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Original Article

Optimization of culture conditions for gamma-aminobutyric acid production in fermented adzuki bean milk

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ABSTRACT

γ-Aminobutyric acid (GABA), a nonprotein amino acid, is widely distributed in nature and fulfills several physiological functions. In this study, various lactic acid strains commonly used to produce fermented milk products were inoculated into adzuki bean milk for producing GABA. The high GABA producing strain was selected in further experiment to improve the GABA production utilizing culture medium optimization. The results demonstrated that adzuki bean milk inoculated with *Lactobacillus rhamnosus* GG increased GABA content from 0.05 mg/mL to 0.44 mg/mL after 36 hours of fermentation, which showed the greatest elevation in this study. Furthermore, the optimal cultural condition to adzuki bean milk inoculated with *L. rhamnosus* GG to improve the GABA content was performed using response surface methodology. The results showed that GABA content was dependent on the addition of galactose, monosodium glutamate, and pyridoxine with which the increasing ratios of GABA were 23–38%, 24–68%, and 8–36%, respectively. The optimal culture condition for GABA production of adzuki bean milk was found at the content of 1.44% galactose, 2.27% monosodium glutamate, and 0.20% pyridoxine. Under the optimal cultural condition, the amount of GABA produced in the fermented adzuki bean milk was 1.12 mg/mL, which was 22.4-fold higher than that of the unfermented adzuki bean milk (0.05 mg/100 mL). The results suggested that the optimized cultural condition of adzuki bean milk inoculated with *L. rhamnosus* GG can increase GABA content for consumers as a daily supplement as suggested.

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1. Introduction

The adzuki bean (*Vigna angularis*), an annual vine widely grown throughout Southern Taiwan, is used mainly in the production of sweets such as mooncakes, tangyuan, and youkan. It is a good source of a variety of minerals, with 100 g of adzuki beans providing 7.1 mg iron, 162 mg magnesium, 442 g potassium, 3.1 mg zinc, and 94.5 mg calcium. It also contains various amino acids, the most abundant being glutamate (3608 mg/100 g), followed by aspartate, leucine, lysine, and arginine [1]. In addition, the adzuki bean contains proanthocyanidins and polyphenols, which are known to have antioxidant effects, attenuate blood pressure elevation, lower serum triglyceride level, suppress hyperglycemia, and prevent cancer metastasis [2]. Although the adzuki bean is used extensively in the treatment of dropsy and beriberi in traditional Chinese medicine [3], it is seldom the focus of functional food development studies.

Gamma-aminobutyric acid (GABA) is a four-carbon nonprotein amino acid that is known to function as an inhibitory neurotransmitter in the brain and spinal cord of mammals. GABA is produced primarily from the α-decarboxylation of glutamate catalyzed by glutamate decarboxylase (GAD) and is known for relieving menopausal syndrome, enhancing immunity, treating cancer, preventing chronic alcohol-related symptoms, and fighting obesity [4]. Although GABA is available in many fruits and vegetables, its concentration in foods is low in nature, ranging from 0.03 to 2.00 μmol/g fresh weight [5]. Many research studies focus on finding new ways to increase GABA content in natural foods that can benefit human health. Environmental stress, additives, and biotransformation have been found to stimulate the accumulation of GABA [6,7]. Microorganism fermentation is an effective and convenient method to ferment and produce GABA in the food industry. The fermentation of *Escherichia coli*, *Aspergillus oryzae*, and *Rhizopus microspores* var. *oligosporus* can produce high-purity GABA, such as Tempeh, and Chinese fermented soybean product [6,8]. Lactic acid bacteria (LAB) have been applied in GABA production over the past few years. For example, *Lactobacillus brevis*, *Lactobacillus paracasei*, and *Lactococcus lactis* are applied to GABA-rich foods and pharmaceuticals synthesis [9–11]. The GABA-producing LAB are mostly cultivated in synthetic or semisynthetic media; however, the purification of GABA after fermentation is necessary, and this will increase the time and cost of production. Application of natural media such as dairy products, fruits, and vegetables for LAB to obtain GABA-enriched food could avoid the previous problems; however, the literature on this topic is limited.

The adzuki bean contains a high level of glutamate and has the potential via fermentation with LAB for enrichment of the GABA content; further development of fermented milk that is suitable for drinking every day and that assists the consumer to take in enough GABA and calcium to experience the conferred health benefits would then be possible.

It is found that the GABA production ability of LAB is influenced by the cultural compositions suitable for GAD reaction, especially by the type of carbon source, nitrogen source, and other components [12–14]. It is important to optimize the medium for enhancing GABA production during fermentation. Traditional optimizing methods involve changing one independent variable while fixing others at given levels, but the single-dimensional search technique often fails to yield optimized conditions because it does not consider possible interactions among factors. Response surface methodology (RSM) is a combination of statistical and mathematical techniques useful for optimization of bioprocesses. It can be used to evaluate the effect of several factors that influence the responses by varying them simultaneously in a limited number of experiments. Based on the key factors influencing the GABA production opted with one independent variable, RSM was applied to optimize the factors of medium for enhancing the GABA production.

The purposes of this study are aimed to evaluate the effects of various LAB commercially used [15,16] in the production of fermented milk on the GABA yield of fermented adzuki bean milk (ABM) and to find out the optimal culture medium of ABM by RSM. This study provides an alternative to the traditional utilization of cooked adzuki beans, enabling the production of a variety of functional adzuki bean products with beneficial effects to health.

2. Materials and methods

2.1. Materials, reagents, and equipment

Adzuki beans (obtained from Wandan Farmer's Association in Taiwan) were washed and soaked in 5-fold volume of water (w/w) at 37°C for 8 hours. After soaking, the adzuki beans and soaking solution were homogenized to a paste and filtered twice through a cotton cloth. Powdered skim milk (5%) was added to the filtrate, and the mixture was homogenized in a blender for 5 minutes and heated in a water bath at 90°C for 1 hour to prepare for the ABM.

2.2. LAB strains

Bifidobacterium adolescentis (BCRC 14606), *Bifidobacterium longum* (BCRC 14634), *Bifidobacterium bifidum* (BCRC 14615), *Bifidobacterium breve* (BCRC 11846), *Lactobacillus rhamnosus* GG (BCRC 16000), *Lactobacillus plantarum* (BCRC 11697), *Lactobacillus acidophilus* (BCRC 14079), and *Streptococcus salivarius* subsp. *thermophilus* (BCRC 14085) were purchased from the Bioresource and Collection Center of the Food Industry Research and Development Institute (HsinChu, Taiwan). LAB were cultured in MRS (de Man, Rogosa and Sharpe) broth (Difco, Detroit, MI, USA) at 37°C in 5% CO_2 for 48 hours, giving a cells number of about 8 log cfu/mL.

2.3. ABM fermentation

ABM was inoculated with 1% activated probiotic strain and fermentation at 37°C in a 5% CO_2 incubator for 6–60 hours. The LAB count, pH, and GABA content in the ABM were analyzed.

2.4. Cultural conditions for GABA production

2.4.1. Carbon source addition

Glucose, fructose, maltose, and galactose with a concentration of 0.5–2% were added to 100 mL ABM. It was inoculated

with 1% *L. rhamnosus* GG BCRC 16,000 (v/v, 8 log cfu/mL) and fermented at 37°C in a 5% CO_2 incubator for 36 hours in order to assay the best suitable conditions for GABA production.

2.4.2. *Nitrogen source addition*

Soytone, soy protein isolate, and monosodium glutamate with a concentration of 1–6% were, respectively, added to the ABM (100 mL) as the nitrogen source. The subsequent step was the same as that described in Section 2.4.1.

2.4.3. *Other source addition*

Pyridoxine, which was filtered through a 0.22-μM sterilized membrane filter (Critical Syringe Filters; Critical Process Filtration Inc., Nashua, NH, USA), together with magnesium sulfate and calcium chloride with concentration of 0.5–2%, were added to ABM. The subsequent step was the same as that described in Section 2.4.1.

2.5. *Response surface methodology*

On the basis of single-factor experiments, the cultural conditions to the cultured ABM, namely, galactose (X_1), monosodium glutamate (X_2), and pyridoxine (X_3), for the GABA yield (Y) in ABM were optimized using RSM (RSREG in the Statistical Analysis System; SAS Inc., Cary, NC, USA). The factors and levels investigated using a Box–Behnken design are listed in Table 1. The experimental results were analyzed by quadratic stepwise regression to fit the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{i<j}^{3} \beta_{ij} X_i X_j, \quad (1)$$

where Y denotes the response observed for treatment combination $X = (X_1, X_2, X_3)$ for p factors, β_0 represents the intercept, and the parameters β_i, β_{ii}, and β_{ij} represent the regression coefficients of variables for linear, quadratic, and interaction regression terms, respectively.

2.6. *Viable cells of LAB*

Viable cells of LAB were quantified using the plate enumeration method. The viable cell numbers were determined after 36 hours of incubation at 37°C under anaerobic condition. Plates with 25–250 colonies were enumerated and recorded as colony forming units per millimeter (cfu/mL) of fermented milk.

2.7. *GABA assay*

Freeze-dried fermented ABM powder of 0.1 g was extracted by the addition of 10 volumes of 70% (v/v) ethanol and ultrasonication at 2°C for 10 minutes. The samples were then centrifuged (18,000*g*, 15 minutes), and the precipitates were extracted twice as described above [12]. The supernatants were pooled and concentrated under vacuum, then redissolved in 0.1M ammonium acetate to 10 mL, filtered through a 0.22-μm membrane filter (Critical Syringe Filters; Critical Process Filtration Inc.), and analyzed for GABA content using the high performance liquid chromatography (HPLC) gradient system with precolumn phenylisothiocyanate (PITC) derivation [17]. A 20-μL sample extract was removed and dried under vacuum. Then, 20 μL of derived reagent A (methanol/water/triethylamine, 2:2:1; v/v) was added. After mixing, the sample was directly dried under vacuum and was then reacted with 30 μL of derived reagent B (methanol/phenylisothiocyanate/triethylamine/water, 7:1:1:1; v/v) at room temperature for 20 minutes prior to drying under vacuum to remove PITC. The derivatized samples were then redissolved in 200 μL of buffer A (0.1M ammonium acetate, pH 6.5) that is used as mobile

Table 1 – Optimization of cultural conditions in fermented adzuki bean milk inoculation with *Lactobacillus rhamnosus* GG using a Box–Behnken design to achieve the greatest GABA content.

Trials	X_1 Galactose (%)	X_2 Monosodium glutamate (%)	X_3 Pyridoxine (%)	GABA content[a] (mg/mL)	
				Observed[b] value	Predicted value
1	1 (2)	1 (3)	0 (0.2)	0.83 ± 0.04	0.91
2	1 (2)	−1 (1)	0 (0.2)	0.73 ± 0.03	0.69
3	−1 (1)	1 (3)	0 (0.2)	0.88 ± 0.02	0.98
4	−1 (1)	−1 (1)	0 (0.2)	0.79 ± 0.06	0.78
5	1 (2)	0 (2)	1 (0.3)	0.66 ± 0.04	0.70
6	1 (2)	0 (2)	−1 (0.1)	0.73 ± 0.06	0.76
7	−1 (1)	0 (2)	1 (0.3)	0.77 ± 0.02	0.79
8	−1 (1)	0 (2)	−1 (0.1)	0.81 ± 0.02	0.83
9	0 (1.5)	1 (3)	1 (0.3)	0.88 ± 0.02	0.92
10	0 (1.5)	1 (3)	−1 (0.1)	0.85 ± 0.05	0.89
11	0 (1.5)	−1 (1)	1 (0.3)	0.61 ± 0.02	0.64
12	0 (1.5)	−1 (1)	−1 (0.1)	0.73 ± 0.06	0.76
13	0 (1.5)	0 (2)	0 (0.2)	1.04 ± 0.05	1.11
14	0 (1.5)	0 (2)	0 (0.2)	1.10 ± 0.05	1.11
15	0 (1.5)	0 (2)	0 (0.2)	1.09 ± 0.02	1.11

ABM = adzuki bean milk; GABA = gamma-aminobutyric acid.

[a] GABA content of ABM without inoculation *Lactobacillus rhamnosus* GG was 0.05 ± 0.01 mg/ml. GABA content in ABM inoculated with *L. rhamnosus* GG at 37°C in 5% CO_2 for 36 hours was 0.44 ± 0.01 mg/mL.

[b] Values are expressed as mean ± SD ($n = 3$).

phase for HPLC and filtered through a Millipore membrane (0.22 μm). A 20-μL sample was injected into an HPLC system using a gradient system of buffer A (100–0% after 50 minutes) and buffer B [0.1M ammonium acetate containing acetonitrile and methanol, 44:46:10, v/v, pH 6.5; (0–100% after 50 minutes)]. C18 reversed-phase column from Phenomenex (C18, 4 μm) was used. The flow rate was 1 mL/min. Absorbance was detected at 254 nm. The results were analyzed by EC2000 data system 1.0 (Analab Co., Taipei, Taiwan). The coefficient of determination (r^2) was greater than 0.99.

3. Results and discussion

3.1. *LAB screening for high GABA production in adzuki milk*

The changes in pH and the LAB counts of the ABM during fermentation are shown in Figure 1. The result showed that after 36 hours of incubation with different lactic acid strains, the pH values of ABM were decreased from 6.3 to the range of 3.81–4.31 (Figure 1A). In the *L. rhamnosus* GG inoculation group, the pH value of ABM was significantly higher than that of the other groups (pH = 4.31). A previous study found that GAD, a critical GABA synthesis enzyme, presents a high ability for synthesizing GABA at pH = 4.4, which suggests that the pH value of fermentation plays an important role in GABA production by regulating the activity of GAD [18]. Our result (Figure 1A) showed that ABM inoculated with *L. rhamnosus* GG after 36 hours may cause a low pH value of 4.31. The low pH value of fermentation may help to increase the production of GABA.

After 36 hours of incubation, the growth of LAB was increased from 6.7 log cfu/mL to 9 log cfu/mL when inoculated with ABM (Figure 1B), which is similar to a previous study [19]. The *L. rhamnosus* GG inoculated ABM group exhibited a rapid growth ability after 36 hours of fermentation (9.53 cfu/mL), and reached the highest growth ability within 42–60 hours compared to the other groups.

The GABA content of ABM milk without probiotic inoculation was approximately 0.05 mg/mL. After fermentation with LAB, the content significantly increased ($p < 0.05$; Table 2), reaching a maximum after 36 hours of fermentation, and then decreased (Table 2). Of the ABM fermented with the different strains of LAB, one that was inoculated with *L. rhamnosus* GG had the highest yield of GABA (0.44 mg/mL; $p < 0.05$) after 36 hours of fermentation, which was approximately 9-fold the original content. Therefore, *L. rhamnosus* GG was selected in following experiments because of its rapid growth and high production of GABA.

3.2. *Effect of culture ingredients on GABA production in fermented adzuki milk*

The effects of carbon source addition in ABM inoculated with *L. rhamnosus* GG on GABA content are shown in Table 3. Without adding a carbon source, the production of GABA was 0.44 mg/mL. Addition of galactose to ABM resulted in a higher GABA content than that of ABM with addition of other carbon source ($p < 0.05$). In 1.5% galactose addition group, GABA

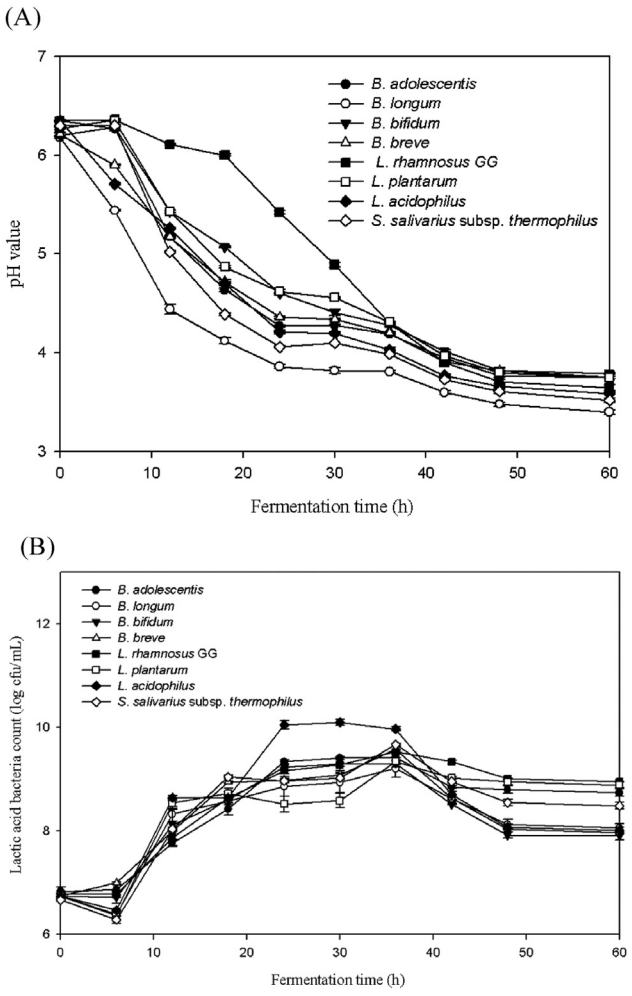

Figure 1 – Changes in (A) pH and (B) lactic acid bacteria count in adzuki bean milk inoculated with different lactic acid bacteria (LAB).

production increased up to 0.61 mg/mL, and no significant increase was observed when a higher concentration (2%) of galactose was added ($p > 0.05$).

GABA production by *L. brevis* K203 was added with 4% maltose with a 16% increase of GABA, compared to the standard MRS medium [20]. Our results showed that the addition of 1.5% galactose to ABM significantly increased GABA content (38.6%). Koskenniemi [21] suggested that *L. rhamnosus* GG could degrade galactose by two different pathways: the Leloir pathway, which converts galactose to glucose-6-phosphate, and the tagatose-6-phosphate pathway, which metabolizes galactose to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. This may explain why the addition of galactose in ABM with *L. rhamnosus* GG can improve the production of GABA. The actual mechanism needs to be investigate in a further study.

With regard to the nitrogen source, addition of monosodium glutamate to ABM during fermentation led to a higher GABA content. Our findings showed that the increasing ratios of GABA with addition of monosodium glutamate as a

Table 2 – Changes of GABA content in adzuki bean milk inoculated with different LAB.

LAB	GABA content (mg/mL)					
	0 h	12 h	24 h	36 h	48 h	60 h
Bifidobacterium adolescentis	$0.05 \pm 0.03^{C,a}$	$0.08 \pm 0.01^{BC,b}$	$0.10 \pm 0.01^{B,d}$	$0.30 \pm 0.03^{A,c}$	$0.29 \pm 0.03^{A,bc}$	$0.10 \pm 0.03^{B,bc}$
Bifidobacterium longum	$0.05 \pm 0.01^{C,a}$	$0.09 \pm 0.02^{B,b}$	$0.10 \pm 0.01^{B,d}$	$0.16 \pm 0.03^{A,f}$	$0.15 \pm 0.02^{A,e}$	$0.04 \pm 0.01^{C,d}$
Bifidobacterium bifidum	$0.05 \pm 0.01^{C,a}$	$0.06 \pm 0.01^{C,b}$	$0.12 \pm 0.03^{B,cd}$	$0.21 \pm 0.01^{A,ef}$	$0.22 \pm 0.02^{A,d}$	$0.04 \pm 0.01^{C,d}$
Bifidobacterium breve	$0.05 \pm 0.01^{D,a}$	$0.07 \pm 0.02^{CD,b}$	$0.14 \pm 0.02^{B,c}$	$0.29 \pm 0.02^{A,c}$	$0.29 \pm 0.04^{A,bc}$	$0.10 \pm 0.04^{C,bc}$
Lactobacillus rhamnosus GG	$0.05 \pm 0.01^{F,a}$	$0.15 \pm 0.02^{E,a}$	$0.28 \pm 0.03^{C,a}$	$0.44 \pm 0.02^{A,a}$	$0.38 \pm 0.02^{B,a}$	$0.20 \pm 0.02^{D,a}$
Lactobacillus plantarum	$0.05 \pm 0.02^{D,a}$	$0.08 \pm 0.02^{CD,b}$	$0.18 \pm 0.03^{B,b}$	$0.37 \pm 0.05^{A,b}$	$0.33 \pm 0.05^{A,b}$	$0.14 \pm 0.05^{BC,b}$
Lactobacillus acidophilus	$0.05 \pm 0.02^{C,a}$	$0.07 \pm 0.01^{C,b}$	$0.11 \pm 0.01^{B,cd}$	$0.23 \pm 0.04^{A,de}$	$0.22 \pm 0.03^{A,d}$	$0.06 \pm 0.02^{C,cd}$
Streptococcus salivarius subsp. *thermophilus*	$0.05 \pm 0.02^{C,a}$	$0.08 \pm 0.01^{C,b}$	$0.13 \pm 0.02^{B,cd}$	$0.27 \pm 0.01^{A,cd}$	$0.24 \pm 0.02^{A,cd}$	$0.06 \pm 0.02^{C,cd}$

Each value was expressed as mean ± SD ($n = 3$).
Different uppercase letters (A–F) in the same row show significant differences; different lowercase letters (a–d) in the same column show significant differences ($p < 0.05$, Duncan test).
GABA = gamma-aminobutyric acid; LAB = lactic acid bacteria; SD = standard deviation.

Table 3 – Effects of the culture ingredients on GABA yield in fermented adzuki bean milk inoculated with *Lactobacillus rhamnosus* GG.

Carbon source	GABA content (mg/mL)				
	0%	0.5%	1.0%	1.5%	2.0%
Glucose	$0.44 \pm 0.02^{A,a,}$	$0.46 \pm 0.02^{A,a,b}$	$0.45 \pm 0.05^{A,b}$	$0.46 \pm 0.02^{A,b}$	$0.48 \pm 0.02^{A,b}$
Fructose	$0.43 \pm 0.02^{B,a}$	$0.39 \pm 0.06^{B,b}$	$0.44 \pm 0.04^{B,b}$	$0.51 \pm 0.04^{A,b}$	$0.52 \pm 0.01^{A,b}$
Maltose	$0.43 \pm 0.03^{A,a}$	$0.44 \pm 0.04^{A,b}$	$0.48 \pm 0.28^{A,ab}$	$0.47 \pm 0.03^{A,b}$	$0.50 \pm 0.03^{A,b}$
Galactose	$0.44 \pm 0.02^{B,a}$	$0.54 \pm 0.03^{B,a}$	$0.54 \pm 0.03^{B,a}$	$0.61 \pm 0.02^{A,a}$	$0.62 \pm 0.01^{A,c}$
Nitrogen source	**GABA content (mg/mL)**				
	0%	1%	2%	4%	6%
Soytone	$0.44 \pm 0.02^{A,a}$	$0.42 \pm 0.02^{AB,b}$	$0.47 \pm 0.04^{A,b}$	$0.41 \pm 0.02^{B,b}$	$0.41 \pm 0.03^{B,b}$
Soy protein isolate	$0.43 \pm 0.02^{A,a}$	$0.39 \pm 0.02^{AB,b}$	$0.41 \pm 0.02^{AB,b}$	$0.42 \pm 0.02^{A,b}$	$0.38 \pm 0.02^{B,b}$
Monosodium glutamate	$0.44 \pm 0.02^{C,a}$	$0.52 \pm 0.05^{B,a}$	$0.64 \pm 0.03^{A,a}$	$0.65 \pm 0.01^{A,a}$	$0.64 \pm 0.02^{A,a}$
Other additives	**GABA content (mg/mL)**				
	0%	0.1%	0.2%	0.3%	0.5%
Pyridoxine	$0.44 \pm 0.02^{B,a,}$	$0.48 \pm 0.05^{B,a}$	$0.60 \pm 0.03^{A,a}$	$0.59 \pm 0.02^{A,a}$	$0.60 \pm 0.02^{A,a}$
Magnesium sulfate	$0.43 \pm 0.02^{C,a}$	$0.44 \pm 0.03^{C,a}$	$0.51 \pm 0.04^{AB,b}$	$0.52 \pm 0.02^{A,b}$	$0.46 \pm 0.01^{BC,b}$
Calcium chloride	$0.44 \pm 0.02^{A,a}$	$0.44 \pm 0.04^{A,a}$	$0.47 \pm 0.02^{A,b}$	$0.49 \pm 0.05^{A,b}$	$0.44 \pm 0.03^{A,b}$

Each value is expressed as mean ± SD ($n = 3$).
Different uppercase letters (A–C) in the same row show significant differences; different lowercase letters (a–c) in the same column show significant differences ($p < 0.05$, Duncan test).
GABA = gamma-aminobutyric acid; SD = standard deviation.

Table 4 – Analysis of variance (ANOVA) for the production of GABA and cultural conditions.

Source	*df*	Sum of squares	Mean of squares[b]	*F*	Prob > *F*
Model[a]	9	0.0300	0.9726	19.70	0.0022
Linear	3	0.0583	0.1892	11.50	0.0111
Quadratic	3	0.2355	0.7643	46.46	0.0004
Cross-product	3	0.0059	0.0191	1.16	0.4118
Total error	5	0.0085	0.0017		
Pure error	2	0.0026	0.0013		
Lack of fit	3	0.0059	0.0195	1.50	0.4240

$R^2 = 97.26\%$
df = degree of freedom; GABA = gamma-aminobutyric acid.
[a] ANOVA for response surface quadratic model.
[b] Determination coefficient.

nitrogen source was 24–68% higher than that of soytone or soy protein isolate. An addition of 2% monosodium glutamate could increase the GABA generation up to 0.64 mg/mL, whereas no significant increase in GABA content was observed as the concentration of monosodium glutamate was increased to 4–6% ($p > 0.05$). Monosodium glutamate can hydrate to form Na^+ and L-glutamic acid, which is the substrate for GABA synthesis. Increasing the added glutamate was aimed to stimulate the production of GABA by GAD via the GABA shunt [22]. For production of GABA by *L. lactis* in MRS broth, increase of monosodium glutamate addition at lower levels (0–20 g/L) can increase GABA yield [23]. Monosodium glutamate concentration above a certain level would inhibit cell growth. As for *L. rhamnosus* YS9, monosodium glutamate concentration should be limited to 200mM. The highest GABA yield was observed with 200mM of monosodium glutamate by

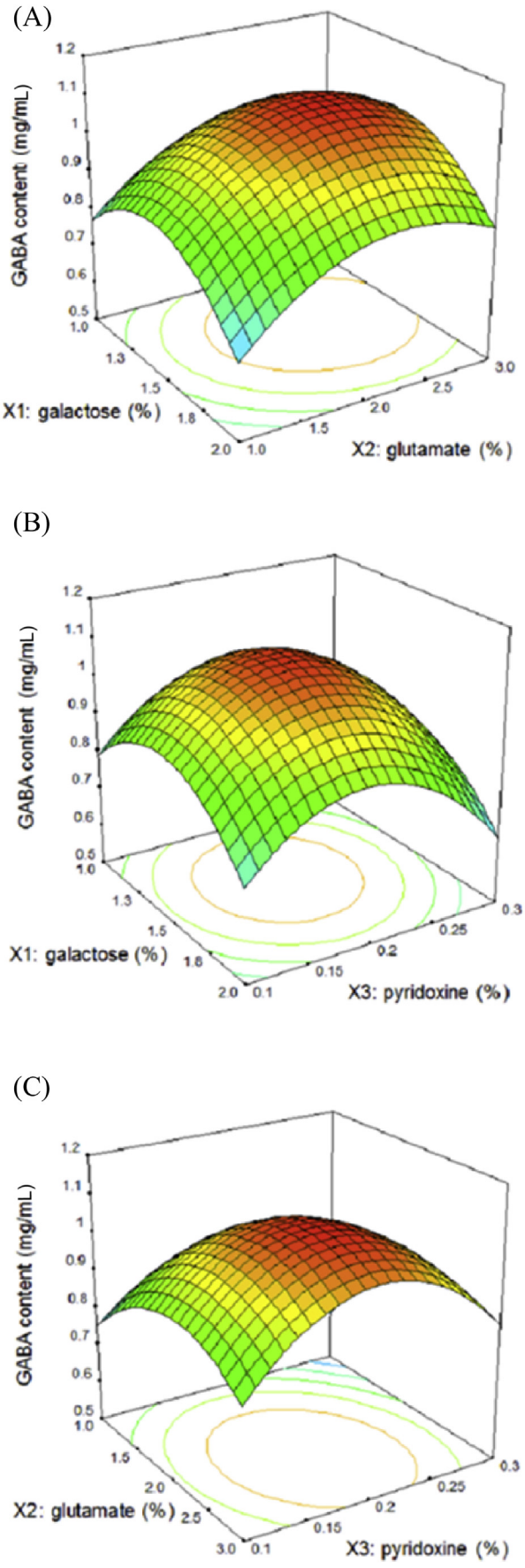

Figure 2 – The response surface for the gamma-aminobutyric acid (GABA) content of fermented adzuki bean milk. (A) Combined effects of galactose and monosodium glutamate with constant pyridoxine (0.2%). (B) Combined effects of galactose and pyridoxine with constant monosodium glutamate (2%). (C) Combined effects of monosodium glutamate and pyridoxine with constant galactose (1.5%).

L. rhamnosus YS9 [18]. Our findings showed that the addition of 2% monosodium glutamate to ABM significantly enhanced its GABA content to 0.64 mg/mL ($p < 0.05$).

With regard to the addition of pyridoxine and mineral salts, 0.2% pyridoxine addition resulted in the highest GABA content, which was considerably higher than that with addition of magnesium sulfate or calcium chloride ($p < 0.05$). Increasing the addition levels of pyridoxine did not induce a higher production of GABA. Pyridoxal phosphate (PLP) plays an important role in stimulating GAD activity because it is a cofactor of enzyme [24]. Thus, the addition of PLP to the culture medium might influence GABA production [25]. With the addition of PLP, GABA production increased and reached 7333 mg/L, 200mM, and 504 mg/kg during the fermentation with *S. salivarius* subsp. *thermophilus* Y2, *L. paracasei* NFRI 74150, and *L. plantarum* C48, respectively [26]. The enhancement effect of GABA production was similar to that of *S. salivarius* subsp. *thermophilus* Y2, *L. paracasei* NFRI 74150, and *L. plantarum* C48 [26]. The addition of sulfate ions increased the GAD activity of *L. brevis* IFO 12005 in a dose-dependent manner, suggesting that the increased GAD activity was attributable to an increased hydrophobic interaction between the subunits [26]. This explains that the addition of 0.3% magnesium sulfate to ABM inoculated with *L. rhamnosus* GG could slightly increase GABA production, which was lower than that with addition of pyridoxine.

3.3. *Optimization of culture conditions for GABA production in fermented adzuki milk*

In the present study, three factors including galactose, monosodium glutamate, and pyridoxine addition in ABM inoculated with *L. rhamnosus* GG on GABA content were selected as independent variables. The Box–Behnken design and the corresponding experimental data are shown in Table 1. Multiple regression analysis of the data demonstrated that the RSM design model was consistent with the second-order polynomial equation (Equation 1). The second-order polynomial model describing the correlation between GABA content and the three variables in this study is presented in Equation 2:

$$Y = -1.37 + 1.73X_1 + 0.49X_2 + 6.77X_3 + 0.01X_1X_2 - 0.15X_1X_3 + 0.38X_2X_3 - 0.60X_1^2 - 0.12X_2^2 - 18.88X_3^2, \quad (2)$$

where Y denotes the predicted GABA production, and X_1, X_2, and X_3 are the uncoded values of initial concentration of galactose, monosodium glutamate, and pyridoxine, respectively.

Table 4 showed that the proposed model was adequate for its purpose and had a satisfactory R^2 value of 0.9726. The closer the value of R^2 was to unity, the better the empirical model fit the actual data. The F value was 19.70, which implied that the model was significant because in model terms, the values of Prob > F was less than 0.01. Meanwhile, the model was adequate in approximating the response surface of the experimental design. Response surface plots were used to illustrate the interactive effects of galactose, monosodium glutamate, and pyridoxine concentration on GABA content. The response surface plots for GABA content are displayed in Figure 2. At a fixed pyridoxine concentration of 0.2%, as shown in Figure 2A, galactose addition led to a slight increase in GABA content; however, GABA content decreased if the addition level was higher. The highest GABA content was found at the galactose concentration of 1.5%. However, if the galactose level was set, the GABA content sharply increased with the addition of monosodium glutamate, in which the optimal addition concentration was 2%. The effects of monosodium glutamate and pyridoxine, at a constant galactose concentration of 1.5%, on GABA content are illustrated in Figure 2B. At the fixed monosodium glutamate concentration, GABA content increased rapidly with the increase in pyridoxine addition at the beginning; after that, a slower rate was observed, which showed the same relation between the GABA yield and the addition of monosodium glutamate. Figure 2C shows the effects of galactose and pyridoxine concentrations on GABA content in ABM at a constant monosodium glutamate concentration of 2%. The GABA content increased when the galactose concentration increased from 1.0% to 1.5%, and decreased thereafter when the galactose concentration was above 1.5%. At a fixed galactose concentration, the GABA content reached a peak value when the addition level of pyridoxine was 0.2%.

According to the results of the RSM test, the optimal additives to the cultured ABM for the highest GABA content were 1.44% galactose, 2.27% monosodium glutamate, and 0.20% pyridoxine. Under these optimal conditions, the predicted maximum GABA content in the fermented ABM was 1.13 mg/mL. Verification of the model represented by Equation 2 was performed under the optimum conditions for maximum GABA content. The average GABA content observed in fermented ABM under the optimal conditions was 1.12 mg/mL, which was in agreement with the predicted value of the model. The GABA content in ABM inoculated with *L. rhamnosus* GG and fermented under the optimum conditions was 22.4-fold that of unfermented ABM (which had a GABA content of 0.05 mg/mL).

In skim milk inoculated with *Lactobacillus helveticus* after 30 hours of fermentation, the GABA content reached 0.165 mg/mL and had a high angiotensin I converting enzyme-inhibitory activity [27]. In female patients with mental symptoms who were administered defatted rice germ enriched with GABA (26.4 mg GABA/d), and symptoms such as sleeplessness, somnipathy, and depression, more than 65% showed improvement [28]. The amount of GABA produced in the fermented ABM in this study was 112 mg/100 mL, which is sufficient to provide the beneficial functions. A previous study demonstrated that the fermentation of beans with GABA provides a successful strategy for producing a functional food with antihypertensive activity [29]. This study showed the alternative utilization of adzuki beans to promote the health benefits of enriched GABA and probiotic bacteria when it was taken by consumers as a dietary supplement to provide the content required for physiological efficiency.

4. Conclusions

In this study, we focused on the development of a novel GABA-enriched adzuki bean fermented milk. Various LAB strains that were commercially used in food fermentation were used to ferment milk with adzuki bean. Among all LAB strains, *L. rhamnosus* GG was selected in the following experiments because of its high GABA production ability. In addition to improved GABA production in adzuki bean fermented milk, the adzuki bean fermented culture medium was also optimized by RSM. The RSM result suggests that the optimal culture medium including 1.44% galactose, 2.27% monosodium glutamate, and 0.20% pyridoxine can help increase GABA production more than 22.4-fold compared to unfermented ABM. Further studies on scaling up the production of adzuki bean fermented milk beverage will be the next challenge in future applications.

Conflicts of interest

The authors declare no conflict of interest.

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