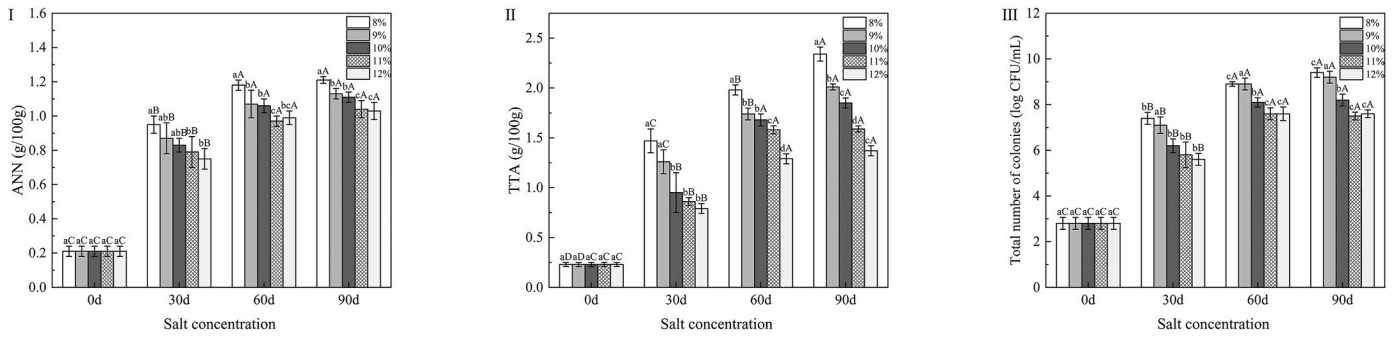

Fig. 6. Physicochemical properties of farmhouse soybean paste in Northeast China.
I-III represent the content of ANN, TTA and the total number of colonies, respectively. The different uppercases(A-D) show significantly different between samples with same salt concentration during the different fermentation time($P < 0.05$), and the different lowercases(a-e) show significantly different between samples with different salt concentrations during the same fermentation time($P < 0.05$).

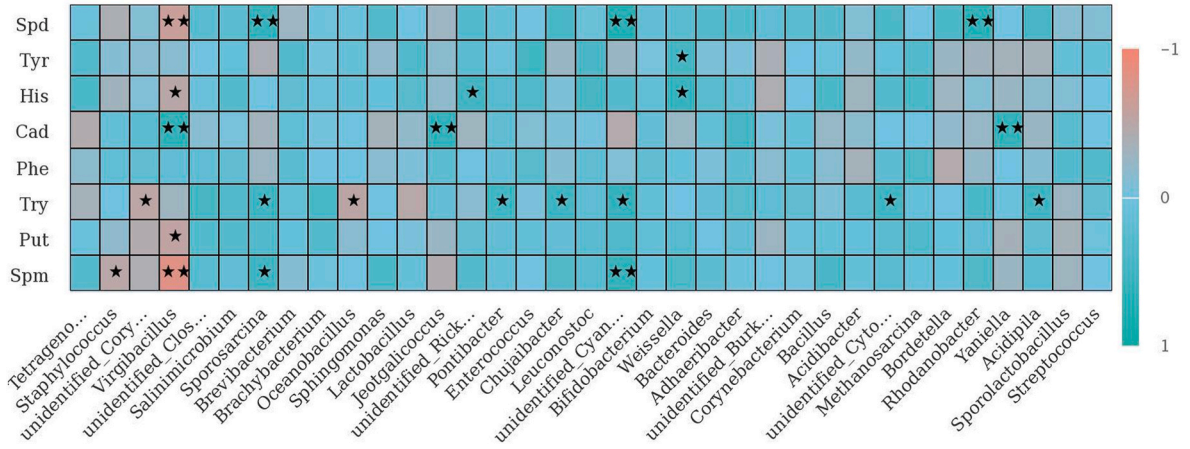

Fig. 7. Correlation heat map of genera and BAs in farmhouse soybean paste in Northeast China.
* $0.01 < P \leq 0.05$, ** $0.001 < P \leq 0.01$.

fermentation progresses, Spm and Spd are no longer produced. At the same time, some genera that can decompose Spm and Spd have also entered the soybean paste, so Spm and Spd have continued to decrease. The other BAs existed from 0 d to 90 d, but their contents decreased with increasing salt concentrations. This variation trend is present in not only soybean paste but also other fermented soy products (Yang et al., 2021) and even fermented meat products (Roseiro et al., 2006; Laranjo et al., 2017; Liu et al., 2020; Roseiro et al., 2017). When the salt concentration dropped reduced to 9% and below, the His content in soybean paste exceeded 100 mg/kg. His is one of the most toxic BAs (Ezzat et al., 2015). There is a study have shown that when His is present at concentrations of more than 100 mg/kg, it produces a serious poisoning reaction (Wang et al., 2016). At the same time, His and Tyr also have synergistic toxicity (del Rio et al., 2017). The European Commission (EC) provides a recommended limit of 100 mg/kg for histamine in food (European Commission, 2003). Therefore, when the salt concentration reduced to 9% and below, His in soybean paste might cause potential harm to humans. TTA and ANN are both metabolites of microorganisms in soybean paste, and they are important indicators for measuring soybean paste quality. ANN, released from the decomposition of soy proteins by protease, can reflect the degree of conversion of nutrients in soybean paste (Kim et al., 2020b), and the content of ANN is affected by not only microorganisms but also raw materials to a large extent. Therefore, by comparing other studies, it can be found that although external conditions such as salt concentration will affect the amount of ANN produced, it is difficult for the maximum amount to exceed 1.5 g/100 g (Kim et al., 2020b; Ryu et al., 2021; Yang et al., 2021). TTA stands for all free acids and acid salts in soybean paste. A high TTA will affect the sensory quality of soybean paste, so the TTA was generally controlled below 2.0 g/100 g. At the same time, the TTA can reflect the overall proliferation of the microbiota in the soybean paste. It can be seen from this study that when the salt concentration is 10%, the microbial growth in the soybean paste is controlled, and the accumulation of TTA can be controlled while ensuring the conversion of ANN. The total number of colonies can reflect the value added of the microorganisms in the soybean paste. During the fermentation process, from 0 to 60 d, due to the rich nutrients in the raw materials, various microbiota continued to proliferate. After 60 d, the microbiotas in the soybean paste reached a dynamic equilibrium, the total amount no longer changed, and may even showed a downward trend (Choi et al., 2007; Bian and Li, 2019).

For the correlation analysis, 12 genera showed positive correlations with different BAs, indicating that they may be the main genera producing BAs in soybean paste. In addition, 4 genera showed negative correlations with BAs. Among them, *Virgibacillus* showed a negative correlation with Spm, Spd, His and Put. Some studies suggested that *Virgibacillus* was negatively related to histidine and arginine metabolism according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis(Jia et al., 2021); these amino acids are the precursors to produce these BAs. Therefore, this may be the reason why *Virgibacillus* have significant negative correlation with these BAs in this study However, there other studies have come to different conclusions on fermented soy products. During the fermentation process of Ganjiang in South Korea, *Virgibacillus* did not show the ability to degrade BAs but

could produce Tyr (Kim et al., 2021). In this study, *Tetragenococcus* did not show correlations with any BAs. However, in the fermentation process of Chinese shuidouchi, *Tetragenoooccus* could produce a large amount of Tyr (Chen et al., 2019). The effect of *Tetragenoooccus* on His production was related to its different strains. Some strains can produce His, and some strains can inhibit the production of His (Kuda et al., 2012). At the same time, *Tetragenoooccus* had a significant inhibitory effect on Cad (Kim et al., 2019). In Chinese sufu, *Streptococcus*, *Enterococcus* and Spd, Put, Try showed significant negative correlations (Liang et al., 2019). Research on other fermented products in wine showed that *Leuconostoc* has a high ability to produce Tyr or His (Aflflaki et al., 2014). In Cheonggukjang, South Korea, *Bacillus* spp. can produce His and Put, and *Enterococcus* spp. can produce Tyr (Jeon et al., 2018). However, these four genera did not show any correlation with any BAs in this study. Other studies have shown that at the end of ripening of fermented meat products, *Staphylococcus* have the ability to degrade Tyr and Put (Schirone et al., 2022). In this study, *Staphylococcus* was significantly positively correlated with Spm. From the above discussion, it can be seen that in different studies, the same genus may have different biogenic amine-producing and biogenic amine-degrading abilities. Therefore it was proposed that the accumulation of BAs might not be related to genus but to different strains (Paulsen et al., 2012). These studies provide a theoretical basis for the rational control of BAs in fermented products in the future. Based on these research results, key strains can be screened for testing to select starter cultures suitable for different fermented products.

5. Conclusion

The results of this study conclude that the addition of salt had significant effects on bacteria richness (Chao1 and ACE), bacterial diversity (Shannon and Simpson), BAs formation and physicochemical properties in farmhouse soybean paste in Northeast China. The bacterial richness and diversity in soybean paste gradually decreased with increasing salt concentration. Except for Spm and Spd, the contents of the other remaining six BAs showed increasing trends. The formation of BAs had a certain relationship with the growth and decline of some specific genera during the fermentation of soybean paste. ANN, TTA and the total number of colonies gradually decreased with increasing salt concentration. When the salt concentration is reduced to 9% and below, the His of more than 100 mg/kg in the soybean paste may cause potential harm to the human body, and the TTA exceeding 2.0 g/100g will affect the quality of the soybean paste. Therefore, from the perspective of food safety and product quality, under the current production conditions, the amount of salt added in the soybean paste can be reduced to 10%. These results provide some basis for deepening the understanding of the subsequent inhibition of the formation of BAs through the control of bacteria in the fermentation process as well as to a certain extent providing theoretical support for the development of reduced-salt soybean paste. However, this article only focused on the impact of salt reduction on soybean paste under natural fermentation conditions. On the basis of this study, follow-up studies should also screen microorganisms with low amine-producing ability or capable of degrading biogenic amines as starter culture. At the same time, with the adjustment of fermentation conditions and processes, while ensuring the safety of soybean paste, it will continue to reduce the amount of salt added in soybean paste.

Credit author statement

Sun B, Xie SY and Li Z designed the research; Xie SY, Li Z and Sun B performed the research; Xie SY, Li Z and Zhang Y did the lab work and acquired data; Xie SY, Li Z and Zhang Y analyzed the data; Xie SY, Li Z and Sun B wrote the manuscript. Xie SY, Li Z and Sun B revised the manuscript.

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.

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