Lim *Applied Biological Chemistry* (2022) 65:5
https://doi.org/10.1186/s13765-021-00664-9

KSABC
The Korean Society for Applied Biological Chemistry

ARTICLE Open Access

Influence of bacteriocin-producing *Bacillus* strains on quality characteristics of fermented soybean product with biogenic amine-forming lactic acid bacteria

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Abstract

This study evaluated the antibacterial activity of bacteriocin produced by *Bacillus* strains against biogenic amines (BA)-forming lactic acid bacteria (LAB), and the applicability of desirable strains as *Bacillus* starters for safety and quality improvement of fermented soybean products. The BA-forming ability of the tested LAB in amino acid decarboxylase broth was mostly strain specific rather that species specific. The bacteriocin produced by specific *Bacillus* strains: *Bacillus* sp. DB407, *Bacillus licheniformis* DB612, and *Bacillus subtilis* DB821 may play a contributory role in the inhibiting the growth of BA-forming strains. In fermented soybean products manufactured using different starter cultures, there was a significant reduction ($p < 0.05$) in the cell counts of LAB by application of a mixed culture containing *B. subtilis* DB821, which showed the highest antimicrobial activity. Specifically, *B. subtilis* DB821 was highly efficient in reducing accumulation of cadaverine, tryptamine, and tyramine produced by *Enterococcus faecium* D12 and *Enterococcus faecalis* D51 during soybean fermentation. In conclusion, the bacteriocin-producing *Bacillus* strains such as *Bacillus* sp. DB 407, *B. licheniformis* DB612, and *B. subtilis* DB821 can be used as a starter culture for the production of BA-controlled soybean products and for the enhancement of the quality and safety of fermented foods.

Keywords: *Bacillus*, Bacteriocin, Biogenic amine, Lactic acid bacteria

Introduction

Fermented soybean foods are rich in some biologically active important compounds like peptides, dietary fiber, isoflavones, and phytic acid. Several studies have documented that these compounds exhibit a wide range of functional properties, such as antioxidant, anti-microbial, anti-diabetic, anti-cholesterol, anti-cancer, anti-genotoxic, and anti-inflammatory effects. On the other hand, amino acids derivatives produced from soybean protein during fermentation commonly lead to the formation of bacterial-related biogenic amines (BA) such as tyramine, histamine, tryptamine and β-phenylethylamine [1].

BA occur naturally and are mainly formed through decarboxylation of specific free amino acids or through amination or transamination of aldehydes and ketones by fermenting microorganisms. Since BA play important roles in the cell proliferation and differentiation, regulation of nucleic acid function, protein synthesis, brain development, nerve growth, and regeneration, these compounds are essential for the normal development, metabolism, and physiological functions of humans. Moderate BA consumption does not generally provoke adverse reactions, because BA are detoxified by acetylation and oxidation reactions enhanced by the BA-metabolizing enzymes such as monoamine oxidase or diamine oxidase [2]. However, an overdose of BA intake is not recommended and may cause significant toxicological implications, including headache, stomach cramp, diarrhea,

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nausea, respiratory distress, hot flush, sweating, heart palpitation, oral burning, and hypo- or hypertension [3]. Therefore, it is necessary to control and monitor the levels of BA that is not only a risk factor for intoxications but is also an indicators of quality and/or acceptability in fermented soybean products, although soy-based foods have been linked to a few potential health benefits [3]. In addition, the presence of some BA, e.g. cadaverine and putrescine may be involved in the formation of carcinogenic nitrosamines in the stomach from dietary nitrite used as curing agents in meat products [4].

Previous research showed that the substantial amounts of BA were found in Doubanjang (185.6 mg/kg of β-phenylethylamine), tofu (> 20 mg/kg of spermidine), natto (457.0 mg/kg of histamine), and Doenjang (1190.7 mg/kg of tyramine) [5]. Many studies have focused on the allowable maximum level of some BA in foods as follows: histamine, 100 mg/kg; tyramine, 100–800 mg/kg; β-phenylethylamine, 30 mg/kg; total BA, 1000 mg/kg [5]. Traditionally, BA accumulation in foods has been prevented, primarily by limiting the growth of amino acid decarboxylase-producing microorganisms through chilling, freezing, smoking, hydrostatic pressure, irradiation, controlled atmosphere packaging, or the use of food additives [6]. However, these processing techniques and treatments may adversely affect the nutritional and sensory quality of the product, including its color, appearance, flavor (taste and aroma), and texture.

Recently, several strategies have been adopted to prevent and minimize the accumulation of excessive BA in fermented foods such as cheese and wine. Cooper [7] reported that the strain of *Lactobacillus plantarum* isolated from fermented sausages facilitates the inactivation of BA in these meat products through the production of amine oxidases, catalyzing the oxidative deamination of amines. Meanwhile, the use of bacteriocin-producing lactic acid bacteria (LAB) strains as antibacterial starter cultures was effective in decreasing the amount of BA in rennet curd [8]. For these reasons, the application of functional and specific strains capable of degrading and/or inhibiting BA formation has attracted considerable interest for use as safe food preservatives in fermented food and beverage industries [9]. Nisin, a bacteriocin produced by several *Lactococcus lactis* strains, have been employed successfully to control various types of pathogens and spoilage microorganisms in foods. No histamine formation was detected in the cheeses made with bacteriocin-producing enterococci and a nisin-producing *Lactococcus lactis* starters [9]. To date, several studies describing the screening and characterization of the antimicrobial substances produced from LAB against BA-forming microorganisms have been reported, however, some species of LAB are considered as the main BA producers in fermented foods [10]. Thus, the objective of this study was to screen bacteriocin-producing *Bacillus* strains from traditional fermented soybean paste and to investigate their applicability as a starter for safety and quality improvement of fermented soybean products.

Materials and methods

Bacterial strains and growth conditions

BA-forming LAB (*Enterococcus faecalis* D08, *Enterococcus faecium* D12, *Enterococcus faecalis* D51, *Enterococcus faecalis* D66, *Leuconostoc* sp. D82) were used as the indicator strains for assessment of antibacterial activity and starter cultures for soybean fermentation. BA-non-forming *Bacillus* strains (*Bacillus* sp. DB108, *Bacillus* sp. DB403, *Bacillus* sp. DB407, *Bacillus thuringiensis* DB513, *Bacillus subtilis* DB517, *Bacillus* sp. DB520, *Bacillus thermoamylovorans* DB605, *Bacillus licheniformis* DB612, *Bacillus pumilus* DB618, *Bacillus* sp. DB730, *Bacillus circulans* DB804, *Bacillus amyloliquefaciens* DB809, *Bacillus* sp. DB814, *B. subtilis* DB821) were used to evaluate the activity of bacteriocin. These strains were identified by 16S ribosomal RNA gene sequencing as well as morphological and biochemical characteristics and confirmed characteristics in our previous study [19–21]. Bacterial stocks were stored at − 20 °C in Brain Heart Infusion (BHI, BD Difco.) broth containing 20% (v/v) glycerol until experimental use.

Preparation of bacteriocin solution

The overnight pre-culture of the test strain was inoculated (0.1% inoculum) in BHI broth supplemented with 0.1% (w/v) glucose and incubated at 35 °C with shaking (150 rpm) for up to 22 h. The cell-free supernatant of the culture medium was collected by centrifugation (7000 × g, 10 min, 4 °C), neutralized to pH 6.5 by the addition of 6 N NaOH to eliminate the inhibitory effect of organic acids, and incubated at 37 °C for 1 h after treating 1 mg/mL catalase (Sigma-Aldrich, St. Louis, MO, USA) to degrade hydrogen peroxide. After heated at 90 °C for 10 min to stop the enzyme reaction, the cell-free supernatant was precipitated with ammonium sulfate at 40% (w/v) saturation overnight at 4 °C with stirring. The precipitates collected by centrifugation (12,000 × *g*, 30 min, 4 °C) were deposited in 20 mM phosphate buffer saline (PBS, pH 6.5) and desalted by using a 1 kDa cut-off dialysis membrane (Spectrum Medical Industries, Inc., CA, USA) within the same buffer for 24 h at 4 °C. The sample solution (100 mL) was vigorously stirred using organic solvents (100 mL) including chloroform–methanol (1:1, v/v) for 20 min and transferred in a separating funnel. The interface layer between the aqueous and organic phase, which contain bacteriocin was harvested, and the residual solvent was removed by speed vacuum.

Bacteriocin activity determination

The antimicrobial activity of crude bacteriocin solution was determined by microtiter plate assay [12]. In detail, the BA-forming strains were grown in BHI broth under aerophilic conditions for 24 h at 37 °C. The cells were harvested by centrifugation (7000×*g*, 10 min, 4 °C), washed two times with PBS (pH 7.0), and adjusted to a density of 1.0×10^5 CFU/mL in BHI broth. The bacteriocin solution was serially diluted twofold with PBS (pH 7.0) and added to each well in the microtiter plate. The bacterial suspension was then placed in the well containing the bacteriocin. The plates were incubated at 37 °C for 24 h and growth was recorded based on optical density changes at 600 nm on a micro-plate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The bacteriocin unit (arbitrary unit, AU/mL) were defined as the reciprocal of the highest dilution that showed inhibition of the indicator strain by 50% as compared to a control culture without the bacteriocin.

Effect of bacteriocin-producing *Bacillus* on amino acid decarboxylase activity of BA-forming LAB

The effect of bacteriocin solution produced by *Bacillus* strains on amino acid decarboxylase activity of BA-forming bacteria was carried out according to the procedures described by Lim [11] with minor modifications. Briefly, a loopful from the test organism was inoculated in decarboxylase basal media (Difco) with 0.5% L-arginine monohydrochloride, L-histidine monohydrochloride monohydrate, L-lysine monohydrochloride, L-ornithine monohydrochloride, L-phenylalanine, L-tryptophan, and L-tyrosine hydrochloride (pH 5.8) supplemented with 0.0005% pyridoxal-HCl (Sigma-Aldrich), and incubated at 37 °C for 48 h. One-milliliter aliquots of these cultures were transferred to fresh tubes containing 9 mL of the assay media supplemented with bacteriocin solutions (300 AU/mL). The plate cultures were incubated under anaerobic condition using anaerobic jar (Anoxomat system, MART Co., The Netherlands) at 35 °C for 72 h. The supernatant fluid obtained after centrifugation (7000×*g*, 10 min, 4 °C) of the cultures was immediately were filtered through a 0.2 μm membrane (Millipore Co., Bedford, MA, USA). One milliliter of the filtered broth cultures was then added to 9 mL of 0.4 M perchloric acid, and the mixture was homogenized by a vortex shaker for 10 s. The homogenates were centrifuged at 3000×*g* for 10 min at 4 °C and the supernatant was filtered through Whatman paper No. 1 (Piscataway, NJ, USA).

Analysis of BA

Stock standard solutions of BA, including cadaverine dihydrochloride, histamine dihydrochloride, putrescine dihydrochloride, tryptamine, and tyramine hydrochloride (all from Sigma), were separately prepared in deionized water at a concentration of 10,000 mg/L. The concentrations of working solutions used were 0, 10, 50, 100, and 1000 mg/L. For derivatization of BA 1 mL of extracts (or standard solution) was mixed with 200 μL of 2 N sodium hydroxide and 300 μL of saturated sodium bicarbonate. Two milliliters of dansyl chloride solution (10 mg/mL in acetone) were added to the reaction mixture, which was then incubated at 40 °C for 45 min. And then 100 μL of 25% ammonium hydroxide (Sigma-Aldrich) was added to remove any residual of dansyl chloride. After completion of the reaction for 30 min at room temperature, the final volume was adjusted to 5 mL by adding acetonitrile. The reaction mixture was centrifuged at 2500×*g* for 5 min, and the supernatant was filtered through 0.2 μm pore-size filters (Millipore) and injected directly into the high-performance liquid chromatography (HPLC). HPLC analysis was performed using a LC-10 system (Shimadzu, Kyoto, Japan) equipped with a Nova-Pack C_{18} 4 μm column (150 × 3.9 mm, Waters, Milford, MA, USA) and fluorescence detector (Waters 2475). The mobile phases consisted of 0.1 M ammonium acetate (Sigma-Aldrich) as solvent A and acetonitrile (Merck) as solvent B, with a constant flow rate of 0.8 mL/min with gradient elution for 35 min. The sample volume injected was 10 μL. The chromatographic column temperature was maintained at 35 °C and the detection was monitored at 254 nm. As shown in Fig. 1, the BA in the fermented soybean products were identified based on chromatographic retention time by comparison with standard solutions.

Preparation of fermented soybean product

The soybeans (500 g, Daepung strain) were washed and soaked with ten volumes of distilled water for 24 h at room temperature, after which they were autoclaved for 30 min at 121 °C. After cooling to 40–45 °C, steamed soybeans were inoculated with different starter cultures, including BA-forming LAB and/or bacteriocin-producing *Bacillus* strain, and a batch without any starter as the control. For preparation of control sample, the steamed soybeans were covered with rice straw, which naturally contains protease producing bacterial strains. The selected starter organisms were separately cultivated for 18 h in BHI broth with shaking (150 rpm) at 37 °C, and cells were recovered by centrifugation at 7000×*g* for 10 min. The precipitated cells were washed 3 times with phosphate buffer saline (PBS, pH 7.0), the cells were inoculated separately into the surfaces of cooked soybeans (200 g) with 1% (v/w) (1×10^6 CFU/g) and thoroughly mixed. Fermentation was proceeded for 72 h at 37 °C with a relative air humidity of 80%. Finally, the fermented

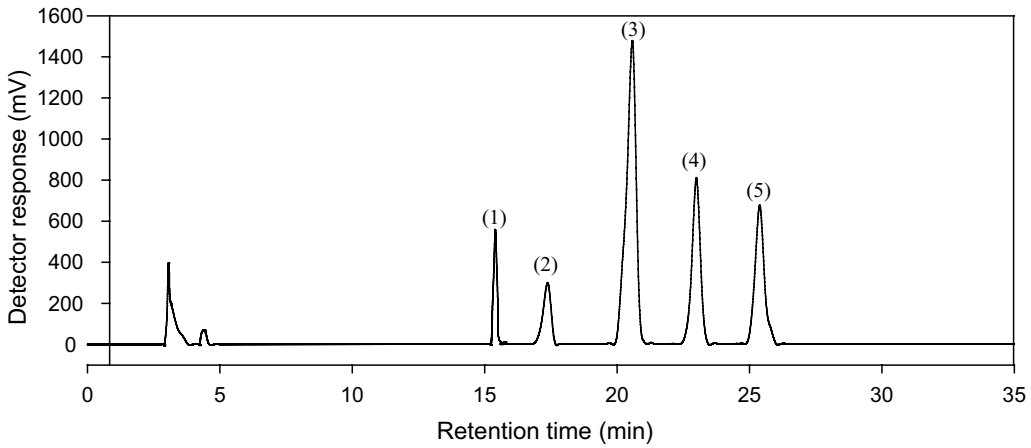

Fig. 1 HPLC chromatograms of mixed standard solution. The numbers on the chromatograms represent the following compounds: (1), tryptamine; (2), putrescine; (3), cadaverine; (4), histamine; (5), tyramine

soybeans were powdered by freeze-drying, homogenization, and sifting, and the powder was kept at − 80 °C until use.

Microbiological and quality properties of fermented soybean product

Microbiological analysis

A 25 g portion of the sample was homogenized with 225 mL of sterile PBS (pH 7.0) for 2 min in a stomacher (3 M). The homogenates were serially tenfold diluted with a sterile PBS (pH 7.0), and 1 mL aliquots of the dilutes were pipetted into petri dishes. Then, molten Mannitol Salt Egg Yolk agar (for *Bacillus* strain) and MRS agar (for LAB) was poured into the dish and mixed thoroughly. After solidification, the plates were incubated at 37 °C for 48 h. Bacterial numbers were expressed as colony-forming units (CFU/g) of sample.

pH

Each sample (10 g) was homogenized in 50 mL of distilled water using a laboratory blender (Waring Laboratory Science, USA) at maximum speed for 2 min. The homogenized sample was then centrifuged at 7000×*g* for 10 min, and pH level of the supernatant was measured using a pH meter (Fisher Scientific, Waltham, MA, USA).

Amino-type nitrogen determination

Amino-type nitrogen content was measured by the formol titration method. Briefly, the sample (5 g) was mixed with 50 mL of distilled water, and the mixture was incubated at 30 °C for 30 min in a shaking water bath. After centrifugation (10,000×*g*, 15 min), 10 mL of supernatant was filtered through a 0.45 μm syringe filter (Whatman, plc, GE Healthcare Life Sciences, Kent, UK). The filtered sample was titrated with 0.1 N NaOH using 0.1% phenolphthalein as indicator. And then, the sample (5 mL) was mixed with 10 mL of distilled water and 10 mL of 35% formaldehyde solution, left to stand for 10 min at room temperature, and titrated to pH 8.3 with 0.1 N NaOH. The amino-type nitrogen content was determined by the amount of 0.1 N NaOH used as shown below. Amino-type nitrogen (mg%) = $[(A - B) \times 1.4 \times F \times D \times 100]/S$, where A is the amount of 0.1 N NaOH required in sample titration (mL), B is the amount of 0.1 N NaOH required in blank titration (mL), 1.4 is the amino nitrogen content value corresponding to 1 mL of 0.1 N NaOH, F is the factor of 0.1 N NaOH, D is the dilution fold, and S is the amount of sample.

Ammonia-type nitrogen determination

To determine ammonia-type nitrogen content, 5 g of the sample was mixed with 50 mL of distilled water, and the mixture was shaken vigorously for 30 s and filtered through sterile gauze to remove large particles. After centrifugation at 10,000×*g* for 10 min, 20 mL of the supernatant was mixed with 0.7 mL of 4% NaOH solution, 0.3 g of magnesium oxide (0.3 g), and a few boiling chips, and then distilled water was added to the mixture to make a final volume of 150 mL. The mixture was distilled to separate the ammonia by using distillation apparatus. The distillate (70 mL) was collected in a flask containing 25 mL of 0.05 N H_2SO_4, and then filled up with distilled water up to 100 mL marking. The mixed indicator of methyl red and bromcresol green was added to the solution, and the mixture was titrated with 0.05 N NaOH to reach the end-point. The ammonia-type

nitrogen content was calculated using the following formula: $(B-A) \times F \times 1000/V \times 0.7$, where A is the amount of 0.05 N NaOH required in sample titration (mL), B is the amount of 0.05 N NaOH required in blank titration (mL), 0.7 is the ammonia nitrogen content value corresponding to 1 mL of 0.05 N NaOH, F is the factor of 0.05 N NaOH, and V is the amount of sample.

BA content

Five-gram samples were homogenized with 20 mL of 0.1 N HCl for 3 min by using a stomacher blender (3 M, Maplewood, MN, USA). The homogenate was then centrifuged at 7000×*g* for 20 min and the supernatant was filtered through Whatman paper No. 1. Sample extracts and standard BA solutions were derivatized with dansyl chloride and analyzed by HPLC according to the same method as described above.

Statistical analysis

All experiments were done in triplicate, and values were expressed as mean ± standard deviation (SD). Statistical analysis was achieved using one-way analysis of variance (ANOVA) with Statistical Package for Social Sciences (SPSS) software version 24.0 (SPSS Inc., Chicago, IL) for window. Duncan's multiple range test was used to determine significant ($p < 0.05$) differences among the mean values.

Results and discussion

BA formation potential of the tested LAB

The BA-forming abilities of LAB isolated from the *Doenjang* were detected in decarboxylase synthetic broth and the results were shown in Fig. 2. The formation of cadaverine was demonstrated in *E. faecalis* D51 (642.3 ± 11.4 mg/L), *E. faecalis* D66 (255.6 ± 9.9 mg/L) and *Leuconostoc* sp. D82 (391.6 ± 15.4 mg/L). Among *Enterococcus* sp., *E. faecalis* D08 (1045.8 ± 33.5 mg/L) and *E. faecalis* D66 (504.7 ± 20.9 mg/L) strains were found to be positive for histamine production. *E. faecalis* D66 (912.4 ± 6.7 mg/L) showed only ornithine decarboxylase activity. The values for the tryptamine production by *E. faecium* D12 and *E. faecalis* D51 were found in the range of 1523.7 ± 40.1 mg/L and 623.4 ± 15.5 mg/L, respectively. *E. faecalis* D08 (237.9 ± 13.2 mg/L) and *E. faecium* D12 (661.4 ± 25.0 mg/L) also produced tyramine in decarboxylase broth supplemented with tyrosine. These results indicated that the BA-forming ability of the tested LAB in amino acid decarboxylase broth was mostly strain specific rather that species specific. However, *Bacillus* strains which used for assessment of bacteriocin activity did not produce BA (data now shown).

Among many different bacterial species isolated from various environments, Gram-negative spoilage organisms belonging to enterobacteria and pseudomonads are known to possess amino acid decarboxylase activity and have the ability to produce BA. BA content in fermented foods has been related to the use of poor quality raw materials and the contamination of decarboxylase

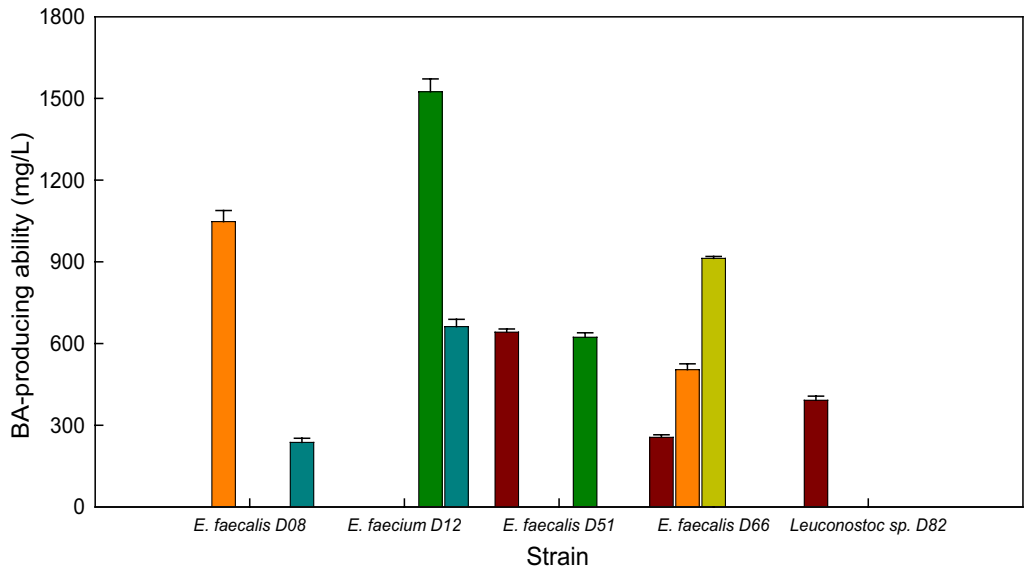

Fig. 2 BA formation potential of the tested LAB. ▪, cadaverine; ▪, histamine; ▪, putrescine; ▪, tryptamine; ▪, tyramine. Data are means ± SD from triplicate determinations

positive spoilage microorganisms during food processing and storage [5]. Therefore, several researchers have proposed microbial quality indices based on food BA content as an indirect indicator of the excessive growth of harmful microbes [4]. Amino acid decarboxylase activity has been described in Gram-positive bacteria, including *Bacillus* sp., *Clostridium* sp., Staphylococci, and LAB [11, 12]. Although LAB strains are classified as the generally recognized as safe (GRAS) and have been the most widely used as probiotics due to their potential health benefits, certain LAB can have the capability to produce BA [10]. For this reason, the attention has been mainly focused on LAB using as starter cultures in several fermented foods.

It is widely known that *Lactobacillus* sp., *Pediococcus* sp., *Streptococcus* sp., and *Leuconostoc* sp. are implicated in BA formation in various fermented products. Among the species of LAB, *Oenococcus oeni*, *Lactobacillus hilgardii*, *Lactobacillus fructivorans*, *Pediococcus parvulus*, and *Lactobacillus brevis* can produce BA by the decarboxylation of amino acids. Moreover, BA formation potential was observed in *Lactobacillus curvatus*, *E. faecalis*, *E. faecium*, *Lactobacillus fermentum*, *L. lactis*, *Streptococcus thermophiles*, and *Lactobacillus paracasei* [10, 13]. Our results partially agree with Barbieri et al. [10], who reported the formation of histamine, tyramine, putrescine, and cadaverine by *E. faecium* and *E. faecalis*. Whereas, *Leuconostoc* sp. D82 had no potential to produce BA except for cadaverine, which is in disagreement with previous reports [10, 14]. Bover-Cid and Holzapfel [10] previously demonstrated that tyramine was produced by *L. mesenteroides* ssp. *mesenteroides* and *Leuconostoc carnosum*, while they did not produced histamine, cadaverine, and putrescine. Barbieri et al. [10] reported that *L. mesenteroides* was histamine, putrescine, and tyramine producer. A potential for formation of tyramine or histamine has also been observed for *L. mesenteroides* isolated from wine. As a results, BA are produced based on microbial substrate-specific enzyme decarboxylation of precursor amino acids and amino acid-decarboxylase activity of LAB is strain dependent rather than genus- or species-specific.

Antibacterial activity of bacteriocins produced by *Bacillus* sp. against BA-forming LAB

As shown in Table 1, the bacteriocin produced by *Bacillus* sp. DB407 was showed antibacterial activity against *E. faecalis* D08 (512 AU/mL). Crude bacteriocin of *B. subtilis* DB821 exerted strong inhibitory activity against *E. faecium* D12 (512 AU/mL) and *E. faecalis* D51 (1024 AU/mL). In addition, the bacteriocin of *B. licheniformis* DB612 had an inhibitory effect on *E. faecalis* D08 (256 AU/mL). These results suggested that the antibacterial spectrums and activities of *Bacillus* strain was highly strain-dependent. The bacteriocin produced by specific *Bacillus* strains may play a contributory role in the inhibiting the growth of BA-forming strains.

Table 1 Potential antibacterial activity of bacteriocin produced by *Bacillus* sp. against BA-forming LAB

Bacteriocin-producing *Bacillus* sp.	Bacteriocin activity (AU/mL)				
	Indicator				
	***Enterococcus faecalis* D08**	***Enterococcus faecium* D12**	***Enterococcus faecalis* D51**	***Enterococcus faecalis* D66**	***Leuconostoc* sp. D82**
Bacillus sp. DB108	ND	ND	ND	ND	ND
Bacillus sp. DB403	ND	ND	ND	ND	ND
Bacillus sp. DB407	512	ND	ND	ND	ND
Bacillus thuringiensis DB513	ND	ND	ND	ND	ND
Bacillus subtilis DB517	ND	ND	ND	ND	ND
Bacillus sp. DB520	ND	ND	ND	ND	ND
Bacillus thermoamylovorans DB605	ND	ND	ND	ND	ND
Bacillus licheniformis DB612	256	ND	ND	ND	ND
Bacillus pumilus DB618	ND	ND	ND	ND	ND
Bacillus sp. DB730	ND	ND	ND	ND	ND
Bacillus circulans DB804	ND	ND	ND	ND	ND
Bacillus amyloliquefaciens DB809	ND	ND	ND	ND	ND
Bacillus sp. DB814	ND	ND	ND	ND	ND
Bacillus subtilis DB821	ND	512	1024	ND	ND

Data are means ± SD from triplicate determinations

ND not detected

Bacteriocins can be defined as ribosomally synthesized antibacterial peptides/proteins displaying antimicrobial activity against other strains of the same or related species, although there are an increasing number of bacteriocins reported to have broad spectrum action. Generally, bacteriocin can cause the formation of pores in the cytoplasmic membrane of the target cells and thus lead to increased efflux of intracellular metabolites, such as potassium and amino acids. This action results in the dissipation of the transmembrane electric potential, the hydrolysis of intracellular ATP, the collapse of the pH gradient at the membrane, and ultimately cell death [15]. Representative strain of *Bacillus* sp., *B. subtilis* is one of the important biocontrol agents due to its ability to produce potent antimicrobial components, such as subtilin, ericin, mersacidin, subtilosin, bacillocin, surfactin, and bacilysin. Bacteriocins of *Bacillus* strains usually kill sensitive cells by making pores in the cytoplasmic membrane, leaking the cell materials, and inhibiting the cell wall biosynthesis. Additionally, these substances that initially bind to specific outer membrane receptors of target cells may disturb cell functions, inactivate enzymes related on the synthesis of nucleic acids and amino acids, and finally lead to death of the bacterial cells [16].

According to previous studies, bacteriocin produced by *B. amyloliquefaciens* LBM 5006 displayed broad-spectrum antibacterial activities against *Listeria monocytogenes*, *Bacillus cereus*, *Serratia marcescens*, and *Pasteurella haemolytica*. *B. amyloliquefaciens* CECT 5940 showed significant antibacterial activities against *Escherichia coli* and *Clostridium perfringens*. The antibacterial substance produced by *B. subtilis* was widely effective against various pathogens such as *S. aureus*, *Pseudomonas aeruginosa*, *B. cereus*, *Streptococcus pyogenes*, and *Candida albicans*. Pumilicin 4 produced by *B. pumilus* WAPB4 exhibited antibacterial activities against vancomycin resistant *E. faecalis* and some Gram-positive strains [17]. Based on these results, the antimicrobial activity and spectrum of bacteriocin produced by *Bacillus* strains vary greatly, depending on bacterial species and strain. Like other *Bacillus* strains, the antimicrobial activity of bacteriocin produced by bacterial strains used in this study was specific for the target strains. So far, several reports proved that bacteriocins obtained from *Bacillus* strains inhibited not only closely related species of the producer but also food-borne bacteria, such as food spoilage bacteria and pathogens. Whereas, there is a lack of experimental data on bacteriocin produced by *Bacillus* strains active against BA-forming microorganisms. Therefore, we focused on the *Bacillus*-derived bacteriocins that exhibit significant potential to inhibit the growth of BA-forming bacteria [18].

Effect of bacteriocin-producing *Bacillus* on amino acid decarboxylase activity of BA-forming LAB

The effect of bacteriocin produced by *Bacillus* strains on the BA-forming abilities of LAB were shown in Table 2. When the bacteriocin of *B. subtilis* DB821 was added to culture medium at a final concentration of 300 AU/mL, the BA (cadaverine and tryptamine) formation of *E. faecalis* D51 were significantly more inhibited than other groups. The amounts of histamine and tyramine formed by *E. faecalis* D08 were reduced by 33.6 ± 0.7% and 39.6 ± 1.8% by bacteriocin of *Bacillus* sp. DB407, respectively. Furthermore, the bacteriocin of *B. licheniformis* DB612 had good effects on preventing the BA formation of *E. faecalis* D08. Based on these results, we realized that the antibacterial compounds produced by *Bacillus* strains were effective in inhibiting of the BA-forming ability of the specific LAB. The most significant BA reduction was observed after treatment of the bacteriocin produced by *B. subtilis* DB821, which showed the highest antibacterial activity.

BA formation in foods can be regulated by several methods such as controlled atmosphere packaging, additives, hydrostatic pressure, irradiation, pasteurization, smoking, starter culture, oxidizing formed biogenic amine, and temperature. In addition, several food additives and natural antimicrobial agents, including glycine, nicotinic acid, potassium sorbate, sodium benzoate,

Table 2 Effect of bacteriocin solution produced by *Bacillus* strains on amino acid decarboxylase activity of BA-forming bacteria

Bacteriocin solution (300 AU/mL) produced by *Bacillus* strain	BA-forming LAB	Inhibition (%)				
		Cadaverine	Histamine	Putrescine	Tryptamine	Tyramine
Bacillus sp. DB407	*Enterococcus faecalis* D08	NT	33.6 ± 0.7	NT	NT	39.6 ± 1.8
Bacillus licheniformis DB612	*Enterococcus faecalis* D08	NT	18.9 ± 1.4	NT	NT	20.7 ± 2.6
Bacillus subtilis DB821	*Enterococcus faecium* D12	NT	NT	NT	25.6 ± 3.0	17.9 ± 1.1
	Enterococcus faecalis D51	43.3 ± 1.0	NT	NT	52.5 ± 0.5	NT

Data are means ± SD from triplicate determinations

ND not detected

sodium chloride, clove, garlic, etc., have been shown to be highly effective in suppressing the growth of BA-forming microorganisms [6].

With consumers becoming more concerned about the negative health effects associated with chemical preservatives, natural antimicrobial agents, such as bacteriocins, can be an attractive alternative to artificial preservatives when it comes to satisfying the increasing consumer demands for health food products. Interestingly, bacteriocins produced by specific strains are now widely used in fermented foods to reduce the amount of toxic substances such as BA and to improve food safety and quality characteristics without the adverse reactions related to chemical food preservative [15, 18, 19].

Some previous studies have shown that LAB bacteriocins are frequently active against BA-forming bacteria [9, 20]. Lim [20] reported that *Pediococcus acidilactici* MCL11, *L. mesenteroides* MCL12, *E. faecium* MCL13, *Lactobacillus sakei* MCL14, and *Lactobacillus acidophilus* MCL15 isolated from *Myeolchi-jeot* were found to produce an antibacterial compound with inhibitory activity against histamine-forming bacteria such as *B. licheniformis* MCH01, *S. marcescens* MCH12, *Staphylococcus xylosus* MCH13, *Aeromonas hydrophila* MCH04, *Morganella morganii* MCH05. Bacteriocin (nisin Z, lacticin 481)-producing *L. lactis* subsp. *lactis* VR84 and EG46 inhibited the growth and the tyramine production of *E. faecalis* EF37. Moreover, the antibacterial substances produced by *L. lactis* subsp. *lactis* VR84 and EG46 significantly reduced the levels of histamine accumulation by *Streptococcus thermophilus* PRI60 in cheese [21]. The growth of histamine- and tyramine-forming *L. buchneri* St2A and *L. brevis* were effectively inhibited by nisin and bacteriocin obtained from eneterococcal, but the treatment of PA-1, bavaricin A and lactococcin A did not affect the content of BA produced by the strains [9]. Meanwhile, *S. xylosus* no. 0538 was found to produce the bacteriocin-like inhibitory substance(s) and have the highest antimicrobial activity against *B. lichniformis* strains defined as BA-producers [22]. Based on peptide structure, molecular weight, or physical–chemical properties, bacteriocins have an interestingly diverse spectrum of antimicrobial activity.

Meanwhile, parallel result was obtained by Lim et al. [23] who reported that the bacteriocin produced by *Bacillus* sp. was active against both Gram-positive pathogenic and spoilage bacteria and LAB such as *L. acidophilusILactobacillus delbrueckii*, *Lactobacillus johnsonii*, *Lactobacillus salivarius*, *Lactobacillus plantarum*, *E. faecalis*, *E. faecium*, *L. delbrueckii* sp. *lactis*, and *L. mesenteriodes*. Unlike the findings in a previous research, in our experiments, the bacteriocin obtained from *Bacillus* strains significantly inhibited the bacterial growth and BA formation of some LAB. Certain strains of *Bacillus* have a major role to play in the health and function of the gastrointestinal tract because of their high metabolic activity and production ability of antibacterial substances. Notably, bacteriocins produced by *Bacillus* sp., which have strong resistance with spore forming ability and wide survival range in nature, have been reported that they have considerable potential for industrial utilization. *Bacillus* bacteriocins can be added to foods in the form of concentrated preparations as food preservatives, shelf-life extenders, additives or ingredients, or they can be produced in situ by bacteriocinogenic starters, adjunct or protective cultures during fermentation [16, 17]. Our results clearly showed that the bacteriocin activity of *B. pumilis* DB618 against BA-forming bacteria was much higher than that of LAB such as *P. acidilactici* MCL11, *L. mesenteroides* MCL12, *E. faecium* MCL13, *L. sakei* MCL14, and *L. acidophilus* MCL15 [20].

Microbiological characteristics and pH values of fermented soybean product manufactured by different starter culture

The viable cell counts of *Bacillus* and LAB and pH levels of fermented soybean product manufactured by different starter culture were shown in Table 3. At the end of 3 days of fermentation, the final counts of bacilli and LAB in non-starter control sample were found to be $2.8 \pm 1.4 \times 10^8$ and $1.7 \pm 0.7 \times 10^7$ CFU/g, respectively. The number of *Bacillus* sp. and LAB in fermented soybean product prepared by the single culture was significantly ($p < 0.05$) higher than that found in the control sample, indicating the growth of starters. The viable cell counts of *Bacillus* sp. in fermented soybean products fermented by the mixed culture of bacteriocin-producing *Bacillus* and BA-forming LAB were approximately $4.0 \pm 0.7 \times 10^9$ to $6.1 \pm 0.3 \times 10^9$ CFU/g, which were not significantly different ($p < 0.05$). Whereas, there was a significant reduction ($p < 0.05$) in the cell counts of LAB by application of mixed culture with *Bacillus* sp. DB407, *B. licheniformis* DB612, and *B. subtilis* DB821 which had the high antimicrobial activity.

These results were partially consistent with published findings [24, 25]. Lee et al. [24] reported that the number of the total mesophilic aerobic and lactic acid bacteria in non-inoculated control samples reached a final number of 1.12×10^8 and 1.40×10^8 CFU/mL, respectively. And total viable counts and LAB concentrations in the soybean samples inoculated with LAB starter culture were 4.10×10^9–7.75×10^9 CFU/mL and 2.85×10^9–4.35×10^9 CFU/mL, respectively. Sarkar et al. [25] demonstrated that *Bacillus* populations were practically identical throughout the fermentations of soybeans in the presence or absence of *Enterococcus* starter and finally reached approximately 10^{10} CFU/g.

Table 3 Viable cell counts of *Bacillus* and LAB and pH levels of fermented soybean product manufactures by different starter culture

Fermentation starter	Viable cell counts (CFU/g)		pH
	Bacillus	LAB	
Non-starter (control)	$2.8 \pm 1.4 \times 10^{8b}$	$1.7 \pm 0.7 \times 10^{7bc}$	6.72 ± 0.20^{e}
Enterococcus faecalis D08	ND	$5.0 \pm 0.8 \times 10^{8a}$	6.76 ± 0.04^{de}
Enterococcus faecium D12	ND	$5.7 \pm 1.7 \times 10^{8a}$	6.90 ± 0.13^{d}
Enterococcus faecalis D51	ND	$4.6 \pm 0.6 \times 10^{8a}$	6.86 ± 0.08^{d}
Bacillus sp. DB407	$5.5 \pm 0.3 \times 10^{9a}$	ND	7.23 ± 0.03^{c}
Bacillus licheniformis DB612	$7.2 \pm 1.1 \times 10^{9a}$	ND	7.75 ± 0.01^{a}
Bacillus subtilis DB821	$4.3 \pm 0.9 \times 10^{9a}$	ND	7.51 ± 0.10^{ab}
Bacillus sp. DB407 + *Enterococcus faecalis* D08	$4.0 \pm 0.7 \times 10^{9a}$	$3.1 \pm 2.5 \times 10^{7b}$	7.19 ± 0.11^{c}
Bacillus licheniformis DB612 + *Enterococcus faecalis* D08	$4.9 \pm 2.1 \times 10^{9a}$	$7.5 \pm 1.5 \times 10^{7b}$	7.40 ± 0.08^{b}
Bacillus subtilis DB821 + *Enterococcus faecium* D12	$5.5 \pm 1.8 \times 10^{9a}$	$4.3 \pm 0.5 \times 10^{7b}$	7.37 ± 0.14^{b}
Bacillus subtilis DB821 + *Enterococcus faecalis* D51	$6.1 \pm 0.3 \times 10^{9a}$	$8.5 \pm 2.2 \times 10^{6c}$	7.11 ± 0.15^{c}

Data are means ± SD from triplicate determinations

[a] [−e]Values within a column with different superscripts are significantly each groups at $p < 0.05$ by Duncan's multiple range test

ND not detected

And *Enterococcus* reached a maximum population level of 10^9 CFU/g and remained at this level throughout the process. Our results were inconsistent with the findings of Saker et al. [25] who reported that *Bacillus* did not significantly affect the growth of *Enterococcus* during fermentation of soybeans. Meanwhile, the number of *Bacillus* in fermented soybean product prepared by mixed starter cultures are similar to the mean total bacterial counts of retail *Cheonggukjang* products described by Park et al. [26], who reported that the wide range of total mesophilic viable bacteria may result from an insufficient standardization of the product manufacturing processes such as different fermentation materials and conditions. The number of LAB was found to be approximately 6.66–8.12 log CFU/g, with an average of 7.09 ± 0.58 log CFU/g, similar to the results obtained in this work. From the observation of Ju and OH [27], the viable cell counts of *Bacillus* strain were similar between the mixed and single culture fermentation. The viable cell counts of *Bacillus* sp. in *Cheonggukjang* prepared by mixed starter cultures were higher than those of LAB. During the period of fermentation, the viable cell number of *B. subtilis* reached a peak (log 9.1 CFU/g) at 24 h, and *L. plantarum* reached a peak (log 7.2 CFU/g) at the same time. However, LAB viable cell numbers gradually decreased after a period of peak value, unlike *B. subtilis*. Although LAB numbers did not exceed 8 log CFU/g at any fermentation period, the bacteria maintained numbers of at least 6 log CFU/g throughout the fermentation process. Similar results were obtained from Lim [11], who demonstrated that the viable cell counts of BA-forming *B. licheniformis* DB102 and *B. subtilis* DB1020 was significantly decreased by the mixed culture with bacteriocin-producing *L. plantarum* DLA205 and *L. fermentum* DLA509. Thus, the bacteriocin producers can serve as the starter culture or be added as an additional protective culture for the reduction of BA accumulation during the manufacture and storage of soybean fermented foods.

Based on our findings, *Bacillus* strain showed a significantly higher probability of growth in soybean fermentation than LAB due to its high availability of soybean protein and its specific antibacterial activity against LAB. Specifically, the specific starter culture producing the bacteriocin was effective in inhibiting the proliferation of BA-forming LAB in fermented soybean product. The lowest LAB counts were detected in fermented soybean product manufactured using the culture of *B. subtilis* DB821 strain that demonstrated the strongest bactericidal potential against BA-forming LAB.

Meanwhile, the pH values of the fermented soybean product were 6.71 ± 0.20 (non-starter), 6.76 ± 0.04–6.90 ± 0.13 (BA-forming LAB starter), and 7.23 ± 0.03–7.75 ± 0.01 (bacteriocin-producing *Bacillus* starter). After 3 days of fermentation, the sample fermented by mixed cultures of *Bacillus subtilis* DB821 and *E. faecalis* D51 had the lowest pH levels when compared to the natural and single-strain fermentation because of microbial metabolism during soybean fermentation. In a similar study on the fermented soybean product, Saker et al. [25] reported that the fermentations inoculated with *Bacillus* and *Enterococcus* exhibited a larger initial pH drop than those inoculated with *Bacillus* alone which is in agreement with the present study. Unlike our results the pH of *Cheonggukjang* manufactured using mixed cultures of *Bacillus* strain and LAB such as *B. breve* ATCC 15700, *L. mesenteroides* ATCC 9135 and *L. plantarum* ATCC 8014

was not significantly different from that of the product prepared with *Bacillus* strain alone [11]. In accordance with our findings, Ju and Oh [27] reported that the pH values of *Cheonggukjang* prepared with the single starter culture of *B. subtilis* CKB were increased probably due to the formation of basic substances such as ammonia during the fermentation process.

Content of amino-type and ammonia-type nitrogen in fermented soybean product

The contents of amino-type and ammonia-type nitrogen in fermented soybean product prepared using the different types of fermentation starters were showed in Table 4. The amino-type nitrogen content (328.31 ± 2.45 mg%) in control sample prepared by natural fermentation without starter cultures exhibited significantly higher value than that in the samples fermented with individual or mixed bacterial cultures. The amino-type nitrogen content of sample produced with *Bacillus* strains (209.40 ± 0.96 mg%–271.01 ± 0.44 mg%) as the starter cultures was greater than that obtained with LAB starter (111.31 ± 0.36 mg%–179.13 ± 1.35 mg%). The results of the current study revealed that fermentation of soybean with mixed culture of *B. subtilis* DB821 and *E. faecium* D12 significantly increased the amino-type nitrogen, which was presumed to be due to the synergistic effect of two starter cultures for the degradation of soybean protein. These results demonstrate that since soybean proteins from the raw materials of fermented soybean product were degraded by the synergistic effect of the proteolytic enzymes of *Bacillus* and LAB, the amino-type nitrogen content was significantly higher when compared with Bacillus strain alone.

Amino-type nitrogen, the nitrogenous compounds (amino acids, peptides and ammonium ions) produced from decomposition of proteins by microbes or autolytic enzymes can be used as an indicator of food quality and approximate index of the protein content, related with the total amount of substances having terminal amino group. According to Korean Food Standards Codex, amino-type nitrogen, which represents the savory taste in fermented soybean foods, is required to contain at least 280 mg%/g [28]. The results obtained from our experiments were in disagreement with those published previously by Shim et al. [29], who suggested that starter doenjang samples showed higher amino-type nitrogen contents than non-starter Doenjang throughout the fermentation period owing to the high enzymatic activities of the selected starter strains. Lee et al. [28] demonstrated that the amino-type nitrogen contents of all fermented soybean products by *Bacillus* sp. were 112.85–227.69 mg% and the highest amino-type nitrogen content was found in the sample fermented with *B. amyloliquefaciens* RD7-7 with high neutral protease-producing capacity. Meanwhile, our data were contrary to the findings of Lee et al. [28] reported that the amino-type nitrogen content of *Cheonggukjang* fermented by *B. subtilis* MC31 and *L. sakei* 383 was less than *Cheonggukjang* fermented by *B. subtilis* MC31 alone. Lee and Chang [30] reported that preferable sensory characteristics of *Cheonggukjang* fermented using *B. subtilis* SN7 had strong positive correlation with amino-type nitrogen content.

Table 4 Contents of amino-type and ammonia-type nitrogen in fermented soybean product prepared using the different types of fermentation starters

Fermentation starter	Amino-type nitrogen contents (mg%)	Ammonia-type nitrogen contents (mg%)
Non-starter	328.31 ± 2.45^{a}	298.47 ± 0.06^{a}
Enterococcus faecalis D08	179.13 ± 1.35^{e}	166.69 ± 0.08^{d}
Enterococcus faecium D12	159.36 ± 0.52^{g}	184.21 ± 0.71^{b}
Enterococcus faecalis D51	111.31 ± 0.36^{h}	176.31 ± 0.19^{bc}
Bacillus sp. DB407	256.72 ± 1.52^{d}	135.14 ± 0.52^{fg}
Bacillus licheniformis DB612	209.40 ± 0.96^{e}	128.21 ± 0.11^{g}
Bacillus subtilis DB821	271.01 ± 0.44^{c}	111.65 ± 0.74^{h}
Bacillus sp. DB407 + *Enterococcus faecalis* D08	267.21 ± 2.11^{c}	141.82 ± 0.24^{f}
Bacillus licheniformis DB612 + *Enterococcus faecalis* D08	248.39 ± 0.99^{b}	155.73 ± 0.38^{e}
Bacillus subtilis DB821 + *Enterococcus faecium* D12	303.11 ± 1.74^{ab}	173.45 ± 0.36^{c}
Bacillus subtilis DB821 + *Enterococcus faecalis* D51	297.58 ± 2.84^{b}	157.51 ± 0.02^{e}

Data are means ± SD from triplicate determinations

[a] [–h]Values within a column with different superscripts are significantly each groups at $p < 0.05$ by Duncan's multiple range test

ND not detected

Meanwhile, the results of the current study revealed that the ammonia-type nitrogen contents were lower in fermented soybean product by single culture of LAB (166.69 ± 0.08 mg%–184.21 ± 0.71 mg%) or *Bacillus* sp. (111.65 ± 0.74 mg%–135.14 ± 0.52 mg%) and by mixed culture of two starters (141.82 ± 0.24 mg%–173.45 ± 0.36 mg%) when compared to the control sample fermented without starter (298.47 ± 0.06 mg%). In particular, the content of ammonia-type nitrogen in fermented soybean product obtained with mixed culture of *B. licheniformis* DB612 and *E. faecalis* D08 was lower than in those obtained with our other starters.

Ammonia-type nitrogen that are volatile and odorous can be generated through excessive decomposition of nitrogenous matter, mostly proteins by microbial enzymes and serves as a fermentation index of fermented foods. The ammonia-type nitrogen was known to be produced by the deamination reaction of amino acids and/or by the oxidative degradation of amines by the oxidase of bacteria. A large amounts of ammonia-type nitrogen, acts as a causative agent in spoilage, can occur from abnormally fermented foods. Fermented products containing more than 120 mg% of the nitrogen related compound become unsuitable to ingest by the consumer [31]. Youn et al. [32] suggested that the content of ammonia-type nitrogen in fermented soybean product was proportional to the growth rate and the protease activity of starter, and the ammonia-type nitrogen content of fermented soybean product in *B. subtilis* was higher than in *B. natto* and *B. licheniformis*. Our findings were similar to those of Lee et al. [28], who provided that the content of ammonia-type nitrogen was significantly lower in *Cheonggukjang* fermented by mixed culture of *B. subtilis* MC31 and *L. sakei* 383 (213.35 ± 0.08 mg%) compared to single culture of *B. subtilis* MC31 (238.74 ± 0.18 mg%), therefore *B. subtilis* MC31 and *L. sakei* 383 as a good candidate for use in mixed starter cultures can be used to produce the desirable quality of fermented soybean paste with reduced off-flavor. Furthermore, similar results for ammonia-type nitrogen contents in *Cheonggukjang* prepared with bacteriocin-producing LAB were reported by Lim [11].

Content of BA in fermented soybean product

As shown in Table 5, the traditional fermented soybean product had average levels of cadaverine of 653.12 ± 4.08 mg%, putrescine of 276.14 ± 15.84 mg%, and tyramine of 399.11 ± 3.58 mg%, whereas histamine and tryptamine were not found in this sample. Significant differences in BA profiles were observed between fermented soybean products made with non-starter and BA-forming LAB starter. Among the LAB starters used in the present study, the levels of cadaverine and histamine were only detected in *E. faecalis* D51 and *E. faecalis* D08-inculated samples, respectively. Tryptamine was highest BA detected in *E. faecium* D12-inoculated sample. Tyramine was significantly higher in *E. faecium* D12-inoculated sample than that in *E. faecalis* D08-inoculated sample. Among the three strains, *E. faecium D12* shows the highest BA-forming ability, which may be due to its high tryptophan and tyrosine decarboxylase activity. However, the BA determined were not detected in any fermented soybean product manufactured with single starter of *Bacillus* strain. Interestingly, a significant decrease in BA levels was observed in fermented soybean product by bacteriocin-producing *Bacillus* sp. DB407, *B. licheniformis* DB612, and *B. subtilis* DB821, suggesting that the bacteriocins seem to directly impact

Table 5 Contents of BA in fermented soybean product prepared using the different types of fermentation starters

Fermentation starter	BA content (mg%)				
	Cadaverine	Histamine	Putrescine	Tryptamine	Tyramine
Non-starter	653.12 ± 4.08	ND	276.14 ± 15.84	ND	399.11 ± 3.58
Enterococcus faecalis D08	ND	895.63 ± 10.55	ND	ND	125.62 ± 9.68
Enterococcus faecium D12	ND	ND	ND	986.32 ± 6.94	312.54 ± 13.04
Enterococcus faecalis D51	470.95 ± 16.94	ND	ND	489.21 ± 20.69	ND
Bacillus sp. DB407	ND	ND	ND	ND	ND
Bacillus licheniformis DB612	ND	ND	ND	ND	ND
Bacillus subtilis DB821	ND	ND	ND	ND	ND
Bacillus sp. DB407 + *Enterococcus faecalis* D08	ND	648.45 ± 7.89	ND	ND	63.94 ± 1.63
Bacillus licheniformis DB612 + *Enterococcus faecalis* D08	ND	776.17 ± 14.51	ND	ND	87.52 ± 8.02
Bacillus subtilis DB821 + *Enterococcus faecium* D12	ND	ND	ND	545.24 ± 25.61	181.31 ± 7.06
Bacillus subtilis DB821 + *Enterococcus faecalis* D51	220.61 ± 5.58	ND	ND	208.51 ± 20.39	ND

Data are means ± SD from triplicate determinations

ND not detected

BA production of *E. faecalis* D08, *E. faecium* D12, and *E. faecalis* D51. Cadaverine concentration produced by a mixed culture of *B. subtilis* DB821 and *E. faecalis* D51 was lower than that obtained with the single cultures of BA-forming LAB. The reduced amount of histamine with 28% decrease was recorded in soybean fermented with *Bacillus* sp. DB407 followed by 14% decrease in soybean fermented with *B. licheniformis* DB612, respectively in comparison with sample fermented by single cultures of *E. faecalis* D08. *B. subtilis* DB821 was significantly efficient in reducing cadaverine, tryptamine, and tyramine accumulation of *E. faecium* D12 and *E. faecalis* D51 during soybean fermentation.

Jeon et al. [33] reported that the contents of aromatic vasoactive amine such as β-phenylethylamine and tyramine in some commercial *Cheonggukjang*, a Korean traditional fermented soybean food exceeded safe levels for consumption. Unlike our results, they noticed that *Bacillus* species are as significant as *Enterococcus* species for BA production in *Cheonggukjang*, including tyramine, and should be controlled accordingly for the reduction of BA contents in the food. BA amounts in our study were higher than those found by Park et al. [34], who reported that the mean values of cadaverine, putrescine, and tyramine determined in twenty-three samples of traditionally available Korean fermented soybean paste samples were found to be 34.24, 70.84, and 126.66 mg%, respectively.

Unlike *Bacillus* used in our study, Mah et al. [18] suggested that *Bacillus* sp. and *Enterococcus* sp. played major roles in tyramine formation in fermented soybean foods, because the deposited genes (*odc*-AZ, *ldc*, *hdc*, *odc*, and *tdc*) encoding amino acid decarboxylases were found in these bacteria. Meanwhile, Han et al. [35] noticed that *B. subtilis* and *B. licheniformis* isolated from *Cheonggukjang* produced high levels of BA including cadaverine, putrescine, histamine and tyramine. Conversely, *B. subtilis* HB-1 showed no BA-forming ability, and *B. subtilis* GD-4 exhibited high BA degradation ability.

Meanwhile, Barbieri et al. [10] noticed that *E. faecium* and *E. faecalis* showed significant histidine, tyrosine, lysine, and ornithine decarboxylase activity. In agreement with our results, high levels of BA were found in fermented soybean product fermented by BA-forming LAB such as *E. faecalis* and *E. faecium* [33]. Accordingly, the individual LAB and *Bacillus* strains showed strain-specific abilities to produce BA.

In particular, our results indicated that the mixed starter cultures of bacteriocin-producing *Bacillus* and BA-forming LAB showed the highest effectiveness in reducing BA accumulation during soybean fermentation compared with the sample fermented without starter culture or with BA-forming LAB as monostarter culture. Consistent with our previous research [11], bacteriocin-producing starters used for product of functional fermented soybean foods showed effectively the reduction of BA production level by BA-forming strains. In a recent study by Lim [36], it was demonstrated that the bacteriocin solution (200 AU/mL) and the cell-free culture supernatants (200 μL/mL) produced by *L. plantarum* FIL20 and *L. paracasei* FIL31 significantly ($p < 0.05$) decreased the bacterial numbers and histamine and tyramine production ability of *S. marcescens* CIH09 and *Aeromonas hydrophilia* RIH28. Similar results were obtained by Lim [11], who reported that the bacteriocin-producing LAB showed antibacterial effect against BA-forming *Bacillus* sp. and BA content of fermented soybean product prepared by mixed culture of the LAB and *Bacillus* sp. were significantly decreased compared to *Bacillus* single starter culture. Joosten and Nunez [9] reported results similar to ours, i.e., they stated that the use of bacteriocin-producing starters prevented the formation of histamine in cheese. Therefore, the use of bacteriocin in the food industry can help extend food shelf life and enhance the food commodities' safety, owing to inhibitory effect of the antimicrobial bacteriocin produced by *Bacillus* strain against BA-forming LAB in fermented soybean product. Mah et al. [18] suggested that in addition to the aforementioned *Bacillus* starter cultures, strains of *E. faecium* and *L. plantarum* can be considered as a useful and accessible tool in soybean fermentation processes because of their abilities to produce bacteriocin or to degrade BA, respectively.

The preservative effect of LAB normally used as starters in fermented products is due to production of several other antimicrobial compounds that prevent the growth of pathogenic and/or spoilage microorganisms. However, LAB starter cultures should take into account the risks posed by the potential of BA in food, because some LAB can have the capability to produce toxic compounds as BA. BA are formed through the decarboxylation of specific free amino acids by exogenous decarboxylases released from the microorganisms naturally present in raw material introduced throughout the processing and indigenous starter cultures. Fermented soybean foods prepared by traditional methods are extremely vulnerable to contamination by toxigenic microorganisms such as BA-forming LAB, because the fermentation processed are carried out in open environments and sterilization processes are not utilized in the manufacturing of the products [5]. Accordingly, the key to control BA is the application of good manufacturing practices according to HACCP (hazard analysis and critical control point) system and the use of high-quality raw material and non-amine forming (amine-negative) or amine oxidizing starter cultures

for fermentation [6]. Interestingly, the use of *Bacillus* starter cultures capable of degrading and/or incapable of forming BA has been proposed as a guaranteed way to reduce BA in fermented soybean foods, considering that *Bacillus* species have been known as fermenting microorganisms responsible for BA formation in the foods [18]. The bacteriocin-producing starter culture can provide a successful prospective alternative strategies for inhibiting BA accumulation in fermented soybean foods. Bacteriocins are attracting considerable interest as safe bio-preservatives to conventional antimicrobials; as they are rapidly digested by proteases in the human gastrointestinal tract, not toxin to normal eukaryotic organism, and not affect sensory and nutritional properties of the foods. Members of the *Bacillus* group can be considered an ideal starter cultures for fermenting soybeans, since they have the ability to produce endospores that can resist to the unfavorable and stressful conditions and to produce antimicrobial compounds that display remarkable broad-spectrum activity [37].

According to these our results, BA were found in high concentrations in the fermented soybean food produced by the traditional method and by specific LAB strains used for starter culture. Accordingly, careful screening for amino acid decarboxylase activity is necessary when selecting LAB as safe starter cultures for the food industry, although LAB are the main vehicle for probiotics that can provide direct benefits to human gastrointestinal health. Since the fermented food products manufactured by traditional method may be contaminated by BA-forming microorganisms from the surrounding environment, the use of bacteriocin-producing *Bacillus* strains can be recommended to successful control BA in fermented soybean products and to protect the consumer's health. In conclusion, the bacteriocin-producing *Bacillus* strains such as *Bacillus* sp. DB 407, *B. licheniformis* DB612, and *B. subtilis* DB821 can be used as starter cultures for the production of BA-controlled soybean products and for the enhancement of quality and safety of fermented foods.

Acknowledgements
This research was supported by the Tongmyong University Research Grants 2020 (2020A044).

Authors' contributions
ESL conceived and designed the analysis, collected the data, performed the analysis, and wrote the paper. The author read and approved the final manuscript.

Declarations

Competing interests
The author declares no competing interests.

Received: 17 August 2021 Accepted: 10 December 2021
Published online: 20 January 2022

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