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Influence of preprocessing methods and fermentation of adzuki beans on γ-aminobutyric acid (GABA) accumulation by lactic acid bacteria

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ABSTRACT

The effects of pre-processes (immersing, germinating, and cold shock) and fermentation conditions of adzuki beans on γ-aminobutyric acid (GABA) accumulation using mixed cultures of *Lactococcus lactis* and *Lactobacillus rhamnosus* were investigated in this study. Among the preprocessing methods, cold shock treatment resulted in the highest observed GABA content (201.2 mg/100 g); a 150-fold increase compared to the non-treated adzuki beans. The LAB strains grew rapidly in cold-treated adzuki bean substrates and reached 10^8 cfu/ml after 24 h of fermentation at 30 °C. After optimization, the GABA yield reached 68.2 mg/100 ml; a 20-fold increase compared to the non-fermentation yield. The viable cell counts of LAB remained above 10^8 cfu/ml after 28 days of storage at 4 °C. Our results suggest that the combination of cold shock pretreatment and fermentation by LAB may be used for the preparation of adzuki beans with high GABA content, which can then be used as a natural resource of functional foods.

1. Introduction

The non-protein amino acid γ-aminobutyric acid (GABA) is ubiquitous and widely distributed among prokaryotic and eukaryotic organisms. Many studies have reported that GABA is the principal inhibitory neurotransmitter in the brain and spinal cords of mammals and is also important in some physiological functions such as regulation of blood pressure (Matsuo, Sato, Park, Nakamura, & Ohtsuki, 2012), prevention of chronic alcohol-related diseases (Oh, Soh, & Cha, 2003) and the inhibition of cancer cell proliferation (Oh & Oh, 2004). In plants, GABA is a metabolic end product and is primarily produced by the decarboxylation of L-glutamic acid, which is catalyzed by glutamate decarboxylase (GAD, EC 4.1.1.15). Various functions of GABA in plants have been described, including involvement in the regulation of cytosolic pH, nitrogen storage, protection against oxidative stress, and in the defense system against phytophagous insects. The stress response activates many enzymes in raw plants, especially glutamate decarboxylase (GAD), an enzyme that catalyzes L-glutamic acid to GABA, breaks down the reserved materials, and synthesizes the structural proteins and cell components after hydration. There is considerable literature showing that GABA is accumulated by stimulating the activity of GAD in a variety

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of plant tissues under several environmental stress conditions, such as: mechanical stimulation, damage, cold shock, heat shock, hypoxia, cytosolic acidification, darkness, water stress, phytohormones, and drought stress (Kinnersley & Turano, 2000). However, there is also growing interest in functional food research related to GABA accumulation prior to consumption. Diverse GABA-enriched foods have been reported, including brown rice (Jannoey et al., 2010), tea (Wang, Tsai, Lin, & Ou, 2006), wheat bran (Youn, Park, Jang, & Rhee, 2011), soybean (Park & Oh, 2007), and lactic acid bacteria (LAB) fermented food (Zbakh & Abbassi, 2012).

The adzuki bean (*Vigna angularis* [Willd] Ohwi & Ohashi) is a major economical legume widely planted in Asia. According to the ancient Chinese medical book Pen-Tsao-Kang-Mu (Li, 1596), the adzuki bean is used as an antidote, and for treating the symptoms of dropsy and beriberi. In Taiwan, adzuki beans are consumed as a dessert or snack, and as whole beans boiled and sweetened for snacks and confectionery items, although the preparations vary from region to region. Adzuki beans are a rich source of carbohydrates, proteins, minerals, vitamins, fiber and nutraceuticals (Tjahjadi, Lin, & Breene, 1988), and are receiving increasing attention due to their health benefits, such as protection against coronary heart disease (Bazzano et al., 2001), inhibition of acetaminophen-induced liver damage (Wu, Wang, Lin, & Chang, 2001), reduction of the serum cholesterol concentration in rats (Han et al., 2003), modulation of blood glucose levels, and protection effect against oxidative damage in diabetic mice (Itoh & Furuichi, 2009).

Many recent studies have reported about the various postharvest processes that increase the GABA content in plant food such as commercial legumes. Bytof, Knopp, Schieberle, Teutsch, and Selmar (2005) reported that GABA is generated in fava beans that are germinated with minerals. Vidal-Valverde et al. (2002) found that GABA contents is influenced by germination time, temperature, pH, and chemical inhibitors through are regulation of the DAO activity in fava beans. Bytof et al. (2005) also reported that germination in the presence of light for lentils, and in darkness for peas can increase the GABA content. Other studies have also showed the biotransformation of cereal proteins to GABA by LAB in sourdough (Stromeck, Hu, Chen, & Gänzle, 2011). GABA-enriched legumes could be exported at higher prices and can be used as nutraceuticals and consumed raw in food with high GABA content. Despite the widespread consumption of adzuki beans, to the best of our knowledge, there is limited information on their GABA content. In this study, we investigated the effects of pre-processes (immersing, germinating, and cold shock) on the accumulation of GABA in adzuki beans. Moreover, the effects of fermentation conditions of adzuki bean milk on GABA accumulation using mixed cultures of *Lactococcus lactis* and *Lactobacillus rhamnosus* were also investigated in this study.

2. Materials and methods

2.1. Materials

The adzuki bean variety Kaohsiung No. 8 (*Vigna angularis*) used in this study was purchased from a local market in Taipei, Taiwan. Two GABA-producing strains, *L. lactis* subsp. lactis and *L. rhamnosus* GG were used as starter culture, which had been kindly provided by Prof. Roch-Chui Yu from the Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan. The strains were cultured in MRS broth and incubated at 37 °C for 18 h. Each activated culture was inoculated into 5 ml of MRS broth and then incubated at 37 °C. After incubation for 24 h, the cell number had increased to 10^8 cfu/ml, which served as the inoculum. All the chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO) and were of at least analytical grade.

2.2. Immersion

The beans were washed with large quantities of tap water. After excess water was removed, 100 g of beans in 500 ml of water were immersed in each of the incubators and respectively maintained at 15, 25, 35, or 45 °C for 4–24 h. At the end of immersion, sample beans were freeze-dried and ground to a particle size of $\leqslant$350 μm (54 mesh screen) for GABA analysis.

2.3. Cold shock of adzuki bean

The adzuki bean was cold shocked as per the partially modified methods of Know et al. (2007). The beans were washed with large quantities of tap water. After excess water was removed, the beans were immersed in water (volume equal to 5 times their dry weight) at 35 °C for 24 h. The immersed beans were then kept in a freezer at −10, −20, −80 °C for 24 h. Afterwards, the beans were transferred into a chamber set at 20 and 30 °C and allowed to stand for 24 h. At the end of cold shock, the beans were dried in a freeze-dryer (Freeze-dryer Alpha 1-2/LD-2, Vacuum pump RZ-5, Christ, Germany), and ground to a particle size of $\leqslant$350 μm. The adzuki bean flour was stored at −80 °C for GABA analysis.

2.4. Germination

The adzuki bean was germinated as per partially modified methods of Vidal-Valverde et al. (2002). Five hundred grams of adzuki beans were soaked in 2500 ml of 0.7% sodium hypochlorite solution for 30 min at room temperature (25 °C). Beans were then drained and washed to neutral pH, and then soaked in distilled water for 16 h. Finally, the imbibed beans were germinated in a chamber by layering them over a moistened filter paper that was continuously watered for 6 days either with or without light. At the end of the germination period, all the samples were freeze-dried and floured for GABA analysis.

2.5. Lactic acid bacteria fermentation

To prepare the adzuki bean milk, the adzuki bean flour was mixed with a volume of distilled water equal to 10 times the dry weight of the adzuki beans, and then homogenized in a blender for 20 min with concomitant heating at 95 °C. The resultant mud was then centrifuged at 3500×*g* for 20 min. The supernatant milk was collected and sterilized for 15 min at 121 °C. Fermentation experiments were conducted in 125 ml Erlenmeyer flasks; 40 ml of adzuki bean milk were transferred to sterile Erlenmeyer flasks and inoculated with 0.4 ml of a single culture of *L. lactis* subsp. lactis and *L. rhamno-*

sus GG. The adzuki bean milk containing the LAB was fermented quiescently at 37 °C for 24 h. GABA content, pH, titratable acidity, and the viable cell count of the fermented adzuki bean milk were all analyzed at the end of the fermentation process. The optimized values of sucrose and yeast extract concentrations for GABA accumulation were then selected in order to carry out shelf life analysis of the fermented product. Fermentation with the optimized composition was carried out in 250 ml Duran bottles containing 50 ml of media for 24 h and then the fermented beverage was stored at 4 °C for 28 days. One bottle of fermented beverage was withdrawn for sampling at regular intervals for 24 h and analyzed for pH, viable cell count, and GABA content. After 24 h of fermentation, the pH, viable cell count, and GABA content in the fermented beverage was determined once every 4 days during the storage period.

2.6. Analysis of GABA in adzuki beans

GABA content was determined following partially modified methods of Rozan et al. (2000). Two grams of ground sample were mulled with 15 ml of 70% (v/v) ethanol. The homogenate was deposited for 18 h at 4 °C in order to sufficiently extract GABA, and then centrifuged at 34,800×*g* for 20 min. The pellets were washed twice with 70% ethanol. The supernatants were pooled and concentrated under vacuum and finally stored in the deep freezer at −20 °C. The GABA contents of the extracts were analyzed by an HPLC gradient system with precolumn phenylisothiocyanate (PITC) derivatization (Khan et al., 1994). Two buffers were used: buffer A (0.1 M ammonium acetate, pH 6.5) and buffer B (0.1 M ammonium acetate containing acetonitrile and methanol, 44:46:10, v/v/v, pH 6.5). For sample preparation, a 50 μL aliquot of extract was removed and dried under vacuum (37 °C, 20 mmHg). Next, 20 μL of a coupling reagent (methanol, water, triethylamine [TEA] [2:2:1, v/v/v]) was added. After mixing, the sample was directly dried under vacuum for 10 min and was then allowed to react with 30 μL of PITC reagent (methanol/PITC/TEA/water [7:1:1:1; v/v/v/v]) at room temperature for 20 min before drying under vacuum to remove PITC. The derivatized samples were then redissolved in 500 μL of buffer A, which was used as the mobile phase for HPLC and filtered through a Millipore membrane (0.22 ím). A 20 μL sample was injected into an HPLC system (Waters model 99 equipped with a photodiode array detector) using a gradient system of buffer A (100–0% after 50 min) and buffer B (0–100% after 50 min). The operating temperature was 39 °C using a C18 reversed-phase column from Alltech (Alltima C18 5 U, 250 × 4.6 mm). Measurements were taken at an absorbance of 254 nm. The UV absorption spectrum was useful for GABA identification. A standard protein amino acid mixture (food hydrolysate A 9656, Sigma) was prepared as above, and quantitative data for GABA content was obtained from a standard curve produced using control (non-immersed) beans.

2.7. Statistical analysis

All the data were expressed as mean ± SD. Student's *t* test was used for statistical analysis performed by comparing treatment groups with the control group. Results were regarded as statistically significant at a p value of <0.05.

3. Results

3.1. Effect of immersion on GABA content

Fig. 1a shows the change in the GABA content of adzuki beans after being soaked for 24 h at different temperatures. The GABA content in the un-soaked adzuki beans is very low, at 1.34 mg/100 g. After soaking for 24 h, the GABA content in the adzuki beans increased to 12.78, 15.38, 28.58, and 43.37 mg/100 g, at 15, 25, 35, and 45 °C, respectively. The GABA content in the adzuki bean seeds therefore appears to increase with the increasing soaking temperatures. The soaking temperatures of 35 and 45 °C are most advantageous to the accumulation of GABA in adzuki beans as the GABA content in the adzuki beans soaked at higher temperature is considerably higher than that at the other soaking temperatures. Fig. 1b shows the effect of different soak pH values on GABA

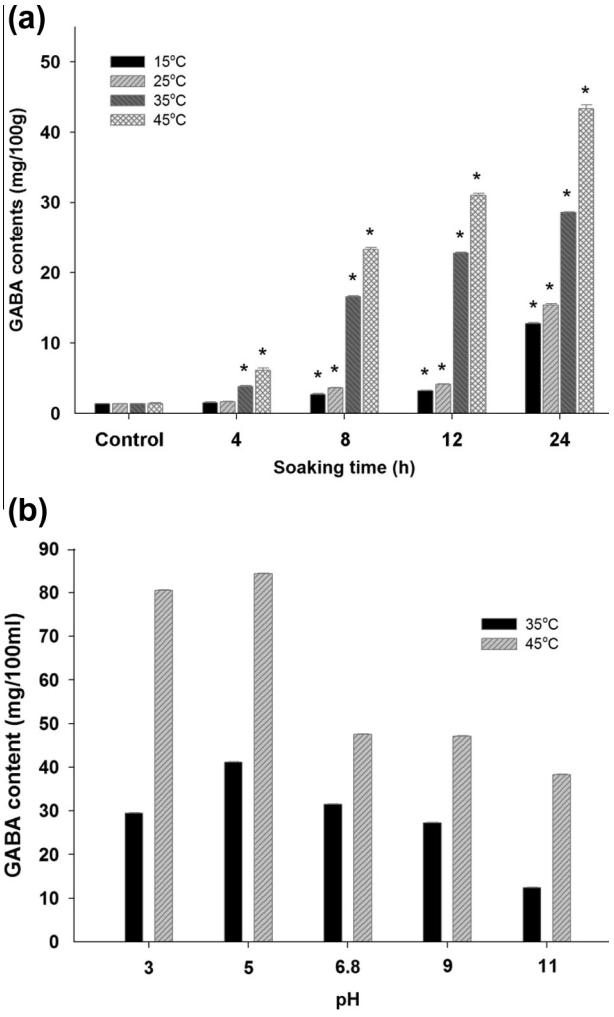

Fig. 1 – (a) The effects of soaking temperature on the GABA content of adzuki beans. The adzuki beans were soaked in distilled water at 15, 15, 35 or 45 °C for 24 h; (b) the effect of pH on the GABA content of adzuki beans during soaking at a temperature of 35 or 45 °C for 24 h. The GABA content was followed by HPLC analysis. Data are expressed as the mean ± SD (n = 3). *p < 0.05 indicates a significant difference from the control group.

content after 24-h treatment of adzuki bean seeds at 35 and 45 °C. The GABA content in adzuki beans varied according to pH. At 35 °C, GABA content is 29.4, 41.46, 30.04, 26.24 and 10.7 mg/100 ml at pH values of 3, 5, 6.8, 9 and 11, respectively; at 45 °C, the GABA content for this same range of pH values is 80.40, 85.18, 48.66, 48.16 and 37.3 mg/100 ml, respectively. The pH value of 5 at either 35 or 45 °C was optimal condition for GABA accumulation in immersed adzuki beans.

3.2. *Effect of cold shock on GABA content*

The adzuki beans were preserved at freezing temperatures of −10, −20, and −80 °C for 24 h, and thawed at different temperatures to examine the influence of cold on the accumulation of GABA. As seen in Table 1, different freezing and thaw temperatures have significant effects on the GABA content in soaked adzuki bean seeds. The GABA content in the untreated adzuki beans was 1.34 mg/100 g, and the GABA content in the adzuki beans treated at different freezing and thaw temperatures increased 100–200 times. GABA content was maximal in the adzuki beans treated by 24-h freezing at −20 °C and 24-h thawing at 30 °C (201 mg/100 g). This represents a 200-fold increase compared to untreated beans and a 3–7-fold increase compared to beans treated with a 24-h soak at 35 °C, which have a GABA content of 28.58 mg/100 g.

3.3. *Effect of germination on GABA content*

The GABA yield was observed over six consecutive days of germination in either continuous illumination or dark. The results are shown in Fig. 2. The yield of GABA in the dark increases with germination time, especially during days 3–4 of germination, when the GABA yield changes significantly. The GABA yield reaches its maximum of 134.5 mg/100 g on day 4, and then decreases gradually. Under constant illumination, the GABA yield also increases with germination time, and reaches a maximum of 26.86 mg/100 g on day 4 of germination. The GABA yield then decreases gradually, and reaches the minimum of 7.63 mg/100 g on day 6 of germination. Overall, GABA content in adzuki bean sprouts that were germinated in dark conditions ranges from 44 to 135 mg/100 g, which is 2–5 times greater than that obtained in illuminated conditions (7–26 mg/100 g).

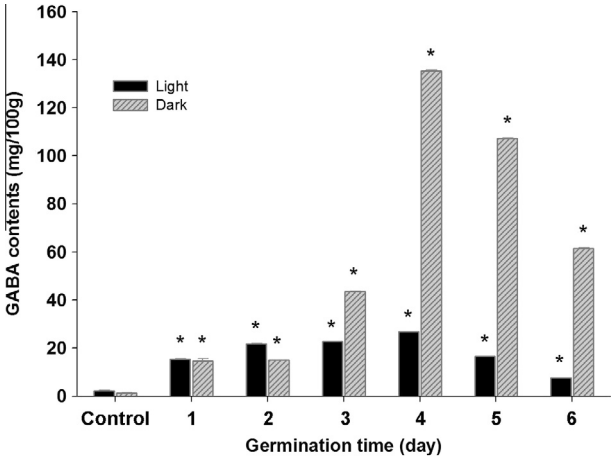

Fig. 2 – The effects of germination time on the GABA content of adzuki beans. The adzuki beans were germinated at 35 °C for 6 days, either under continuous light or continuous dark, and the GABA content was analyzed by HPLC. Data are expressed as the mean ± SD ($n = 3$). $^{*}p < 0.05$ indicates a significant difference from the control group.

3.4. *Effect of fermentation on GABA content*

The results above indicate that pretreatment processes (soaking, germination, and cold shock) before fermentation can all increase the GABA content in adzuki beans effectively. Because the cold shock pretreatment process appears to have a more substantial effect than other processes, the cold shock treated adzuki beans were used for subsequent fermentation experiments. The GABA content in the untreated and cold impact treated adzuki bean substrates before fermentation was 3.43 and 14.13 mg/100 ml, respectively, and the GABA content derived from 24-h fermentation with mixed strains of *L. lactis* and *L. rhamnosus* GG increased to 16.46 mg/100 ml. In addition, preliminary experiments servealed that multiple additional carbon and nitrogen sources could influence the growth of LAB. The mixed strains *L. lactis* subsp. lactis and *L. rhamnosus* GG could use saccharose and yeast extract effectively, and therefore increase the GABA content effectively. After 24-h fermentation of the adzuki bean substrate with additional 4% saccharose and 4% yeast extract, the GABA content increased

Table 1 – The effects of cold shock conditions on the GABA content of adzuki beans.[a]

Soaking temperature (°C)	Freezing temperature (°C)	Thawing temperature (°C)	GABA contents (mg/100 g)[b]
4	–	–	1.34 ± 0.002 h
35	–	–	28.58 ± 0.001 g
35	−10	20	188.00 ± 0.009 b
		30	123.69 ± 0.009 d
	−20	20	113.12 ± 0.012 e
		30	201.23 ± 0.003 a
	−80	20	103.05 ± 0.006 f
		30	174.81 ± 0.008 c

a Adzuki beans were frozen at −10, −20 or −80 °C for 24 h and then transferred into a chamber set at 20 or 30 °C for 24 h, followed by HPLC analysis for GABA content.
b Results were expressed by the mean value of triplicate experiments ± standard deviation.

to 49.44 mg/100 ml, but there is no significant influence of the lactic acid bacteria content on the fermentation liquor pH value, and acidity (Table 2). The effects of fermentation time on the GABA content are shown in Fig. 3a. During the 24 h of the fermentation process of mixed strains *L. lactis* subsp. lactis and *L. rhamnosus* GG, the GABA yield in the fermentation liquor increased with the fermentation time. The GABA yield after 12 h was only 39.67 mg/100 ml, and then increased rapidly, reaching a maximum of 68.81 mg/100 ml after 24 h of fermentation, and became stable as the fermentation time extended. Fig. 3b shows that the content of active lactic acid bacteria increases with fermentation time, and reaches the maximum of 8.32 log cfu/ml after 24 h of fermentation. The bacteria content then decreases slightly as the fermentation time extends. The pH value decreases as the fermentation time extends, reaching a minimum of 3.36 after 24 h of fermentation. Therefore, although the GABA yield reaches the maximum after 24 h of fermentation, the lactic acid bacteria content decreases, and is slightly different from the GABA yield after 24 h. In addition, the fermented adzuki bean milk was tested during storage in order to measure variations in the pH value, the content of active lactic acid bacteria and GABA content throughout the storage period. The results are shown in Fig. 3a. The GABA content in the fermented adzuki bean milk does not decrease during the storage period, but rather increases slowly. GABA content increased particularly quickly during the first 12 days, in which the yield was 67.96 mg/100 ml, and then slowed down. By the end of the storage period on day 28, the GABA yield was 68.21 mg/100 ml. Active LAB content increases continuously with the storage time (Fig. 3b). The active bacteria content reached its maximum of 8.51 log cfu/ml on day 16, and then slowed down so that the active bacteria content was 8.36 log cfu/ml by the end of the storge period, at day 28. The pH value decreased as the storage time extended, reaching a minimum of 3.33 by the end of the storage period on day 28 (Fig. 3c).

4. Discussion

Pharmaceuticals and functional foods have been developed using GABA. Although GABA is present in many fruits and vegetables, the natural concentrations are relatively low, ranging from 0.03 to 2.00 μmol/g fresh weight. Due to the increasing commercial demand for GABA, diverse foods containing both biologically and chemically produced GABA have been reported. For example, GABA-enriched green tea is produced by an anaerobic treatment, which in rice germ, it is produced by soaking in water, by a high-pressure treatment and germination in brown rice, and in tempeh-like fermented soybean and black raspberry juice, GABA enrichment is achieved by fermentation by *Lactobacillus brevis* (Kim, Lee, Ji, Lee, & Hwang, 2009). This study aimed to increase the GABA content in adzuki beans by testing various recipes, in addition to various preprocessing and fermentation conditions. The results clearly indicate that it possible to produce GABA enriched adzuki bean food through cold shock treatment combined with LAB fermentation. Previous studies have revealed that in plants, GABA is rapidly produced in response to anaerobic conditions, low pH, low or high temperatures and darkness, and by mechanical manipulation (Akihiro et al., 2008). In all the test recipes, GABA content in the adzuki bean seeds increased with the soaking time, suggesting that the adzuki beans have a metabolic reaction when they absorb water. GABA is a metabolic endproduct and is primarily produced by the decarboxylation of L-glutamic acid, which is catalyzed by glutamate decarboxylase. While the enzyme system related to endogenous enzymes and glutamate decarboxylase (GAD) are activated, major components such as starches and proteins may be degraded, and result in the production of some secondary metabolites such as GABA and nutrients (Xu, Hu, Duan, & Tian, 2010). As mature seeds contain less moisture, the first step of germination is moisture absorption. Once the seeds have absorbed adequate moisture, they begin to sprout, the physiological metabolic activity is initiated, thereby increasing enzyme activity, metabolism begins, and ultimately accelerating the GABA formation rate. The reason may be related to the size of the seeds that are germinated. In this study, it was observed that the optimum germination temperature for increasing the GABA content of adzuki beans was 45 °C (Fig. 1). At different soaking temperatures, the adzuki beans soaked at 45 °C for 24 h showed the maximum GABA content (43.37 mg/100 g), which is approximately a 40-fold increase as compared with the GABA content in the untreated adzuki beans (1.34 mg/100 g). This result is

Table 2 – The effects of nutrient source on GABA content, biomass, pH, and titratable acidity of adzuki bean substrates fermented by L. lactis and L. rhamnosus.

Nutrient sources	Concentration (%)	Viable count (log/ml)	pH	Titratable acidity (%)	GABA content (mg/100 ml)
–	–	7.80 ± 0.02 b	5.11 ± 0.04 a	0.153 ± 0.02 c	12.95 ± 0.07 g
Yeast extract	1	7.86 ± 0.02 b	3.66 ± 0.02 d	0.79 ± 0.06 b	22.81 ± 0.14 c
	2	7.90 ± 0.01 a	3.79 ± 0.04 c	0.95 ± 0.03 b	32.89 ± 0.04 b
	4	8.05 ± 0.03 a	3.97 ± 0.05 b	1.24 ± 0.06 a	49.44 ± 0.33 a
Sucrose	1	7.84 ± 0.02 b	3.41 ± 0.03 e	0.74 ± 0.04 b	14.81 ± 0.06 f
	2	7.77 ± 0.05 b	3.38 ± 0.02 f	0.75 ± 0.02 b	16.85 ± 0.12 e
	4	7.79 ± 0.04 b	3.35 ± 0.05 f	0.75 ± 0.02 b	17.92 ± 0.08 d

The adzuki bean substrate was treated by cold shock, different concentration of nutrient source was added, and then fermented by mixed culture *L. lactis* subsp. lactis and *L. rhamnosus* GG at 37 °C for 24 h. After fermentation, the GABA content of fermented adzuki bean milk was analyzed by HPLC, and biomass was measured by serial dilution-agar plate method. The pH values were measured using pH meter and titratable acidity, expressed as lactic acid, was determined by methods of AOAC. 1999.

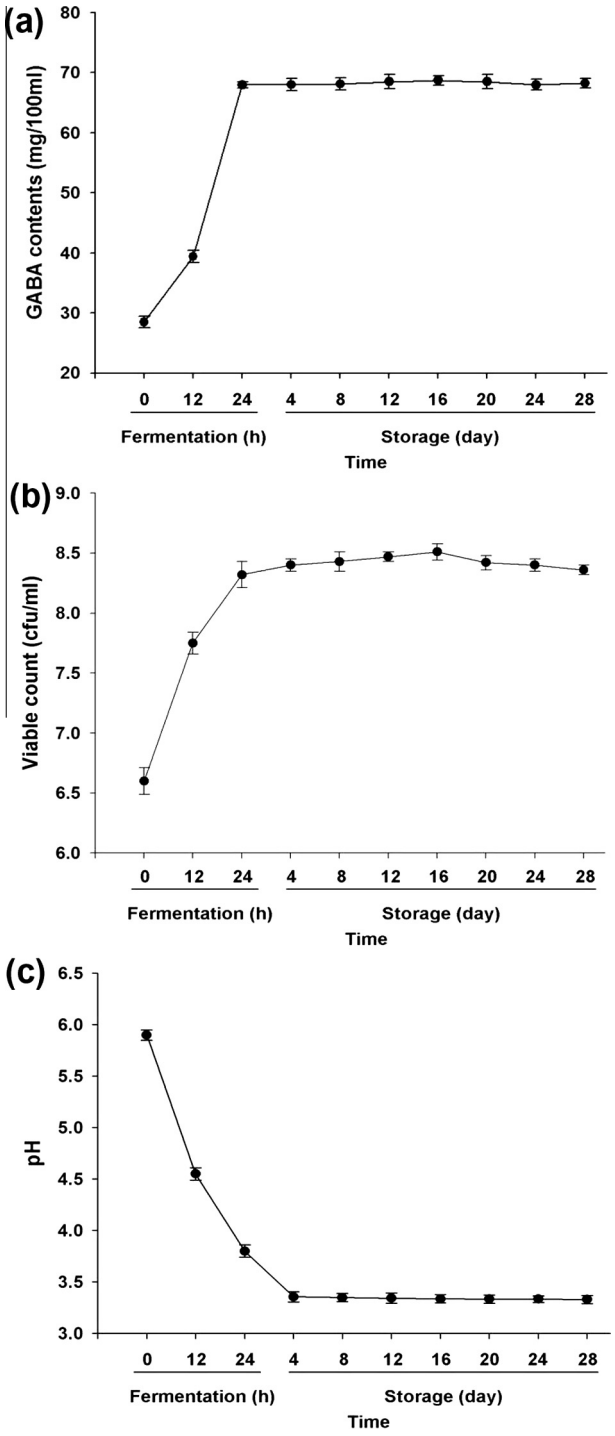

Fig. 3 – (a) GABA content; (b) viable counts; and (c) pH changes during adzuki bean milk fermentation (24 h at 37 °C) and cold storage (28 days at 4 °C). Bars on data points represent standard errors.

opposite to previous studies that showed an optimal temperature of 30–35 °C for GABA accumulation in germinated rice (Komatsuzaki et al., 2007), fermented black raspberry juice (Kim et al., 2009), and germinated foxtail millet (Bai, Fan, Gu, Cao, & Gu, 2008). It suggests that the optimum generation temperature of GAD differs among different plants. Li, Bai, Jin, Wen, and Gu (2009) showed that GABA content in the fava bean was 2.41 mg/g DW when germinating at 33.6 °C, an amount representing 3.17-fold of the results obtained in the present study (0.76 mg/g DW). According to Fig. 1b, the GABA content varies with the pH value. In an acidic environment (pH 3–5), the GABA in adzuki beans increases gradually with increasing pH value of the soaking environment. In neutral and alkaline environments (pH 6.8–11), the GABA in adzuki beans decreases gradually as the pH value of soaking environment increases. Li et al. (2009) reported that the optimal culture conditions for increasing GABA in fava beans is a temperature below 33.6 °C and a pH of 3.19. In addition, the GABA content in the adzuki beans soaked at 45 °C under a variety of pH values. The adzuki beans soaked at 45 °C and pH 5 showed the maximum GABA content of 85.18 mg/100 g. This result is consistent with the findings of in vitro experiments, which indicated that GAD activity is optimal at a pH value of about 5.8. In an alkaline environment and under the soaking condition of pH 11, the GABA content in adzuki beans at 35 or 45 °C is very low (10.7 and 37.3 mg/100 g, respectively). This is likely due to the low activity of GAD in alkaline environments. In summary, both the soaking temperature and pH will influence the GABA content in adzuki beans.

This study also demonstrated that cold shock is helpful to the accumulation of GABA. Cytosolic levels of calcium ions are elevated in response to cold shock, so that when the GAD catalyzes the formation of GABA from glutamic acid, it is further activated by increases in the cytosolic concentration of Ca^{2+} (Kinnersley & Turano, 2000). Cholewa, Cholewinski, Shelp, Snedden, and Bown (1997) also reported that cold shock initiates a signal transduction pathway in which increased cytosolic Ca^{2+} stimulates calmodulin-dependent L-Glu decarboxylase activity and γ-aminobutyric acid synthesis. Bown and Shelp (1997) found a rise in GABA levels in soybean leaves of 20- to 40-fold within 5 min in response to cold shock or mechanical stimulation. The GABA content in unsprouted adzuki beans is 1–1.72 mg/100 g and is increased greatly in sprouted beans with a 10–100-fold amplitude increase. The GABA content after germination is 10–134 mg/100 g, and is particularly high in dark conditions. The amplitude of increase in adzuki bean sprouts reaches its maximum on day 4 of germination, and the GABA yield reaches a maximum of 134.5 mg/100 g. There have been reports on improving the GABA contents in plants by germination, since activation of the GAD and protease in the seeds leads to accumulation of GABA. Complex physiological and biochemical reactions occur during the sprouting process of adzuki beans, therefore activating various enzymes, and intensifying metabolic activity. The GABA content is apparently higher during germination in the dark than in the adzuki bean sprouts exposed to continuous illumination. The yield of GABA is significantly positively correlated with the sprouting degree and GAD activity. These results were consistent with those of previous studies examining the GDA activity and GABA accumulation in foxtail millet (Bai et al., 2009), fava bean (Yang, Chen, & Gu, 2011), and rice grains (Jannoey et al., 2010) during germination. Although all of the above three treatment processes were experimentally shown to significantly increase the GABA content in adzuki beans, the soaking treatment is the fastest and most convenient, but results in the minimum GABA content among

the three processes. Although the operating time of germination and cold impact treatment is longer, and the operation procedure of the sprouting treatment is more complex, this treatment increases the GABA content greatly; 1–2.3 times more than that of soaking treatment. Compared to the germination treatment alone, the operation procedure of the cold impact treatment is simpler and the treatment time is shorter, and results in a greater content of GABA, which is 1.5 times greater than the germination treatment.

GABA can be produced by microorganisms including bacteria, fungi and yeasts. Several GABA-producing LAB species have been reported in recent decades, including *L. brevis* (Zhang et al., 2012), *Lactobacillus reuteri*, *Lactobacillus rossiae* (Stromeck et al., 2011), *L. plantarum* (Tung, Lee, Liu, & Pan, 2011) and *Lactobacillus farciminis* (Thwe et al., 2011). The GABA-producing ability of LAB varies widely among the strains and is significantly affected by culture conditions, such as the growth medium. It is therefore important to optimize the medium for enhancing GABA production during fermentation. Li, Qiu, Gao, and Cao (2010) found that glucose, soya peptone, Tween-80 and $MnSO_4{\cdot}4H_2O$ were the key factors affecting GABA production using the response surface methodology. In the present study, the cold shock-treated adzuki bean substrate was fermented by LAB, in which the carbon source is the carbon skeleton forming bacterial synthesis. The LAB strains can use glucose effectively as an energy source, but use other saccharides, such as sucrose, fructose and lactose, to different extents. According to the results, when the mixed strains *L. lactis* subsp. lactis and *L. rhamnosus* GG use sucrose as the carbon source, the fermented adzuki bean milk has a higher GABA content, with a yield of 15.42 mg/100 ml. The GABA yield from bacteria is also influenced by the nitrogen sources. In which different nitrogen sources added in the fermentation substrate result in different GABA yields. The organic nitrogen sources, including pancreatic protein, yeast extract, peptone and skim milk powder, were used in this study for the fermentation test. The results shows that the mixed strains *L. lactis* subsp. lactis and *L. rhamnosus* GG can use these organic nitrogen sources to grow and produce GABA. When 4% yeast extract is used as single nitrogen source, the fermented adzuki bean milk has a maximum GABA yield of 49.44 mg/100 ml, which is 14 times higher than the yield of the fermented adzuki bean milk without pretreatment (3.43 mg/100 ml). The GABA is transformed from glutamic acid by GAD, and GAD is a pyridoxal enzyme which is a requisite co-factor for the reaction of GABA (Kato, Kato, Fuaukawa, & Hara, 2002). The pyridoxal phosphate is one of the activated forms of vitamin B6, because the additional yeast extract contains a rich vitamin B complex, which can increase the content of GABA in fermented adzuki bean milk effectively by activating GAD. Komatsuzaki, Shima, Kawamoto, Momose, and Kimura (2005) demonstrated that the addition of pyridoxal phosphate could increase the content of GABA. The lactic acid bacteria produces GABA depending on the decarboxylase transformation system of glutamic acid, as GAD is only active in alkaline environments, with an optimum pH of approximately 4–4.5, and in the LAB fermentation period. As the pH of the fermentation liquor decreases gradually, the activity of GAD increases gradually. The activity of GAD begins to be inhibited as the pH value decreases continuously. The GAD derived from different strains has different tolerances depending on the pH, resulting in significant differences between the initial pH values of the fermentation liquor of different strains (Cho, Park, Kim, Ryu, & Park, 2011).

In conclusion, this study used adzuki beans as the fermentation substrate to develop a functionally healthy fermented beverage. In this study, the adzuki beans were treated by soaking, germination and cold impact before fermentation to prepare the fermentation substrate. The experimental results showed that the three treatment processes can increase the content of GABA in adzuki beans effectively, with the cold impact pretreatment process showing the best performance in increasing the GABA content (201.23 mg/100 g), by approximately 150 times greater than that the GABA produced by untreated adzuki beans. The soaked and cold impacted adzuki beans have intact appearances, and the operation is convenient with low processing costs; thus, the method is applicable for the food processing industry for complete adzuki beans. This method could increase the GABA content in adzuki beans, thus enhancing the added value of processed food. The treatment process is convenient, and is applicable to average consumers, who can treat adzuki beans at home to prepare food with high GABA content, such as adzuki bean soup and adzuki bean bread. Moreover, healthy constituents such as GABA can be produced in the germination process. This study therefore successfully developed a fermented adzuki bean milk beverage with high GABA content, high lactic acid bacterial count, and high stability using adzuki beans as the fermentation substrate.

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