Original paper

Optimized Production of GABA and γ -PGA in a Turmeric and Roasted Soybean Mixture Co-fermented by *Bacillus subtilis* and *Lactobacillus plantarum*

Jong-Soon LIM¹, Coralia V. GARCIA² and Sam-Pin LEE^{1,2*}

¹The Center for Traditional Microorganism Resources (TMR), Keimyung University, Daegu 42601, Korea

²Department of Food Science and Technology, Keimyung University, Daegu 42601, Korea

Received June 8, 2015 ; Accepted July 28, 2015

A turmeric and roasted soybean flour mixture (TRSF) was successfully fermented in solid-state using *Bacillus subtilis* HA and *Lactobacillus plantarum* K154. The co-fermentation was optimized using the turmeric mixture (turmeric: RSF=1:1), 5% MSG, and a 2-fold volume of water. The TRSF fermented by *B. subtilis* HA showed a consistency of 1.23 Pa.sⁿ and 3.57% (w/w) mucilage. The co-fermented TRSF exhibited pH 5.94 and 1.94% acidity after the second lactic acid fermentation. The viable cell counts of *Bacillus* sp. and *Lactobacillus* sp. were 4.50×10^7 CFU/g and 8.00×10^9 CFU/g, respectively. In addition, the co-fermented TRSF exhibited a higher GABA content (1.78%) than that of the single strain-fermented TRSF. Serial co-fermentation using two bacterial strains can thus provide novel turmeric-based ingredients fortified with γ -PGA, GABA, peptides, and probiotics.

Keywords: turmeric, soybean, GABA, *Bacillus subtilis*, *Lactobacillus plantarum*

Introduction

Turmeric (*Curcuma longa* L.) is a tropical plant that belongs to the family Zingiberaceae. Turmeric is grown mainly in South Asia and China, and is an important component of the cuisine and traditional medicine of those regions (Tyagi *et al.*, 2007). The yellow pigment of turmeric was identified as curcumin (diferuloylmethane) (Roughley and Whiting, 1973). Apart from giving turmeric its characteristic color, curcumin is responsible for most of the biological activities of this spice, including its antiinflammatory, anticancer, antioxidant, and antibacterial activities (Araújo and Leon, 2001). Fermented turmeric was reported to exhibit higher antioxidant (Kang *et al.*, 2009) and antiinflammatory (Kim *et al.*, 2011) activities than those of fresh turmeric; thus, fermentation could be an effective approach to enhance the health benefits of this spice.

Food products made from soybeans, such as tofu, soy sauce, and soybean paste, have been consumed in Asia for a long time. In Western countries, soy protein is used in processed products such

as sausages, hamburgers, breads and pastries (Fukushima, 1981). Soy products are recognized as healthy foods rich in protein and low in saturated fats, which also contain bioactive compounds such as isoflavones and saponins (Messina, 2010). Fermented soy products are popular in Asian cuisines; however, they generally require a long processing time. Nevertheless, soybean fermentation using *Bacillus subtilis* can be achieved in a short time, and the products generated are rich in polypeptides and peptones, resulting in easy digestion and absorption. Examples of such products are the Japanese natto and Korean chungkookjang. Fermented soybean products have been reported to have antihypertensive (Shin *et al.*, 2001), antioxidant (Wang *et al.*, 2008), fibrinolytic (Kim *et al.*, 2006) and anticancer (Zhao *et al.*, 2013) activities. However, the strong smell and taste of these fermented products is a barrier for their generalized consumption (Park *et al.*, 2012). Therefore, improving the organoleptic qualities of fermented soy products is desirable. A co-fermentation with *B. subtilis* MC31 and *Lactobacillus sakei* 83 was reported to enhance the GABA content

*To whom correspondence should be addressed.

E-mail: splee@kmu.ac.kr

and flavor of chungkookjang (Lee *et al.*, 2014).

The lactic acid bacteria (LAB) used to make fermented food products generate functional compounds such as oligosaccharides and peptides in addition to lactic acid (Leroy and De Vuyst, 2004). One of the functional compounds that can be generated by fermentation with LAB is γ -aminobutyric acid (GABA), a nonprotein amino acid that acts as an inhibitory neurotransmitter and that also exhibits hypotensive effects (Dhakal *et al.*, 2012). In LAB, GABA acts as a defense against acidic environments and as an alternative energy source (Kook *et al.*, 2010a). Fermentation with *B. subtilis* generates mucilage containing γ -polyglutamic acid (γ -PGA) and fructan, which give the sticky consistency to natto (Nagai *et al.*, 1994; Park *et al.*, 2012). γ -PGA is a biodegradable, water soluble and edible biopolymer, which can be composed of either the L or D isomer of glutamic acid or both. γ -PGA is a promising biological material that could be used in medicine as a drug delivery agent, in food as an encapsulating agent and fat replacement, and in skin care products as a humectant (Ogunleye *et al.*, 2015). γ -PGA has also been used as a bitterness relieving agent in Japan (Murase *et al.*, 2000). Moreover, γ -PGA exhibits immune-boosting and anticancer activities. However, the production cost of γ -PGA is still high; hence, more research on the microbial production of this biopolymer is needed (Kim *et al.*, 2014b; Ogunleye *et al.*, 2015).

In this study, the solid-state fermentation of a turmeric/roasted soybean flour mixture (TRSF) was optimized to enhance its organoleptic properties as well as to fortify it with bioactive compounds. The aim was to develop a turmeric-based ingredient enhanced with functional compounds. The novel co-fermentation was achieved continuously using *Bacillus subtilis* HA and *Lactobacillus plantarum* K154 in the presence of a glutamate-enriched substrate, aiming to efficiently produce both γ -PGA and GABA. The final fermented TRSF was analyzed to determine the production of bioactive compounds including γ -PGA, GABA, peptides, and probiotics.

Materials and Methods

Materials Powdered turmeric (*Curcuma longa*) rhizomes were purchased from a local farm (Ingreen Co., Pochen, Korea). Roasted soybean flour (RSF) was bought from Ilho (Daegu, Korea). GABA and monosodium L-glutamate (MSG) standards were obtained from Yakuri Pure Chemicals Co., Ltd. (Kyoto, Japan). Curcumin ($\geq 98\%$) and ninhydrin were purchased from Sigma (St. Louis, MO, USA). MRS broth (Difco™ Lactobacilli MRS) was obtained from Becton, Dickinson and Company (Sparks, MD, USA). All chemicals used were analytical grade reagents.

Strains and starter culture *Bacillus subtilis* HA (KCCM 10775P) was used for the first solid-state fermentation. This strain is used to produce traditional Korean fermented soybeans (Kim *et al.*, 2010) and is an effective producer of γ -PGA (Seo *et al.*, 2008),

so it was chosen for the solid-state fermentation of the TRSF. The bacteria were inoculated on an MRS agar plate previously sterilized in an autoclave (MLS-3020, Sanyo Electric Co., Ltd., Osaka, Japan) at 121°C for 15 min. The bacteria were cultured at 42°C for 24 h. Subsequently, a single colony was taken and cultured in 5% (w/v) defatted soy milk, using a shaking incubator (SI-900R, JEIO TECH, Co., Daejeon, Korea) at a constant temperature of 42°C for 24 h and 160 rpm, and used as a starter.

Lactobacillus plantarum K154 (KACC 91727) was used for the second fermentation; this strain is recognized as an effective GABA-producing LAB isolated from fermented kimchi (Park *et al.*, 2014). *L. plantarum* K154 was cultured in stationary MRS agar plates at 30°C for 24 h. Subsequently, a single colony was taken and cultured in the MRS broth, and used as a starter.

Efficient production of GABA by co-fermentation with *L. plantarum* K154, using TRSF fermented with *B. subtilis* HA MSG solutions were prepared in 80 mL of water at concentrations of 0%–7%. Subsequently, the MSG solutions were combined with TRSF (turmeric:RSF = 1:1; 40 g) to produce culture media. The media were sterilized at 121°C for 15 min, followed by inoculation with *B. subtilis* HA starter (1%, v/v; 1.0×10^7 CFU/mL). The solid-state fermentation was performed at 42°C for 1 day. For the second LAB fermentation for GABA production, the first-fermented semi-solid culture was inoculated with *L. plantarum* K154 starter (5%, v/v; 5.0×10^7 CFU/mL), and cultured at 30°C for 7 days.

pH and acidity The pH was measured using a pH-meter (model 420+, Thermo Orion, USA). To determine titratable acidity, 1 mL of sample was dissolved in 9 mL of distilled water, and 0.1 N NaOH was added until reaching a pH of 8.3. Acidity was expressed as percentage of lactic acid (% v/v).

Viable cell count measurement To determine viable cells of *B. subtilis*, 20 μ L of serial diluted cell suspensions were plated onto MSR agar plates and cultured at 42°C for 24 h to yield colony forming units (CFU)/g. To selectively determine the viable cell counts of *L. plantarum* K154 during the second fermentation, the MRS agar plate with mixed strains was cultured at 30°C for 24 h for delaying the growth of *B. subtilis* (Kook and Cho, 2013).

Mucilage determination Five grams of viscous broth were diluted with 20 mL of distilled water, and centrifuged for 20 min at 6296 $\times$ g (SUPRA21K, Hanil, Incheon, Korea). The supernatant was recovered, and mixed with twice the volume of isopropanol. The precipitate was recovered by centrifugation, and then washed using 95% ethanol. The precipitate was dried at 50°C for 24 h, and the dry weight was determined.

Tyrosine content measurement Proteolytic activity was determined using a modified Anson's method (Oh *et al.*, 2006). The fermented TRSF (2 g) was homogenized in 18 mL of distilled water, and centrifuged at 22,250 $\times$ g for 15 min. Subsequently, the supernatant was mixed with 0.7 mL of a 0.44-M trichloroacetic acid solution, and incubated at 37°C for 30 min. The precipitate was removed by centrifugation at 22,250 $\times$ g for 10 min, and the

supernatant (1 mL) was mixed with 2.5 mL of 0.55 M Na_2CO_3 and 0.5 mL of Folin-phenol reagent (3-fold-diluted stock solution), followed by incubation at 37°C for 30 min. The absorbance was measured on a spectrophotometer (Ultrospec®21400 pro, Amersham Biosciences, Piscataway, NJ, USA) at 660 nm, and one unit of proteolytic activity was defined as the amount of enzyme producing 1 μg tyrosine/min. The tyrosine content was calculated from a standard curve.

GABA and glutamic acid content analysis For the qualitative analysis of MSG and GABA, a silica TLC plate (10 × 20 cm) and glass chamber (30 × 25 × 10 cm) was used for solvent development. Standards of 0.25%, 0.5%, and 1% MSG and GABA were used. The solvent (*n*-butyl alcohol: glacial acetic acid: distilled water; 3:1:1, v/v) was poured into the chamber and allowed to saturate the atmosphere for 4 h at room temperature before use. The sample was diluted 3-fold, and a volume of 2 μL was spotted at the bottom of the TLC plate. The distance between spots was 10–15 mm. After elution, the plate was allowed to dry at room temperature, and further dried at 50°C. Subsequently, a 0.2% ninhydrin solution was sprayed, and the plate was developed at 100°C for 5–10 min or until spots appeared clearly.

High-performance liquid chromatography analysis Free amino acids were analyzed on an Agilent 1290 Infinity HPLC (Santa Clara, CA, USA) fitted with an Agilent SB-C18 (2.1 × 150 mm, 1.8 μm) column. The mobile phase consisted of Solvent A (140 mM NaOAc, 0.15% TEA, 0.03% EDTA, 6% CH_3CN ; pH 6.1) and Solvent B (60% CH_3CN , 0.015% EDTA), pumped at a flow rate of 0.4 mL/min. The detector was an Agilent 1290 Infinity diode array detector (DAD) operated at 254 nm. The sample was previously dried at room temperature for 30 min, dissolved in solvent A, and filtered through a 0.45- μm syringe filter before analysis.

The elution profile was as follows: 0.1% at the beginning, gradually increased to 40% B at 14.5 min, then increased to 100% B at 15.6 min and maintained at 100% B until 17.9 min, then decreased to 0.1% B at 18 min, and maintained at 0.1% B until 20 min.

Statistical analysis The Statistical Package for the Social Sciences (SPSS, Ver. 20.0, SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Results were expressed as mean $\pm$ SE. A one-way analysis of variance (ANOVA) and Duncan's test was performed to determine whether the groups exhibited significant differences. Statistical significance was set at $p < 0.05$.

Results and Discussion

Solid state fermentation of the turmeric and roasted soybean flour mixture For the first fermentation with *B. subtilis* HA, solutions with concentrations of 0%–7% MSG were added to the TRSF to make a paste. A concentration of 50% turmeric was used because concentrations above 70% could not be fermented by *B. subtilis* (unpublished result). The consistency of the fermented

Fig. 1. Effect of the MSG concentration on (a) the consistency index of the co-fermented TRSF, (b) mucilage content and consistency index of the TRSF fermented by *B. subtilis* HA

TRSF thickened substantially after the first day of fermentation, indicating the production of mucilage containing substances such as γ -PGA. The production of γ -PGA by alkaline fermentation is affected by the nutrients in the medium, strain used, fermentation temperature and time (Kim *et al.*, 2014b). It was reported that a *Bacillus* strain produced γ -PGA and levan simultaneously using a defined medium containing L-glutamic acid and sucrose (Shi *et al.*, 2006). In the current study, adding 1% MSG enhanced the production of mucilage by *B. subtilis* HA, reflected in the high consistency (3.68 Pa·s) and mucilage content (3.47%) obtained. However, the consistency decreased for MSG concentrations above 3%, resulting in the lowest consistency at 7% MSG (Fig. 1a), even though the mucilage content decreased only slightly when 5% or more MSG was added (Fig. 1b). *Bacillus* sp. are glutamate-dependent strains; thus, increasing the glutamate content up to a certain point results in an increase in the consistency index because mucilage is produced. However, excess glutamate in the medium results in an incomplete conversion to γ -PGA and a lowered consistency index (Oh *et al.*, 2007). Furthermore, it has been reported that a high sodium concentration results in γ -PGA with a low molecular weight and a broth with a low viscosity (Sung *et al.*, 2005). Hence, using a high concentration of MSG may also decrease the consistency because excess sodium is added to the medium. Adding sodium may also cause contraction of the molecules in the mucilage, decreasing viscosity (Capitani *et al.*, 2015). It is also possible that the excess of glutamate acts as an inhibitor for the production of γ -PGA as the phenomenon of

Table 1. Physicochemical properties of the TRSF after the first fermentation using *Bacillus subtilis* HA, according to MSG content

MSG (%)	pH	Acidity (%)	Viable cell count (CFU/g)	Tyrosine content (mg/g)
0	6.24 ± 0.01	1.12 ± 0.04	1.80 × 10 ⁹	3.60 ± 0.16
1	6.54 ± 0.03	0.95 ± 0.01	1.20 × 10 ⁹	3.89 ± 0.15
3	6.42 ± 0.03	0.84 ± 0.01	2.60 × 10 ⁹	2.85 ± 0.03
5	6.50 ± 0.03	0.75 ± 0.01	1.10 × 10 ⁹	2.84 ± 0.01
7	6.64 ± 0.06	0.72 ± 0.07	6.50 × 10 ⁸	2.56 ± 0.05

substrate inhibition is well known. The medium lacking MSG exhibited an intermediate consistency index because *Bacillus* sp. can produce sticky mucilage when fermenting soybeans; however, without glutamate addition, γ -PGA production is low (Oh *et al.*, 2007). In addition, the protease activity of the TRSF fermented by *B. subtilis* decreased as the MSG concentration increased (unpublished result).

Some studies have reported the successful fermentation of low concentrations of turmeric (2%–5%) using *Bacillus* and *Lactobacillus* strains (Kang *et al.*, 2009; Kim *et al.*, 2011). The production of a fermented milk containing up to 3% turmeric was also reported (Gereltuya *et al.*, 2015). In the current study, an alkaline fermentation of a high concentration of turmeric (50% of the turmeric/soybean substrate) was successfully carried out by *B. subtilis*. The ability of lactic acid bacteria to ferment turmeric reveals that the antimicrobial activity of curcumin and other turmeric compounds is selective, affecting pathogenic bacteria but having little effect on various LAB strains (Lee, 2006). Compared to controls lacking turmeric, milk containing turmeric and fermented using lactic acid bacteria was reported to exhibit similar (Hosny *et al.*, 2011) or even higher (Gereltuya *et al.*, 2015) viable cell counts. Our results suggest that a mixture of turmeric and soybean could be a suitable substrate for alkaline fermentation to produce bioactive compounds. Moreover, MSG is a valuable substrate for producing bioactive compounds including γ -PGA in a defined medium, because strains such as *B. subtilis* HA are glutamate dependent. MSG also acted as a precursor for GABA production during the second fermentation with *L. plantarum* K154 (Kim *et al.*, 2014b), which was carried out for 7 days. During the solid-state fermentation, the number of viable cells of *Bacillus* sp. was in the range of $1.80 \times 10^9 - 6.50 \times 10^8$ CFU/g, depending on the MSG concentration used (Table 1). The lowest viable cell counts corresponded to a concentration of 7% MSG. *Bacillus* species are used as probiotics; thus, their presence in a fermented product is considered to provide health benefits (Kim *et al.*, 2014b).

The tyrosine content in the fermented TRSF ranged from 2.56 to 3.89 mg/g, depending on the MSG concentration.

The highest tyrosine content (3.89 ± 0.15 mg/g) was obtained when 1% MSG was used, and the lowest, when 7% MSG was used (Table 1). The presence of tyrosine indicates the effective hydrolysis of the soy protein, with the resulting generation of

peptides (Park *et al.*, 2012). The results obtained suggest that high MSG concentrations may inhibit the growth of *Bacillus* sp. during the solid-state fermentation, resulting in decreased viable cell counts and protein hydrolysis; thus, 5% MSG was added to the TRSF.

Lactic acid fermentation of the TRSF mixture, using L. plantarum K154 The physicochemical changes after the second fermentation with *L. plantarum* K154 are summarized in Table 2.

The change in pH and acidity during fermentation depended on the concentration of MSG supplemented. When 1% MSG was added, the pH decreased from 4.94 at the start of the second fermentation to 4.84 by 7 days, whereas acidity increased to 1.98% by the end of the second fermentation. By contrast, when 7% MSG was added, the pH increased from 5.08 in the first day to 6.72 by the end of the second fermentation, whereas acidity decreased to 0.72% by 7 days. When a 5% MSG concentration was used during the second fermentation, acidity gradually decreased to a final value of 1.14% by 7 days. Furthermore, the viable cell counts of *B. subtilis* HA and *L. plantarum* K154 were 4.50×10^7 and 8.00×10^9 CFU/g, respectively, by the end of the second fermentation. The increase in the numbers of the lactic acid-producing *L. plantarum* K154 thus resulted in a decrease in the viable cell count of the *Bacillus* strain, which coincided with the acidification of the broth. Lower MSG contents resulted in lower viable cell counts, in particular that of *B. subtilis* HA, as a result of the increased acidity.

The consistency of the broth became thinner during the second (lactic acid) fermentation (Fig. 1a). This outcome was due to the acidic pH, which affected the degree of ionization of the γ -PGA biopolymer, resulting in structural changes of the mucilage (Do *et al.*, 2001; Kim *et al.*, 2014b).

The tyrosine content changed according to the MSG concentration. When no MSG was added, the tyrosine concentration decreased during the second fermentation. However, at 3%–7% MSG, the tyrosine content increased during the second fermentation. The highest tyrosine concentration (3.43 ± 0.09 mg/g) was obtained when 5% MSG was used. Tyrosine was generated by the proteolytic enzymes produced by *B. subtilis* HA with highly viable cells, and was an indicator of peptide generation (Park *et al.*, 2012).

GABA production by co-fermentation The free amino acid content in the TRSF containing 5% MSG, before and after fermentation, is listed in Table 3. The content of glutamic acid

Table 2. Physicochemical properties of the TRSF after the second fermentation using *Lactobacillus plantarum* K154, according to MSG content

MSG (%)	Day	pH	Acidity (%)	Viable cell count (CFU/g)		Tyrosine content (mg/g)
				<i>Bacillus</i> sp.	<i>Lactobacillus</i> sp.	
0	1	4.75 ± 0.01	1.73 ± 0.00	2.00 × 10 ⁶	5.85 × 10 ⁹	2.68 ± 0.07
	3	4.74 ± 0.00	1.84 ± 0.02	6.00 × 10 ⁵	4.60 × 10 ⁹	2.59 ± 0.04
	5	4.70 ± 0.00	1.92 ± 0.01	5.03 × 10 ⁵	1.45 × 10 ⁹	2.69 ± 0.08
	7	4.74 ± 0.00	1.90 ± 0.00	4.50 × 10 ⁵	1.50 × 10 ⁹	2.60 ± 0.01
1	1	4.94 ± 0.01	1.55 ± 0.02	7.50 × 10 ⁶	7.60 × 10 ⁹	2.66 ± 0.03
	3	4.85 ± 0.00	1.79 ± 0.01	2.50 × 10 ⁶	6.35 × 10 ⁹	2.51 ± 0.04
	5	4.79 ± 0.00	1.95 ± 0.03	5.50 × 10 ⁶	3.60 × 10 ⁹	2.61 ± 0.07
	7	4.84 ± 0.00	1.98 ± 0.00	2.00 × 10 ⁶	3.30 × 10 ⁹	2.52 ± 0.05
3	1	5.16 ± 0.02	1.46 ± 0.02	1.00 × 10 ⁸	8.35 × 10 ⁹	2.75 ± 0.11
	3	5.30 ± 0.00	1.50 ± 0.01	2.63 × 10 ⁸	7.60 × 10 ⁹	3.15 ± 0.09
	5	5.23 ± 0.02	1.63 ± 0.03	2.60 × 10 ⁷	7.45 × 10 ⁹	2.91 ± 0.10
	7	5.17 ± 0.00	1.67 ± 0.01	2.70 × 10 ⁷	3.90 × 10 ⁹	3.37 ± 0.19
5	1	5.13 ± 0.01	1.68 ± 0.01	1.65 × 10 ⁷	3.60 × 10 ⁹	2.12 ± 0.04
	3	5.99 ± 0.00	0.92 ± 0.00	8.85 × 10 ⁷	7.45 × 10 ⁹	2.76 ± 0.05
	5	5.95 ± 0.00	1.00 ± 0.01	4.40 × 10 ⁷	8.00 × 10 ⁹	3.05 ± 0.01
	7	5.94 ± 0.00	1.14 ± 0.01	4.50 × 10 ⁷	8.00 × 10 ⁹	3.43 ± 0.09
7	1	5.08 ± 0.02	1.91 ± 0.01	4.05 × 10 ⁷	3.30 × 10 ⁹	2.09 ± 0.09
	3	6.26 ± 0.00	0.86 ± 0.01	1.10 × 10 ⁸	3.90 × 10 ⁹	2.94 ± 0.02
	5	6.34 ± 0.00	0.86 ± 0.01	2.45 × 10 ⁸	4.66 × 10 ⁹	3.05 ± 0.06
	7	6.72 ± 0.01	0.72 ± 0.02	3.80 × 10 ⁷	5.70 × 10 ⁹	2.81 ± 0.06

decreased from 4.18% before fermentation to 3.83% after the first fermentation, and 0.27% after the second fermentation by LAB. By contrast, the content of GABA increased from 0.01% before fermentation to 1.78% after the second fermentation. The changes in the glutamic acid and GABA contents indicate that MSG was used as a precursor for GABA production. The high level of GABA obtained (1.78%) by fermenting the TRSF mixture was notable. The content of most free amino acids in the TRSF mixture was low, except for glutamic acid, which was added as MSG. However, the first alkaline fermentation was able to generate free amino acids in the culture broth, resulting in an increase in the content of 19 amino acids. Although the total amino acid content decreased after the second fermentation because of the decrease in glutamic acid, the concentrations of all the other 19 amino acids increased. In foods, the GABA content varies from 0.01–0.04 mg/g in white rice to 0.1–1 mg/g in sprouted brown rice, 0.35–2.05 mg/g in green tea, and 2.5–7.0 mg/g in foods produced by lactic acid fermentation (Hwang, 2011). A study (Lee *et al.*, 2014) reported that the co-fermentation of chungkookjang, using *B. subtilis* MC31 and *L. sakei* 383 resulted in a GABA concentration of 0.47 mg/g and improved organoleptic characteristics. By contrast, another study reported that co-fermenting milk with *B. subtilis* and *L. lactis* resulted in a GABA content of only 0.18 µg/mL (Lim and Lee,

2014). These results indicate that microbial GABA production depends on the strains used and fermentation conditions.

The effect of the co-culture on GABA production was substantial as demonstrated by the ten-fold increase in GABA concentration after the co-fermentation. By contrast, the single strain fermentation failed to generate GABA, indicating that the first fermentation with *B. subtilis* MC31 was a prerequisite to attain suitable culture conditions, such as moderate pH and acidity, for *L. plantarum* K154 to use glutamate as a substrate for producing GABA (Kim *et al.*, 2014).

Effect of the co-culture on the production of functional compounds In the current study, serial application of *B. subtilis* HA and *L. plantarum* K154 to co-ferment a mixture of roasted soybean flour and turmeric fortified with MSG resulted in an enhanced production of functional compounds such as mucilage, GABA, and peptides. The highest GABA production was obtained when a 5% MSG concentration was added to the TRSF co-fermented for 3 days.

To determine the effectivity of the co-fermentation, we designed an experiment comparing the GABA production obtained by the co-fermentation with that obtained by a single strain lactic fermentation. As previously described, for the co-fermentation, the first fermentation process was performed at 42°C for 1 day, using *B. subtilis* HA, followed by the second fermentation at 30°C for 7

Table 3. Free amino acid pattern of TRSF co-fermented by *B. subtilis* HA and *L. plantarum* K154

AA (μg/g)	Non ¹⁾	1 st ²⁾	2 nd ³⁾
CYS	0.00	0.00	0.00
ASP	281.99	218.82	612.86
GLU	41889.94	38318.64	2782.12
ASN	0.00	79.80	296.54
SER	0.00	192.16	210.20
GLN	87.33	98.42	180.28
GLY	36.95	79.57	275.71
HIS	0.00	129.91	215.96
ARG	0.00	446.63	947.37
THR	0.00	213.80	263.87
ALA	226.99	377.45	602.93
GABA	113.65	178.32	17821.30
PRO	61.60	65.24	322.98
TYR	0.00	405.24	765.52
VAL	0.00	384.85	795.29
MET	0.00	71.37	180.29
ILE	0.00	399.79	841.62
LEU	0.00	909.17	1555.70
PHE	0.00	677.09	1037.64
TRP	0.00	138.47	285.78
LYS	0.00	536.30	760.74
TOTAL	42698.46	43921.03	30789.40

¹⁾Non-fermented TRSF²⁾TRSF after the 1st fermentation by *B. subtilis* HA³⁾TRSF after the 2nd fermentation by *L. plantarum* K154

days, using *L. plantarum* K154. By contrast, the single strain fermentation was only performed at 30°C for 7 days, using *L. plantarum* K154. The changes in the pH and acidity of both the single strain and co-fermented TRSF are displayed in Fig. 2(a). The co-fermented TRSF exhibited a slight increase in acidity, in contrast to the single strain-fermented TRSF. The pH of the single strain-fermented TRSF was also substantially lower. This outcome may be explained by the production of lactic acid by *L. plantarum*, which acidified the culture broth; however, in the co-fermentation, the first step using *B. subtilis* resulted in suitable conditions for producing GABA, which in turn increased the pH and decreased the acidity of the culture broth (Kook *et al.*, 2010a; Seo *et al.*, 2013).

By 7 days, the pH of the co-fermented TRSF (5.94 ± 0.00) was significantly higher than that of the single strain-fermented TRSF (4.71 ± 0.01), whereas the acidity of the co-fermented TRSF ($1.14\% \pm 0.01\%$) was significantly lower than that of the single strain-fermented TRSF ($2.03\% \pm 0.03\%$). In particular, the acidity of the co-fermented TRSF markedly decreased during the second LAB fermentation, even though LAB produced lactic acid. A similar outcome was observed in a co-fermentation using a mushroom and GABA-producing LAB (unpublished result). The changes in the viable cell counts of both *B. subtilis* HA and *L. plantarum* K154 during the fermentations are displayed in Fig.

2(b). During the co-fermentation, the viable cell count of *B. subtilis* decreased at the beginning, but then increased by the seventh day, during the second fermentation, whereas the viable cell count of *L. plantarum* K154 gradually increased to 8.00×10^9 CFU/g by the seventh day. By contrast, in the single strain fermentation, the viable cell count of *L. plantarum* K154 increased in the first day, and then gradually decreased to a value of 2.00×10^8 CFU/g by the seventh day. This decrease in the viable cell count of *L. plantarum* K154 coincided with the acidification of the broth, suggesting that the low pH hindered the growth of this strain.

Furthermore, it is likely that the thick consistency of the TRSF fermented by *B. subtilis* HA also contributed to the growth of *L. plantarum* K154 in the co-culture, because *L. plantarum* K154 is a facultative homofermentative strain able to thrive in anaerobic environments. In addition to being a mucilage component, γ -PGA has been reported to exhibit cryoprotective effects, which could increase the viability of probiotics such as *L. plantarum* and *Bacillus* sp. during freeze-drying (Bhat *et al.*, 2013). Therefore, the initial fermentation with *B. subtilis* was an essential step to provide adequate conditions for the growth of *L. plantarum* K154, including a thick consistency (anaerobic medium), moderate pH, and low acidity (Kim *et al.*, 2014b; Park *et al.*, 2014).

Figure 2(c) shows the changes in the tyrosine content of both the single strain-fermented and co-fermented TRSF. The tyrosine in the single strain-fermented TRSF remained at low concentrations during the fermentation (0.76 mg/g by the seventh day). By contrast, the tyrosine in the co-fermented TRSF increased substantially in the first fermentation, and then gradually during the second fermentation, reaching a value of 2.66 mg/g by 7 days. This result suggests that LAB fermentation generates weak protease activity, resulting in a low production of peptides. On the other hand, the co-fermented TRSF may create a favorable environment for the generation of peptides including tyrosine by *B. subtilis* HA. The proteases in *B. subtilis* are known to have optimal pH values in the neutral and alkaline range. Foods produced using *B. subtilis* strains, such as natto and chungkookjang, are the result of alkaline fermentation and their pH values are close to neutral (Zhao *et al.*, 2013). Therefore, inhibition of proteases in the acidic environment of the single strain-fermented TRSF, resulting in a decreased tyrosine production, would be expected (Prestidge *et al.*, 1971).

TLC analysis was performed to compare the GABA production in the single strain-fermented and co-fermented TRSF. After a 3-fold dilution with water, the supernatant samples were spotted onto a silica TLC plate, which after being developed clearly showed that co-fermentation resulted in a substantial production of GABA (approximately 2% by the third day), whereas the GABA content in the single strain-fermented TRSF was negligible (Fig. 3). This result suggests that in the co-cultured mixture both strains act synergistically to boost GABA production (Watanabe *et al.*, 2011).

GABA is generated from L-glutamate as part of the mechanism of acid resistance of LAB (Kook and Cho, 2013). The acid

Fig. 2. Changes in (a) pH and acidity, (b) viable cell counts, and (c) tyrosine content of the TRSF fermented with a single strain (*L. plantarum* K154) and the TRSF co-fermented with *B. subtilis* HA and *L. plantarum* K154; 5% MSG was added for the fermentation. Different letters indicate significant differences (Duncan's multiple range test, $p < 0.05$)

generated by anaerobic fermentation is counteracted by the consumption of protons during decarboxylation (Maras *et al.*, 1992). The optimal pH for the genes required to control the expression of the GAD enzyme, which is responsible for the decarboxylation of glutamate to GABA, is approximately 4 in the case of *gadA* and *gadB* and 5 in the case of *aidA*. Thus, GABA production is decreased if the pH is out of this range (Kim, 2009).

Changes in the acidity of the broth during the first 3 days of the second fermentation were noticeable, in particular in the co-fermented TRSF, which experienced a change in acidity from

$1.68\% \pm 0.01\%$ in the first day to $0.92\% \pm 0.00\%$ in the third day. By contrast, the acidity of the single-strain-fermented TRSF decreased slightly, from $2.18\% \pm 0.05\%$ in the first day to $2.03\% \pm 0.01\%$ in the third day, during the lactic acid fermentation (Fig. 2(a)). Similarly, as GABA production increased to 1.78% during the co-fermentation, the pH increased because of the consumption of protons and the exchange of glutamate for the more alkaline GABA (Cho *et al.*, 2007; Kook *et al.*, 2010b). This result confirms that the efficient GABA production in LAB fermentation is closely related to the decrease in acidity.

Fig. 3. Changes in GABA production in the single strain- and co-fermented TRSF (dilution $\times 3$). The arrow indicates the final GABA concentration (1.78%).

¹⁾ MSG standard concentration

²⁾ GABA standard concentration

³⁾ Non-fermented

⁴⁾ Co-fermented by *B. subtilis* HA and *L. plantarum* K154

⁵⁾ Fermented by *L. plantarum* K154

Conclusion

The co-fermentation of a mixture of turmeric and roasted soybean flour, using *B. subtilis* HA and *L. plantarum* K154 produced bioactive compounds, and the product generated could be used as a turmeric-based functional ingredient. The TRSF was successfully fermented, producing increased concentrations of GABA, peptides, γ -PGA, and probiotics. Furthermore, the fermented TRSF had a pleasant aroma and mild astringent taste, probably as a result of the co-fermentation and interaction between γ -PGA and amino acids. The co-fermentation was more effective than a single strain fermentation, indicating that the first fermentation with *B. subtilis* HA was not only necessary for generating mucilage rich in γ -PGA, but also for providing favorable conditions for the growth of the facultative anaerobe *L. plantarum* K154, which in turn produced GABA. Therefore, co-fermentation with bacterial strains that act synergistically could be a promising strategy for generating functional food ingredients containing γ -PGA, GABA, peptides, and probiotics.

Acknowledgements This research was supported by the Agricultural Biotechnology Development Program of the Ministry of Agriculture, Food and Rural Affairs, and was partially supported by the Ministry of Trade, Industry and Energy through the Center for Traditional Microorganism Resources at Keimyung University, Republic of Korea.

References

- Araújo, C. and Leon, L. (2001). Biological activities of *Curcuma longa* L. *Mem. Inst. Oswaldo Cruz*, **96**, 723-728.
- Bhat, A.R., Irorere, V.U., Bartlett, T., Hill, D., Kedia, G., Morris, M.R., Charalampopoulos, D., and Radecka, I. (2013). *Bacillus subtilis* natto: a non-toxic source of poly- γ -glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *AMB Express*, **3**, 36-36.
- Capitani, M.I., Corzo-Rios, L.J., Chel-Guerrero, L.A., Betancur-Ancona,

- D.A., Nolasco, S.M., and Tomás, M.C. (2015). Rheological properties of aqueous dispersions of chia (*Salvia hispanica* L.) mucilage. *J. Food Eng.*, **149**, 70-77.
- Cho, Y.R., Chang, J.Y., and Chang, H.C. (2007). Production of γ -aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from kimchi and its neuroprotective effect on neuronal cells. *J. Microbiol. Biotechnol.*, **17**, 104-109.
- Dhakal, R., Bajpai, V.K., and Baek, K.-H. (2012). Production of GABA (γ -aminobutyric acid) by microorganisms: a review. *Braz. J. Microbiol.*, **43**, 1230-1241.
- Do, J.H., Chang, H.N., and Lee, S.Y. (2001). Efficient recovery of γ -poly (glutamic acid) from highly viscous culture broth. *Biotechnol. Bioeng.*, **76**, 219-223.
- Fukushima, D. (1981). Soy proteins for foods centering around soy sauce and tofu. *J. Am. Oil Chem. Soc.*, **58**, 346-354.
- Gereltuya, R., Son, J.Y., Magsar, U., Paik, S.-H., Lee, J.Y., and Nam, M.S. (2015). Fermentation properties and inflammatory cytokines modulating of fermented milk with *Curcuma longa* L powder. *J. Life Sci.*, **25**, 75-83.
- Hosny, I.M., El Kholy, W.I., Murad, H.A., and El Dairouty, R.K. (2011). Antimicrobial activity of Curcumin upon pathogenic microorganisms during manufacture and storage of a novel style cheese 'Karishcum'. *J. Am. Sci.*, **7**, 611-618.
- Hwang, J. (2011). Selection of superior functionality soybeans and changes of isoflavone and GABA contents according to soymilk lactic acid fermentation conditions. M.Sc. Thesis, Andong National University, Andong.
- Kang, J.K., Kang, H.J., Seo, J.H., Kim, S.O., Choi, J.H., Cho, D.Y., Park, C.G., and Lee, H.Y. (2009). Effects of fermented turmeric (*Curcuma longa*) by *Bacillus natto* supplementation on liver function and serum lipid parameters in mice. *J. Korean Soc. Food Sci. Nutr.*, **38**, 430-435.
- Kim, D. (2009). Study on the condition of fermentation in lactic acid bacteria for the production of γ -aminobutyric acid. M.Sc. Thesis, Hannam University, Daejeon.
- Kim, J.-E., Hwang, K., and Lee, S.-P. (2010). ACE inhibitory and hydrolytic enzyme activities in textured vegetable protein in relation to the solid state fermentation period using *Bacillus subtilis* HA. *Food Sci. Biotechnol.*, **19**, 487-495.
- Kim, J.E., Kim, J.S., Song, Y.C., Lee, J., and Lee, S.P. (2014). Novel bioconversion of sodium glutamate to γ -poly-glutamic acid and γ -amino butyric acid in a mixed fermentation using *Bacillus subtilis* HA and *Lactobacillus plantarum* K154. *Food Sci. Biotechnol.*, **23**, 1551-1559.
- Kim, S.-B., Lee, D.-W., Cheigh, C.-I., Choe, E.-A., Lee, S.-J., Hong, Y.-H., Choi, H.-J., and Pyun, Y.-R. (2006). Purification and characterization of a fibrinolytic subtilisin-like protease of *Bacillus subtilis* TP-6 from an Indonesian fermented soybean, Tempeh. *J. Ind. Microbiol. Biotechnol.*, **33**, 436-444.
- Kim, S.B., Kang, B.H., Kwon, H.S., and Kang, J.H. (2011). Antiinflammatory and antiallergic activity of fermented turmeric by *Lactobacillus johnsonii* IDCC 9203. *Korean J. Microbiol. Biotechnol.*, **39**, 266-273.
- Kook, M.-C. and Cho, S.-C. (2013). Production of GABA (gamma amino

- butyric acid) by lactic acid bacteria. *Korean J. Food Sci. An.*, **33**, 377-389.
- Kook, M.-C., Seo, M.-J., Cheigh, C.-I., Lee, S.-J., Pyun, Y.-R., and Park, H. (2010a). Enhancement of γ -aminobutyric acid production by *Lactobacillus sakei* B2-16 expressing glutamate decarboxylase from *Lactobacillus plantarum* ATCC 14917. *J. Korean Soc. Appl. Bi.*, **56**, 816-820.
- Kook, M.-C., Seo, M.-J., Cheigh, C.-I., Pyun, Y.-R., Cho, Y.R., and Park, H. (2010b). Enhanced production of γ -aminobutyric acid using rice bran extracts by *Lactobacillus sakei* B2-16. *J. Microbiol. Biotechnol.*, **20**, 763-766.
- Lee, G.-Y., Kim, S.-I., Jung, M.-G., Seong, J.-H., Lee, Y.-G., Kim, H.-S., Chung, H.-S., Lee, B.-W., and Kim, D.-S. (2014). Characteristics of chungkookjang that enhance the flavor and GABA content in a mixed culture of *Bacillus subtilis* MC31 and *Lactobacillus sakei* 383. *J. Life Sci.*, **24**, 1102-1109.
- Lee, H.-S. (2006). Antimicrobial properties of turmeric (*Curcuma longa* L.) rhizome-derived ar-turmerone and curcumin. *Food Sci. Biotechnol.*, **15**, 559-563.
- Leroy, F. and De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.*, **15**, 67-78.
- Lim, J.-S. and Lee, S.-P. (2014). Production of set-type yogurt fortified with peptides and γ -aminobutyric acid by mixed fermentation using *Bacillus subtilis* and *Lactococcus lactis*. *Korean J. Food Sci. Technol.*, **46**, 165-172.
- Maras, B., Sweeney, G., Barra, D., Bossa, F., and John, R.A. (1992). The amino acid sequence of glutamate decarboxylase from *Escherichia coli*. *Eur. J. Biochem.*, **204**, 93-98.
- Messina, M. (2010). A brief historical overview of the past two decades of soy and isoflavone research. *J. Nutr.*, **140**, 1350S-1354S.
- Murase, K., Sakai, K., and Sonoda, C. (2000). Bitterness relieving agent. Japan patent WO0021390.
- Nagai, T., Nishimura, K., Suzuki, H., Banba, Y., Sasaki, H., and Kiuchi, K. (1994). Isolation and characterization of a *Bacillus subtilis* strain producing natto with strong umami-taste and high viscosity. *Nippon Shokuhin Kogyo Gakkaishi*, **41**, 123-128 (in Japanese).
- Ogunleye, A., Bhat, A., Irorere, V.U., Hill, D., Williams, C., and Radecka, I. (2015). Poly- γ -glutamic acid: production, properties and applications. *Microbiology*, **161**, 1-17.
- Oh, S.-M., Jang, E.-K., Seo, J.-H., Ryu, M.-J., and Lee, S.-P. (2007). Characterization of γ -polyglutamic acid produced from the solid-state fermentation of soybean milk cake using *Bacillus* sp. *Food Sci. Biotechnol.*, **16**, 509-514.
- Oh, S.-M., Kim, C.-S., and Lee, S.-P. (2006). Characterization of the functional properties of soy milk cake fermented by *Bacillus* sp. *Food Sci. Biotechnol.*, **15**, 704-709.
- Park, M.-J., General, T., and Lee, S.-P. (2012). Physicochemical properties of roasted soybean flour bioconverted by solid-state fermentation using *Bacillus subtilis* and *Lactobacillus plantarum*. *Prev. Nutr. Food Sci.*, **17**, 36-45.
- Park, S.-Y., Lee, J.-W., and Lim, S.-D. (2014). The probiotic characteristics and GABA production of *Lactobacillus plantarum* K154 isolated from kimchi. *Food Sci. Biotechnol.*, **23**, 1951-1957.
- Prestidge, L., Gage, V., and Spizizen, J. (1971). Protease activities during the course of sporulation in *Bacillus subtilis*. *J. Bacteriol.*, **107**, 815-823.
- Roughley, P.J. and Whiting, D.A. (1973). Experiments in the biosynthesis of curcumin. *J. Chem. Soc., Perkin Trans. 1*, 2379-2388.
- Seo, J.-H., Kim, C.-S., and Lee, S.-P. (2008). Physicochemical properties of poly- γ -glutamic acid produced by a novel *Bacillus subtilis* HA isolated from cheonggukjang. *Prev. Nutr. Food Sci.*, **13**, 354-361.
- Seo, M.-J., Nam, Y.-D., Park, S.-L., Lee, S.-Y., Yi, S.-H., and Lim, S.-I. (2013). γ -Aminobutyric acid production in skim milk co-fermented with *Lactobacillus brevis* 877G and *Lactobacillus sakei* 795. *Food Sci. Biotechnol.*, **22**, 751-755.
- Shi, F., Xu, Z.N., and Cen, P.L. (2006). Efficient production of polyglutamic acid by *Bacillus subtilis* ZJU-7. *Appl. Biochem. Biotechnol.*, **133**, 271-282.
- Shin, Z.-I., Yu, R., Park, S.-A., Chung, D.K., Ahn, C.-W., Nam, H.-S., Kim, K.-S., and Lee, H.J. (2001). His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. *J. Agric. Food Chem.*, **49**, 3004-3009.
- Sung, M.-H., Park, C., Kim, C.-J., Poo, H., Soda, K., and Ashiuchi, M. (2005). Natural and edible biopolymer poly- γ -glutamic acid: synthesis, production, and applications. *Chem. Rec.*, **5**, 352-366.
- Tyagi, R., Agrawal, A., Mahalakshmi, C., Hussain, Z., and Tyagi, H. (2007). Low-cost media for in vitro conservation of turmeric (*Curcuma longa* L.) and genetic stability assessment using RAPD markers. *In Vitro Cell. Dev. Biol. - Plant*, **43**, 51-58.
- Wang, D., Wang, L.-J., Zhu, F.-X., Zhu, J.-Y., Chen, X.D., Zou, L., Saito, M., and Li, L.-T. (2008). In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). *Food Chem.*, **107**, 1421-1428.
- Watanabe, Y., Hayakawa, K., and Ueno, H. (2011). Effects of co-culturing LAB on GABA production. *J. Biol. Macromol.*, **11**, 3-13.
- Zhao, X., Song, J.-L., Wang, Q., Qian, Y., Li, G.-J., and Pang, L. (2013). Comparisons of Shuidouchi, Natto, and Cheonggukjang in their physicochemical properties, and antimutagenic and anticancer effects. *Food Sci. Biotechnol.*, **22**, 1077-1084.