Scientific and Social Research, 2025, Volume 7, Issue 1
https://ojs.bbwpublisher.com/index.php/SSR
Online ISSN: 2981-9946
Print ISSN: 2661-4332

Study on the Effect of Mixed Strains of Bacteria on the Fermentation Quality of Natto Beans

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Abstract: During the fermentation process of natto, the decomposition of soy protein by *Bacillus subtilis* produces ammonia-nitrogen-like substances, resulting in a finished product with an ammonia-fishy odor, which affects consumer acceptance. In this study, *Bifidobacterium bifidum*, *Lactobacillus lactis,* and *Bacillus subtilis* were mixed for natto fermentation experiments, respectively, aiming to improve its flavor characteristics. The experimental results showed that fermentation with mixed strains significantly changed the volatile matter composition of natto. Among them, the electronic nose analysis showed that the content of nitrogen oxides and inorganic sulfides increased significantly in the fermented samples, while the ammonia nitrogen species decreased relatively. In addition, the amino acid analyzer test revealed that the total amount of free amino acids was significantly increased after fermentation of the mixed strains, while several flavor-improving amino acids also occupied a larger proportion. The experimental results indicated that the addition of Bifidobacterium and *Lactobacillus* could effectively regulate the metabolites during natto fermentation, reduce the ammonia-fishy odor, and improve the fermentation quality, which has potential application value.

Keywords: *Bifidobacteria*; *Lactobacillus*; *Bacillus subtilis*; Natto; Fermentation quality; Amino acids

Online publication: January 23, 2025

1. Introduction

1.1. Origin and nutrition of natto

Natto is a traditional fermented soybean product, usually made by fermenting soybeans with *Bacillus subtilis*. It has a unique flavor and viscosity, and not only retains the nutritional value of soybeans but also improves the digestibility and absorption of proteins. Under the action of microorganisms, natto produces a variety of physiologically active substances, such as nattokinase, soybean flavonoids, phytosterols, superoxide dismutase, and several biologically active peptides from raw soybeans, and regular consumption of natto improves immunity and physical fitness [1]. However, during the fermentation process of natto, due to the hydrolysis of rich soy protein under the action of protease, ammonia nitrogen-like substances with ammonia fishy odor are often produced, of which the ammonia taste and bitter taste are often difficult for people to accept [2].

1.2. Research conception

Bacillus subtilis is a subspecies of *Bacillus subtilis*, which is the main microorganism for fermentation of natto at home and abroad. The secreted extracellular protease and other extracellular enzymes can decompose various macromolecules to produce various nutrients and physiologically active substances with healthcare functions [3].

Bifidobacteria are important beneficial microorganisms in the intestinal tract. As beneficial microorganisms, Bifidobacteria have biological barriers, nutritional support, anti-tumor and immune-enhancing effects on human health, improve the function of the gastrointestinal tract, and can also play an anti-aging role [4].

Lactobacillus is a collective term for bacteria that can utilize fermentable carbohydrates to produce large amounts of lactic acid. It can improve the function of the human gastrointestinal tract, restore the balance of bacterial flora in the human intestinal tract, form antimicrobial biological barriers, maintain human health; regulate immunity, and can enhance human immunity and resistance [5].

1.3. Research conception

The flavor is an important factor affecting the quality and value of food, and in natto, the flavor of natto has been the focus of research because of the special ammonia-fishy odor [6–8]. The flavor of natto mainly depends on microorganisms and fermentation conditions, and the formation of flavor substances is a complex and variable biochemical process. In recent years, there have been a lot of studies on improving the flavor of natto, mainly using the following methods: raw material screening and matching, strain screening, strain improvement, mixed-bacteria fermentation, control of fermentation conditions, seasoning, and freeze-drying, etc. All of these methods can, to a certain extent, play a great role in the improvement of the flavor and quality of natto [9–11].

During the fermentation of natto, due to the hydrolysis of rich soy protein under the action of protease, ammonia and nitrogen-like substances with ammonia-fishy odor are often produced, and most of these substances are alkaline compounds. In this experiment, *Bifidobacterium bifidum*, *Bacillus subtilis*, and *Lactobacillus* single and mixed bacteria were utilized to ferment natto. It is hoped that the various flavor substances and acidic components produced by the fermentation of *Lactobacillus* and *Bifidobacterium* lactis, such as lactic acid and acetic acid, can be neutralized with alkaline compounds acid and alkaline to a certain extent, thus alleviating the undesirable odors of the natto, and at the same time enriching the flavor qualities of the natto.

2. Materials and methods

2.1. Experimental materials

2.1.1. Bacterial strains

100 g of each of *Bifidobacterium bifidum*, *Bacillus subtilis*, and *Lactobacillus* were purchased and stored in a refrigerator at 4°C in the freezer for spare use (Production company: Xi'an Jushengyuan Biotechnology Co., Ltd., China), as shown in **Figure 1**. For convenience, KC stands for *Bacillus subtilis*, SQ stands for *Bifidobacterium bifidum*, and RS stands for *Lactobacillus* in the following experiments.

Bacillus subtilis *Bifidobacteria* *Lactobacilli*

Figure 1. Experimental strains of bacteria

The organic soybeans (Origin: Jilin, China Grade: Grade I) used are shown in **Figure 2**.

Figure 2. Experimental soybean

2.1.2. Experimental apparatus

Natto fermenter (Model: RS-G29 Brand: Rongshida, China)
Pulverizer (Model: LL-A Brand: Liren, China)
Incubator (Model: Huaguan Digital Oscillation Incubator, China)
Automatic amino acid analyzer (Model: L-8900, China)
Electronic Nose (Model PEN3 from AIRSENSE, Germany)
Digital Food Thermometer (Wenzhou Mittel Intelligent Technology Co., Ltd., China)

2.2. Methods

2.2.1. Pre-treatment of bean seeds

(1) Selection: Pick out the beans that are half, peeled, scabbed, and other conditions, and keep the soybeans that have bright outer skin color, clean skin, and full and neat grains (**Figure 3**).

(2) Cleaning: Wash with water more than three times, until the water color is clear

(3) Soaking: Soak in water at 19°C water temperature for 12 hours.

Pouring beans Washing beans Picking beans Soaking beans

Figure 3. Pre-processing

2.2.2. Fermentation experiment of natto beans

Soaked soybeans were divided into conical flasks according to 200 g per portion, covered with a sealing film, and sterilized in an autoclave at 121°C for 20 minutes (**Figure 4**). After removal, it was placed on the ultra-clean bench to dry to room temperature.

Figure 4. Samples of natto to be fermented

According to the preparation method in the table below (**Table 1**), the bacterial powder was weighed separately and dissolved in 10 ml of sterile water to prepare a single bacterial solution and mixed well.

Table 1. Bacterial solution preparation table

Sample No.	**Strain and amount of bacteria added**		
	***Bifidobacterium bifidum* (g)**	***Bacillus subtilis* (g)**	***Lactobacillus* (g)**
KC	0	3	0
SQ	3	0	0
RS	0	0	3
KC-SQ-1	1	3	0
KC-SQ-2	2	3	0
KC-SQ-3	3	3	0
KC-RS-1	0	3	1
KC-RS-2	0	3	2
KC-RS-3	0	3	3

2.2.3. Sensory evaluation test of natto samples

The fermented natto samples were subjected to preliminary sensory observations in five aspects: color, aroma, taste, length of pull, and amount of mucus.

2.2.4. Electronic nose testing experiment

The samples were homogenized and sent to the Beijing Food Research Institute for testing. The electronic nose took readings every 1s through 10 sensors for the 10 main volatile substances in **Table 2** for a total of 90s consecutive readings.

Table 2. Table of main substances measured by different sensors

Sensor name	Main detected substances
W1C	Aromatic ingredients
W5S	Nitrogen oxides
W3C	Ammonia
W6S	Hydrides
W5C	Short-chain alkanes
W1S	Methyls
W1W	Inorganic sulfides
W2S	P-alcohols, aldehydes and ketones
W2W	Organic sulfides
W3S	Long-chain alkanes

2.2.5. Detection of free amino acids in natto

After the samples were crushed and homogenized, they were sent to the laboratory of the Beijing Academy of Agricultural and Forestry Sciences for testing the free amino acid content of natto using the fully automatic amino acid analyzer method with reference to the national standard of GB/T30987-2020 “Determination of Free Amino Acids in Plants” in China.

3. Results and analysis

3.1. Sensory evaluation

From **Table 3**, it can be seen that when a single strain was fermented, *Bifidobacterium bifidum* was better than *Bacillus subtilis* and *Lactobacillus* in terms of color, taste, and all aspects, the length of the draw was longer and there was more mucus. However, the ammonia smell was heavier. When the ratio of *Bacillus subtilis* and *Bifidobacterium bifidum* was 1:1, the color was improved, the surface was glossy, and the taste was changed, but at the same time, the ammonia smell was aggravated. When *Bacillus subtilis* and *Lactobacillus lactis* were paired in the ratio of 1:1 the ammonia smell was reduced, which indicated that the mixed-bacteria fermentation could significantly improve the flavor and color of the natto, but it did not have much effect on the taste, drawing, and mucus effect of the fermented natto.

Table 3. Effect of fermentation of three bacteria on the quality of natto beans

Strain	Color	Incense	Flavor	Wire drawing	Mucous
SQ	Bright color, shiny skin	Heavy ammonia odor	Softer, wetter, slightly bitter aftertaste	12–20	More
KC	Dull color, no luster	General ammonia odor	Softer, drier, more bitter aftertaste	6–10	General
RS	Dull color, no luster	A little ammonia.	Softer, drier, more bitter aftertaste	0–5	No
KC-SQ mixing	Bright color, shiny skin	Heavy ammonia odor	Softer, wetter, slightly bitter aftertaste	6–15	More
KC-RS mixing	Bright color, shiny skin	A little ammonia.	Softer, wetter, slightly bitter aftertaste	0–5	General

3.2. Evaluation of electronic noses

As shown in **Figure 5**, the odor signal data of natto samples detected by the electronic nose were analyzed by PCA, and the 0-coordinate boundaries of the two principal components were taken as the axes to show that the natto fermented with different strains was in a dispersed state, and except for the mixture of *Bacillus subtilis* with *Lactobacillus* and *Bifidobacterium bifidum* 1;1, there was an intersection of the detected odors, and the other components of the mixed-strain components were more dispersed than those of the single component, which indicated that the natto fermented with the mixed strain had a significant difference from that of the single component in terms of the odors. This shows that a single component had a significant difference.

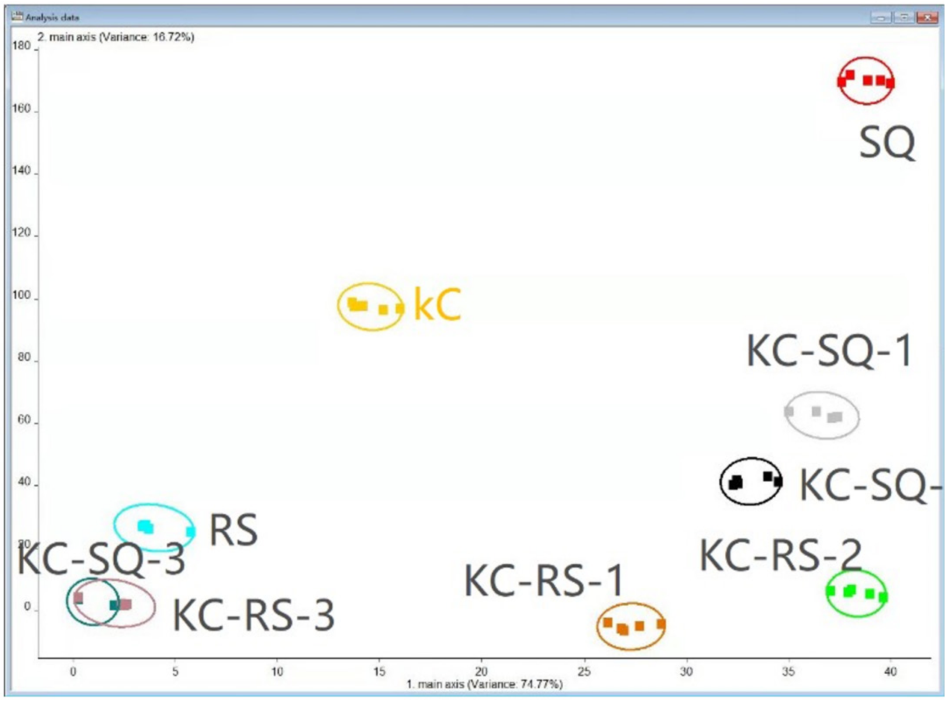

Figure 5. PCA of odor characteristics of fermented natto with different strains of bacteria

As can be seen in **Figure 6**, by analyzing the fermented natto of three single fractions of *Bacillus subtilis*, *Bifidobacterium bifidum,* and *Lactobacillus lactis* through the electronic nose, it can be seen that, for the content of two types of substances, nitrogen oxides (W5S) and inorganic sulphides (W1W), *Bifidobacterium bifidum* was significantly higher than *Bacillus subtilis* and *Lactobacillus lactis*, and it is initially judged that *Bifidobacterium bifidum* produces special gases under the action of anaerobiosis (**Table 4**).

Table 4. Evaluation of single-strain fermented natto e-nose

	W1C	W5S	W3C	W6S	W5C	W1S	W1W	W2S	W2W	W3S	
KC	0.4582	0.4582	0.4582	0.4582	0.4582	0.4582	0.4582	0.4582	0.4582	0.4582	AVG
SQ	0.5678	43.9647	0.6784	1.1106	0.9451	5.2652	25.7794	4.9617	5.6848	1.2155	AVG
RS	0.6298	10.2546	0.7789	1.0420	0.9638	3.6339	5.9424	2.8392	2.3592	1.2359	AVG

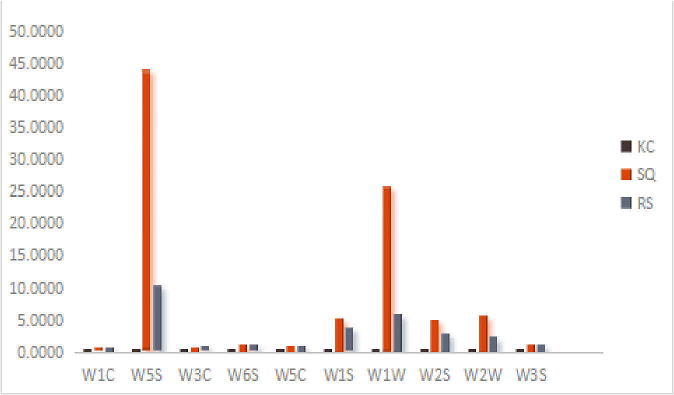

Figure 6. Electronic nose analysis of single-strain fermented natto

As can be seen in **Figure 7**, the results of analyzing the mixture of *Bacillus subtilis* and *Bifidobacterium bifidum* according to different ratios by the electronic nose revealed that for the contents of two types of substances, nitrogen oxides (W5S) and W1W (inorganic sulphides), when the ratio of *Bifidobacterium bifidum* to *Bacillus subtilis* reaches 2:3, the measured concentration is significantly higher than that of *Bifidobacterium bifidum* to *Bacillus subtilis* with the ratios of 1:3 and 1:1 (**Table 5**).

Table 5. Mixed strains for fermentation of natto e-nose

	W1C	W5S	W3C	W6S	W5C	W1S	W1W	W2S	W2W	W3S	
KC	0.9811	1.1937	0.9887	1.0072	0.9883	1.0763	1.0568	1.3578	0.9240	1.1326	AVG
KC-SQ-1	0.5700	23.1542	0.6931	1.0399	0.9453	4.5912	16.1879	3.7403	4.4268	1.2451	AVG
KC-SQ-2	0.6258	39.6239	0.7181	1.0762	0.9454	4.3552	25.8619	3.8373	5.3190	1.1836	AVG
KC-SQ-3	1.0373	1.1507	1.0355	1.0212	0.9943	1.1122	0.9124	1.4363	0.7743	1.1431	AVG

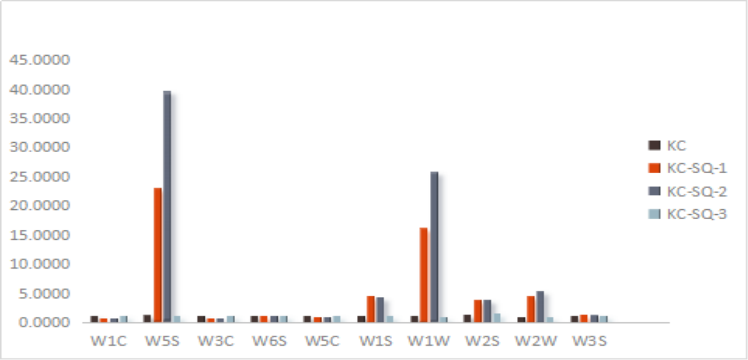

Figure 7. Comparison of e-nose assay after mixed fermentation

As can be seen in **Figure 8**, when *Bacillus subtilis* and *Lactobacillus* were mixed, the content of both nitrogen oxides (W5S) and inorganic sulfides (W1W) appeared to increase significantly, and when the ratio of *Lactobacillus* to *Bacillus subtilis* reached 1:3, the measured concentrations were significantly higher than the ratio of *Lactobacillus* to *Bacillus subtilis* of 2:3 and 1:1 (**Table 6**).

Table 6. Evaluation of *Bacillus subtilis* and *Lactobacillus* hybrid strains for fermentation of natto e-nose

	W1C	W5S	W3C	W6S	W5C	W1S	W1W	W2S	W2W	W3S	
KC	0.9811	1.1937	0.9887	1.0072	0.9883	1.0763	1.0568	1.3578	0.9240	1.1326	AVG
KC-RS-1	0.5311	62.3764	0.6383	1.0624	0.9364	5.0098	27.9089	4.4150	6.1031	1.2112	AVG
KC-RS-2	0.6110	46.8356	0.7030	1.1231	0.9396	4.7436	26.1585	4.7252	5.4600	1.2065	AVG
KC-RS-3	0.6039	37.1963	0.7104	1.0608	0.9474	4.3427	23.4015	3.8464	5.0550	1.1975	AVG

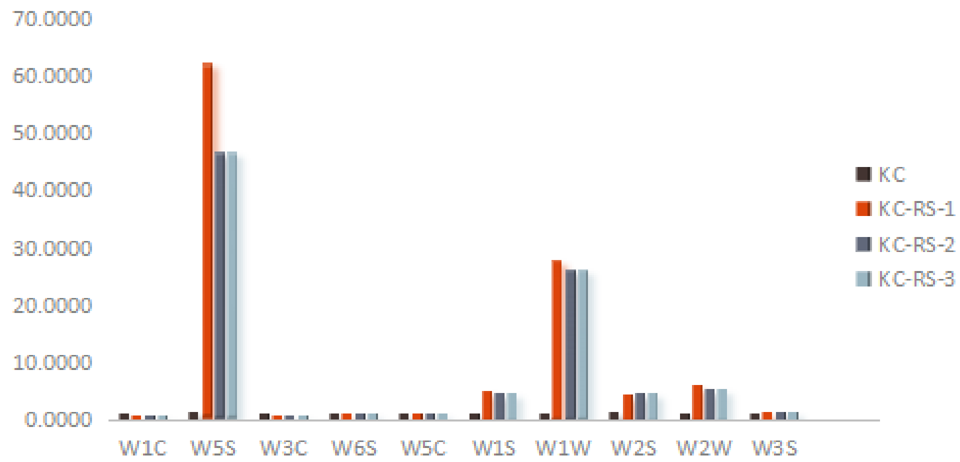

Figure 8. Flavor analysis of mixed fermentation of *Bacillus subtilis* with lactic acid bacteria

3.3. Free amino acid composition analysis

From **Figure 9**, it can be seen that the overall total amount of amino acids in natto fermented with mixed strains was higher than that in natto fermented with *Bacillus subtilis* alone, and the content of free amino acids in natto fermented with a 1:1 mixture of *Bifidobacterium bifidum* and *Bacillus subtilis* when fermented with a mixed strain was also higher than that of the amino acid content of the mixture of strains with other ratios. As can be seen from **Figure 10**, the proportion of essential amino acids accounted for by the single-strain fermented natto was higher than that of the mixed-strain fermented natto, indicating that the total amount of amino acids was improved by the mixed fermentation, but it did not improve the content of essential amino acids (**Table 7**).

The sensory quality of food is closely related to the type and content of amino acids [13–14]. There are still great differences in the flavor presentation of different kinds of amino acids. Glutamic acid and aspartic acid are important fresh-flavored amino acids, especially glutamic acid, the sodium salt of which is the main ingredient of MSG used in daily life, and the addition of bifidobacteria and lactobacilli increased the content of both glutamic acid and aspartic acid, and glycine and alanine presented a sweet flavor [15]. These four amino acids account for about 50% of the total free amino acids in the mixed fermented natto and have a positive effect on the taste improvement of fermented natto.

Table 7. Compositional analysis of free amino acids in samples (unit: mg/100g, essential amino acids:*)

Aspect / Sample number	KC	RS	SQ	KC-RS-1	KC-RS-2	KC-RS-3	KC-SQ-1	KC-SQ-2	KC-SQ-3
Aspartic acid	5.1	8.96	6.12	5.11	7.09	7	1.68	0.77	3.6
Serine	2.38	3.11	7.35	0.96	0.71	0.97	1.64	0.37	4.05
Glutamic acid	19.84	43.82	49.55	34.12	42.63	40.48	23.09	11.68	37.85
Glycine	2.2	3.16	4.54	2.03	2.89	2.82	3.26	3.03	4.16
Alanine	9.3	8.72	13.96	8.91	9.69	9.09	6.62	4.94	13.08
Tyrosine	1.36	5.42	5.23	2.76	4.56	4.49	1.74	0.22	7.51
Arginine	tr	0.82	0.77	0.69	1.15	1.33	2.19	15.07	8.31
Proline	5.34	5.45	4.29	6.51	6.78	5.59	4.99	4.55	5.18
**Threonine	2.45	1.48	5.25	1.23	1.23	1.31	1.59	0.5	4.19
*Valine	16.11	10.47	3.16	12.33	11.84	11.09	12.82	9.09	0.96
Methionine	1.39	0.52	4.19	0.64	0.54	0.63	1.1	0.4	3.08
*Isoleucine	2.6	0.51	6.02	1.69	0.78	0.71	1.83	2.12	4.34
*Leucine	7.36	1.56	21.37	1.29	1.19	1.54	2.33	0.17	8.59
*Phenylalanine	2.65	6.3	13.49	3.33	4.57	4.53	2.11	0.39	9.8
*Lysine	6.77	4.97	19.96	4.04	5.92	5.18	4.5	4.53	8.16
Histidine	0.51	2.07	2.64	1.15	1.97	1.82	0.46	0.33	1.6
Total acids	85.36	107.34	167.89	86.79	103.54	98.58	71.95	58.16	124.46
Non-essential Amino Acids/%	53.30%	74.00%	54.70%	70.40%	72.90%	72.80%	62.80%	69.90%	67.30%
* Essential Amino Acids/%	46.70%	26.00%	45.30%	29.60%	27.10%	27.20%	37.20%	30.10%	32.70%

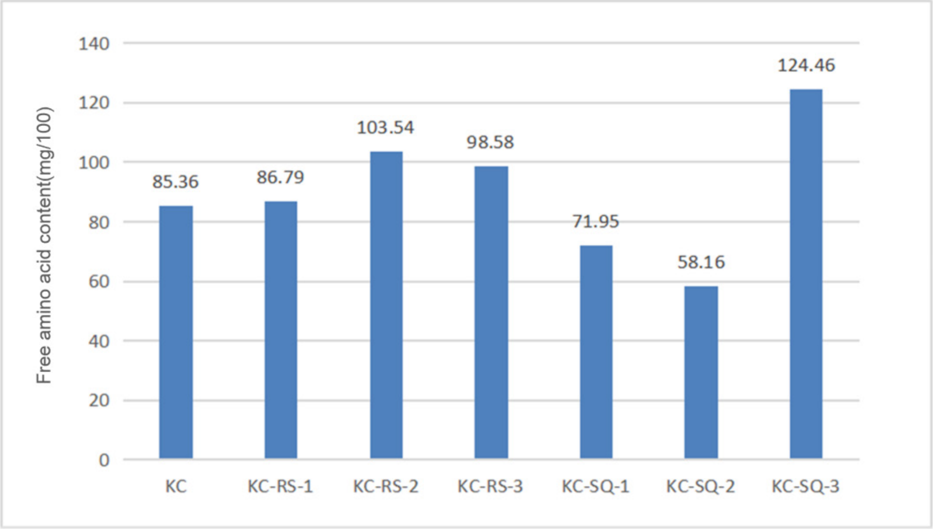

Figure 9. Comparison of amino acids between single and mixed strains of bacteria

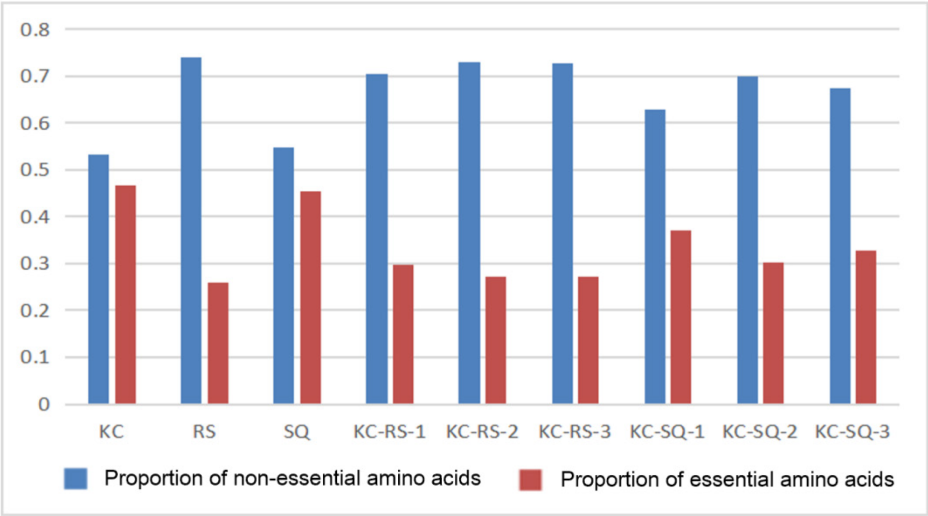

Figure 10. Proportion in fermented natto with single and complex strains of bacteria

4. Conclusion

The ammonia-fishy odor of its natto can be changed and the fermentation quality can be improved by mixing *Bacillus subtilis* with *Lactobacillus* and *Bifidobacterium* in different ratios for fermentation.

Firstly, mixed-strain fermentation increased the total free amino acids, but there was no significant change in the proportion of essential amino acids.

Secondly, the taste of the mixture was indeed significantly different from the fermentation of a single strain as detected by the electronic nose.

Thirdly, the amino acid content of the mixed fermentation portion was increased, which had a positive effect on the improvement of taste.

It can be concluded that various flavor substances and acidic components such as lactic acid and acetic acid produced by fermentation of lactobacilli and bifidobacteria can be neutralized to a certain extent with alkaline compounds, thus reducing the bad odor of natto and at the same time enriching the flavor components of natto.

Disclosure statement

The authors declare no conflict of interest.

References

[1] Pang YX, Xie YH, Jin JH, et al., 2021, Screening of Bacillus subtilis SH21 with Low Purine and High Nattokinase Activity and Optimisation of Fermentation Conditions. Food and Fermentation Industry, 47(11): 194–199.

[2] Lan GQ, 2020, Effect of Mixed-bacteria Fermentation on Sensory Properties and Functionality of Natto, thesis, Guizhou University.

[3] Yang LN, Cao SF, Xie MS, et al., 2023, Improvement of the Preparation Process of Natto and its Thrombolytic and Intestinal Function. Food and Fermentation Science and Technology, 59(1): 134–139.

[4] Gao ZX, Wang CS, Huang X, et al., 2014, Optimisation of Conditions for the Production of Tempeh by Mixed

Fermentation of Two Bacteria. China Brewing, 33(9): 49–52.

[5] Zheng DN, 2020, Research on the Process of Multi-strain Composite Fermentation of Low Ammonia Flavour Natto, thesis, Wuhan University of Light Industry.

[6] Gao YX, Zhang MR, Hou LZ, et al., 2022, Research Progress on the Undesirable Flavour of Natto. Food Industry Science and Technology, 43(1): 445–450.

[7] Zhang J, Zhao ZF, JIN Y, et al., 2019, Comparative Study of Processes for Improving the Flavour and Nutritional Properties of Natto. China Seasoning, 44(4): 11–15 + 22.

[8] Rong PX, He XQ, Gan RY, 2022, Progress in the Study of Flavour, Active Components and Functional Properties of Tempeh. Food Science and Technology, 47(9): 217–223.

[9] Sun JD, Chen S, Yang L, et al., 2016, Optimisation of Conditions for Mixed Fermentation of Natto with Two Strains of Bacteria. Journal of Shenyang Agricultural University, 47(1): 35–40.

[10] Huang ZW, Shangguan XC, Cheng JH, et al., 2005, Research on Mixed Fermentation Technology of Natto. Chinese Journal of Food Science, 2005(4): 70–73.

[11] Liu MJ, Chen SW, Wei XT, 2016, Rapid Screening of Nattokinase-producing Bacteria and their Fermentation Characteristics of Natto. Food Science, 37(21): 131–135.

[12] Guan ZW, 2007, Study on the Fermentation of Bacillus subtilis for Nattokinase Production, thesis, Shandong Institute of Light Industry.

[13] Huang TT, Luo YF, Chang C, et al., 2022, Correlation between Sensory Quality and Amino Acid Composition of Sheep Bone Soup. China Seasoning, 47(6): 5–9.

[14] Liu Y, Su H, Song HL, et al., 2016, Comparative Study on Volatile Aroma Components of Eight Types of Natto. Food Industry Science and Technology, 37(5): 302–307.

[15] Jin H, Shi J, Guan P, et al., 2016, Study on the Effect of Soybean Variety and Mixed Bacteria Fermentation on the Quality and Flavour Improvement of Natto. Agricultural Product Processing, 2016(17): 4–7.

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