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Characteristics of Potential Gamma-Aminobutyric Acid-Producing Bacteria Isolated from Korean and Vietnamese Fermented Fish Products

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Gamma-aminobutyric acid (GABA) is a neurotransmitter that exerts several physiological functions and positive effects on human health. The aim of this study was to isolate and characterize the strains that had GABA-producing abilities from various fermented fish products. A total of 91 acid-producing strains were isolated from 41 samples of fermented fish products, and 27 strains showing GABA-producing abilities were identified by the 16S rDNA sequences. Among the strains, 31% strains tolerated at high-salt environment of 10–20% throughout the fermentation of fish sauces. The 27 isolates that produced GABA at various concentrations did so in the range of 5 to 454 mM. These GABA-producing isolates were identified as lactic acid bacteria of 14 strains, which included twelve *Lactococcus lactis*, one *Enterococcus faecium*, and one *Lactococcus pentosus*; eight *Bacillus cereus* group, which included seven *B. thuringiensis* and one *B. cereus*; and five *Staphylococcus* spp. Interestingly, with Vietnamese fish sauces, we mostly identified species of *B. thuringiensis* and *Staphylococcus* spp., while with Korean fermented fish products, the majority of the strains identified belonged to *L. lactis*. Among the strains, *B. thuringiensis* LH2134 produced the highest levels of GABA at 366 mM among the strains identified from Vietnamese fish sauces, whereas *L. lactis* LA43, a new strain isolated from Korean jeotgal (salted shrimp paste), produced the highest amount of GABA at 454 mM and the glutamate concentration in the medium was essential for GABA accumulation. Therefore, such the isolates might serve as good starters for development of more GABA-reinforced foods among fermented fish products.

Keywords: Gamma-aminobutyric acid, fermented fish products, glutamate, lactic acid bacteria, *Bacillus*, *Staphylococcus*

Introduction

Fermented fish products are valuable nutritional condiments made by combining fish and sea salt. They are generally used as a main ingredient for traditional foods in various cuisines in Asia. In Vietnam, they are called nuoc mam (fish sauce) and are one of the few traditional products manufactured on a large scale throughout the country, with an annual output of approximately 220 million products. In Korea, they are called aekjeot, and are typically used in kimchi to accelerate the fermentation process. On some occasions, they are also used in Korean side dishes and soup or stew to give extra umami (flavor). Thus, they are considered one of the most important sources of dietary proteins. Fish sauce contains up to 20 g/l nitrogen, 80% of which is in the form of essential amino acids. Glutamic acid, aspartic acid, and lysine are three amino acids mostly present in fish sauce [1], which contains particularly high amounts of glutamic acid (23 mg/ml) [2]. Glutamic acid is a precursor of gamma-aminobutyric acid (GABA), and also helps to give the characteristic flavor of fish sauce.

GABA is a four-carbon, non-protein amino acid that is widely present in bacteria, plants, and vertebrates. It is principally formed by an α-decarboxylation reaction of L-glutamic acid or its salts, and catalyzed by glutamic acid

decarboxylase, whose biochemical properties have been reported previously [3]. GABA is served as a bioactive compound in foods and is seen as a great bioactive natural compound for human health since it exerts several physiological functions and also has positive antioxidant, anti-diabetes, hypotensive, and anxiety reduction effects [4]. GABA could delay or inhibit the invasion and metastasis of various types of cancer cells in the mammary gland, colon, and hepatic cancer cells [5]. Indeed, GABA-enriched food is required because the GABA content in the typical daily human diet is relatively low [6]. As a result, the development of functional foods containing GABA has been actively increased with a vast variety of GABA-enhanced food products including cereals, sourdough, breads, cheeses, fermented sausages, teas, vegetables, legumes, dairy soy products, alcohol beverages, and especially traditional Asian fermented foods [7].

A high amount of GABA is found mainly in fermented products, especially fermented dairy products [8]. GABA produced by fermentation with microorganisms has been reported in bacteria, fungi, and yeasts [9]. The most interesting and practical group of bacteria for GABA production is lactic acid bacteria (LAB), including strains of *Lactobacillus (L.)*, and *Lactococcus (Lc.)*, of which *L. brevis* was isolated from many fermented foods such as Chinese traditional paocai [10], fresh milk [11], soya yogurt [12], and black raspberry juice [13]. The strains *L. delbrueckii, L. plantarum,* and *L. paracasei* were isolated from cheese and Japanese traditional fermented fish [14, 15], respectively. The best GABA-producing LAB strains were *L. paracasei*, *L. delbrueckii, L. lactis,* and *L. brevis* isolated from a variety of Italian cheeses, and *Lc. lactis* sp. were screened from cheese starters with the highest level of GABA production (391 mg/kg) [16]. A total of 61 GABA-producing LAB strains were identified in cheese [9]. Other GABA-producing LAB were considered as potential candidates for fermentation in skim milk [16].

For GABA producers in fermented foods in Vietnam, remarkably, three GABA-producing strains were isolated from *Nem chua* (sour fermented pork), and these were *L. plantarum* NCDC3, *L. plantarum* BAC52, and *L. brevis* NCTH24. One isolated from dua chua (sour fermented vegetables) was *L. plantarum* LD3, and one isolated from *Com ruou* (fermented white rice paste) was *L. plantarum* LV1. These five strains were all LAB strains, four of which belonged to *L. plantarum* and another that belonged to *L. brevis* [17]. Similarly in Korea, GABA-producing strains isolated from kimchi including *L. brevis* NCL912 [18], *Lactobacillus buchneri* MS [19], and *Lc. lactis.* subsp. *lactis* B produced the highest amount of GABA (3.68 g/l) [20].

GABA-producing LAB strains have mostly been found in fermented foods including cheese, skim milk, raw milk, and yogurt as well as fermented fish products such as fish sauce. *L. paracasei* has been found in Japanese traditional fermented fish with strain NFRI 7415 producing GABA at a concentration of 302 mM [15]. However, there have been few strains as GABA producers isolated from Vietnamese or Korean fermented fish products up to now.

The aim of this study was to screen various types of GABA-producing bacteria from fermented fish products in Vietnam and Korea as well as those used as possible starters in the production of fermented foods. The new GABA-producing strains were hypothesized to enhance the development of functional fermented foods containing GABA.

Materials and Methods

Microbiological Analysis

Forty-one samples of fermented fish products including 29 fish sauces, five anchovy pastes, two sour shrimp pastes, and five salted shrimp pastes were obtained from markets in Vietnam and Korea. Viable bacterial cells in fish samples were enumerated by a conventional counting method. The fish samples were homogenized in saline solution and spread-plated on plate count agar-bromocresol purple (PCA-BCP) agar supplement with 2.5 mg/l amphotericin B. The agar plates were incubated at 30°C for 2 days under anaerobic condition using BD BBL GasPak anaerobic and CO_2 indicators (Becton, Dickinson and Company, USA). Colonies that formed a yellow halo were selected, and then screened on De Man, Rogosa and Sharpe (MRS) agar (Oxoid, England) for identification as acid-producing bacteria. Furthermore, isolates were harvested and purified again on MRS medium and stored frozen at −80°C with 20% glycerol for further characterization [21].

Phenotypic Characterization of Isolated Bacteria

All of the isolates were initially tested for Gram reaction by the KOH method, while catalase reaction, gas production from glucose, and fermentative type were also determined by culture on homofermentative-heterofermentative differential (HHD) medium [22]. MRS broth was used to measure the growth at pH values ranging from 2 to 9. The salt tolerance tests were carried out with MRS broth medium containing 3, 6.5, 10, 15, 20, and 25% NaCl (w/v), and the range of growth temperature was assayed at 4, 10, 15, 37, 45, 50, and 15 min-heat shock at 60°C in MRS broth, respectively [23]. Using these tests, the isolates were grouped based on their characteristics.

GABA-Producing Analysis by Thin-Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC)

GABA production was analyzed qualitatively by the aluminum TLC silica gel plate (Aluminum Sheets Silica gel 60 F254, Merck, Germany) [24]. Cells were cultivated in MRS broth supplement with 1% monosodium glutamate (MSG) at 30°C for 72 h. Culture supernatants were obtained by centrifugation at 9,000 ×*g* for 10 min and then subjected to TLC analysis. An 0.75-μl aliquot of sample supernatant and standard at a concentration of 2 mg/ml was spotted on the TLC plate. Plates were air-dried and subjected to a TLC solvent mixture (1-butanol : acetic acid : distilled water at 5:3:2 ratio) dissolved directly with 0.4% (w/v) ninhydrin as the mobile phase. TLC plates were developed for 2 h, and then dried in the convection oven at 60°C for 15 min to yield a red-purple color.

The GABA production was quantified by HPLC basically following the method of Kim [25] with some modifications. Culture supernatants and GABA standard solutions were derivatized with phenylisothiocyanate (PITC) [26]. Briefly, aliquots of 500 μl of culture supernatant and GABA standards were dried using a speed-vacuum concentrator (Vacuum Concentrators VC2124, Gyrozen, Korea). The residue was dissolved in 100 μl mixture of ethanol-water-triethylamine (2:2:1 v/v/v) and evaporated to dryness. An aliquot of 150 μl ethanol-water-triethylamine-PITC (7:1:1:1 v/v/v/v) was added to the residue and incubated for 20 min at room temperature to form phenylthiocarbamyl-GABA. The dry residue was dissolved in 500 μl of the mobile phase consisting of 80% solution A (1.4 mM sodium acetate, 0.1% triethylamine, 6% acetonitrile) and 20% solution B (60% acetonitrile). The solutions were filtered through 0.45 μm membranes and then subjected to HPLC analysis. The GABA analysis was performed using an UltiMate 3000 Standard Dual HPLC System equipped with an Acclaim 120 C18 column (Thermo Fisher, Korea). The column was eluted for 50 min with a linear gradient of 0–100% solution B at a flow rate of 1 ml/min. A sample volume of 20 μl was injected and monitored at a wavelength of 254 nm. Sample peak areas were measured and compared with the calibration curve standard of GABA at known concentrations (2.5, 5, 7.5, and 10%) in order to quantify GABA concentrations. The conversion rate of glutamate to GABA was calculated based on the produced GABA and initial glutamate content.

Genetic Analysis of GABA-Producing Microorganisms

Analyses of the 16S rRNA gene sequence were performed to identify the GABA-producing bacterial isolates. Total genomic DNA was extracted from the isolates using the genomic DNA extraction kit (Bioneer, Korea). Amplification of the 16S rRNA gene was performed using universal primer set 27F (5′ AGAGTT TGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTAC GACTT-3′) [27]. The PCR was performed in 20-μl reaction mixtures containing a premix (Bioneer) with 1 μl template DNA, 1 μl of 5 μM each primer, and 17 μl distilled water. The cycle conditions for the 16S rRNA gene amplification were as follows: initial denaturation at 95°C for 5 min; followed by 32 cycles at 95°C for 45 sec, 48°C for 45 sec, and 72°C for 75 sec, and a final extension at 72°C for 5 min. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany), then sequenced and analyzed by comparing the consensus sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) using the basic local alignment search tool (BLAST) [28].

Identification of *Bacillus* spp.

Based on the 16S rRNA gene sequencing data and reference strains from GenBank, *Bacillus* strains were distinguished from each other preliminarily by the reference culture media of mannitol-egg yolk-polymyxine agar (MYPA, Difco, USA), polymyxin-egg-yolk-mannitol-bacillus agar (PEMBA, UK), and phenol red dextrose agar (PRDA, Oxoid). In addition, the characteristics of hemolysis and lecithinase for the *B. cereus* group were tested. The hemolytic zones formed were observed and classified based on lytic activities of red blood cells in the media around and under the colonies. The lecithinase activity was determined by pink colonies with opaque halos on the MYPA medium. The extracted DNA was subjected to PCR using the primer pair BC1/BC2r to classify as part of the *B. cereus* group [29]. *B. cereus* strains were distinguished from *Bacillus thuringiensis* strains by PCR with the following primer pairs targeting the *gyrB* gene: BT1/BT2r, BFW1/BCrevnew [30]. The following *Bacillus* exotoxin genes were also confirmed by the primer pairs ETF/ETR used to detect *becT* of enterotoxin T [31], and EntFM-F/EntFM-R [32] and ENTA/ENTB [33] were used to detect *entFM* of enterotoxin FM. Finally, the *Bacillus* isolates were confirmed by PCR with the K3/K5 primer pair targeting the *cry* gene on the basis that *B. cereus* does not produce crystal toxin, whereas *B. thuringiensis* does [34]. *B. cereus* KCCM 1034 and *B. thuringiensis* KCTC 1094 type strains were used as a reference control for PCR. Microscopy observations were carried out to detect crystal proteins (δ-endotoxins) for the distinction of *B. thuringiensis* among the *B. cereus* group strains. The simple staining procedure with TB carbol-fuchsin ZN (BD BBL Difco) was used for staining the cells [35]. The crystal proteins were examined with an optical microscope (Optika, Italy).

Identification of *Staphylococcus* spp.

The first key coagulase test was carried out to divide *Staphylococcus* into two main groups: coagulase positive *Staphylococci* (CPS) and coagulase negative *Staphylococci* (CNS). Bacterial cultures of *Staphylococci* spp. were incubated at 37°C for 16 h in tryptic soy broth (TSB, Oxoid). The coagulase-positive organism caused the plasma to form a clot in the test tube whereas, the coagulase-negative organism did not [36]. Further, CPS and CNS were differentiated by biochemical test on mannitol salt agar (MSA, Oxoid). The production of yellow colonies due to the high salt content of media and fermentation of mannitol is regarded as a presumptive tool for the identification of CPS [37]. The PCR amplification was performed to identify *Staphylococcus* spp. for

nuc gene target which CPS encoded while CNS did not, by using the pair of primers Sa1 and Sa2 [38]. The strains *S. aureus* ATCC 19095 and *S. epidermis* KCCM 40416 were used as positive and negative controls, respectively. Finally, 16S rRNA gene sequencing was done again to confirm the subspecies of *Staphylococcus* isolates using the universal primer set 27F and 1492R as decribed above.

GABA Production on Glutamate-Reinforced Medium

The best GABA-producing strain would be chosen for further investigation of the maximum GABA accumulation under glutamate-reinforced conditions. Four desired conditions for strain cultivation for enhancing GABA levels were as follows: MRS containing 500 mM MSG, incubated at 30°C for 72 h; MRS containing 700 mM MSG, pH 5, 30°C for 72 h; MRS containing 700 mM MSG supplement with 2% maltose and 3% tryptone, pH 5, incubated at 30°C for 72 h; and MRS containing 400 mM MSG with 3.5% glucose, pH 5, incubated at 32°C for 48 h [18]. The GABA content would be determined and compared with the basic culture condition of 500 mM MSG in MRS by HPLC as described above.

Results and Discussion

Microbiological Analysis and Characteristics

Viable cell counts in all samples were various from 0 to 10^7 CFU/ml. In the Korean fermented fish samples, the density of microorganisms was 1.94×10^7 CFU/ml, whereas in the Vietnamese fermented fish samples, the maximum density was 5.17×10^5 CFU/ml. This proportion was consistent with previous studies reporting an average mesophilic bacterial counts at 3.6×10^7 on PCA agar as well as a similar viable cell count of 10^5 in fermented seafood products in Indonesia [39, 40]. Among them, 91 colonies of acid-producing bacteria were selected by the presence of yellow zones, and then they were classified in 9 groups (A–

Table 1. Characteristics of acid-producing bacteria isolated from Korean and Vietnamese fermented fish products.

Characteristics	A	B	C	D	E	F	G	H	I
Number of isolates	11	15	4	6	17	4	4	16	14
Shape	Cocci	Cocci	Rod	Rod	Rod	Cocci	Cocci	Rod	Rod
Gram stain	+	+	+	+	+	+	+	+	+
Gas from D-glucose	7/11	9/15	+	+	11/17	+	+	12/16	+
Catalase	-	-	-	-	-	+	+	+	+
Fermentation type	Homo	Homo	Hetero	Homo	Homo	Hetero	Homo	Hetero	Homo
Heat shock at 60°C, 15 min	w	w	w	w	w	+	+	+	+
Growth at temperature (T°C)									
$10 \leq T \leq 37$	+	+	+	+	+	+	+	+	+
Growth at pH									
3	-	w	-	w	-	+	-	+	-
5	+	+	+	+	+	+	w	+	+
7	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+
9	6/11	w	w	w	16/17	-	3/4	13/16	2/14
Growth in NaCl (%)									
1	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	1/4	+	+
6.5	+	+	+	+	+	+	3/4	+	+
10	2/12	7/12	2/4	-	3/17	3/4	+	+	+
15	-	2/12	1/4	-	-	-	1/4	11/16	8/14
20	-	-	-	-	-	-	-	10/16	2/14
25	-	-	-	-	-	-	-	-	-

Gram stain: +, Gram-positive; -, Gram-negative. Gas from glucose: +, producing gas from glucose; -, no gas produced.
Catalase: +, producing hydrogen peroxide; -, no hydrogen peroxide produced. Homo, homofermentative; Hetero, heterofermentative
Growth: +, normal growth; w, low growth rate; -, no growth

I) based on biochemical and physiological tests (Table 1). Gram staining revealed 37% with a coccoid shape and 63% with a rod shape, and homo- and hetero-fermentative properties were also identified in these strains. Furthermore, more than 75% of the isolates could ferment glucose and produce CO_2, and 29 strains grew well in 10% NaCl, while 23 stains could tolerate 15% NaCl and 12 strains could tolerate up to 20% NaCl. Those salt-tolerant strains belonged to groups B (3 strains), G (1 strain), H (11 strains), and I (8 strains). All of these strains were isolated from Vietnamese fish products, except for two strains from Korean jeotgal, suggesting that mostly halophilic strains were present in Vietnamese fish products. The fact that 31% of strains tolerated a high-salt environment (10–20%) throughout the fermentation of fish sauces revealed that these strains might play an active role in the fermentation process of fish sauces in Vietnam. This was in agreement with a previous study reporting that halophilic LAB were dominant at the final stage of fish sauce fermentation when color, aroma, and flavor were fully developed [41]. This has led to a prediction that halophilic LAB could play a significant role in the characteristics of fish sauces.

Qualitative and Quantitative Analyses of GABA Production by TLC and HPLC

Next, the 91 isolates were tested for their GABA-producing ability on TLC plates. Diluted GABA standards were used as a positive control, and MSG was used as negative control. Among the 91 acid-producing isolates, 27 (30%) showed GABA spots on the TLC plates (Fig. 1). This was higher than the detection ratio of 10 strains in the 53 total strains isolated from traditional fermented foods of Ishikawa Prefecture in Japan (19%), and also much higher than 12 of the 130 strains (9%) that were isolated from Myanmar fishery products fermented with boiled rice [27, 42].

The GABA-production by 27 isolates were then quantitatively confirmed by HPLC. According to the gradient elution experimental results, the HPLC GABA standard curve was presented as $Y = 0.2707X + 66.449$ with a linear relationship ($R^2 = 0.982$). HPLC chromatograms of the GABA standard solution and GABA produced by isolates were obtained with a retention time at 33.06 min. All 27 GABA-producing isolates that produced GABA at various concentrations are indicated in Table 2.

All 27 isolates produced GABA at various concentrations in the range of 5 to 454 mM. In fish sauces, GABA was produced at concentrations of 10 to 435 mM, where the highest producer was by the isolate LA51 from Korean jeotgal. In shrimp pastes, GABA reached the highest concentration of 454 mM, where isolate LA43 was considered as the best producer. The GABA level produced in anchovy pastes was lowest at 18 to 65 mM. In general, isolates from Korean fermented fish products could produce GABA much more than those from Vietnam in the range of 45–454 mM, while the concentration of GABA in fermented fish products from Vietnam ranged from 5–366 mM. The highest producers of GABA in Vietnamese and Korean fermented fish products belonged to isolate LH2134 in the Vietnamese fish sauce *nuoc mam* and isolate LA43 in the Korean shrimp paste jeotgal, respectively.

Genetic Analysis of GABA-Producing Microorganisms

To identify the species, all isolates were subjected to comparative 16S rRNA gene sequencing analysis using the BLAST search program at the NCBI website. The 16S rRNA

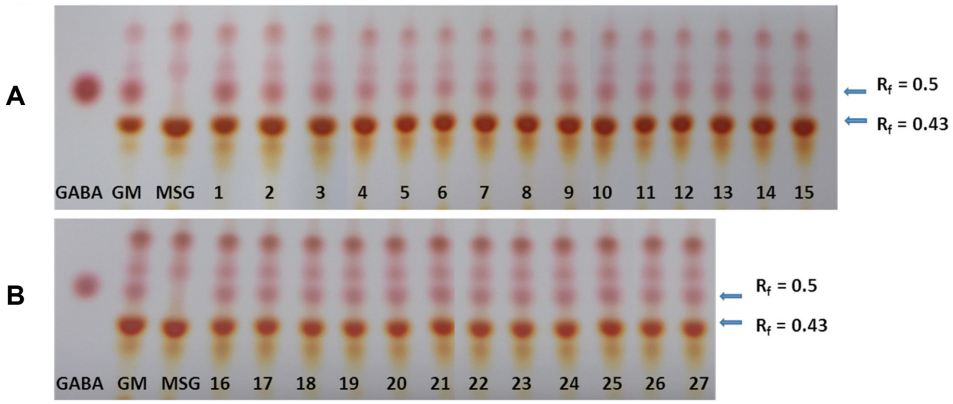

Fig. 1. Thin layer chromatogram of GABA production.
Lane GABA, GABA standard at concentration of 2 mg/ml; Lane GM, mixture of GABA and MSG; Lane MSG, monosodium glutamate in MRS (negative control) at concentrations of 1%; 1A, lanes 1–15; 1B, lanes 16–27 presented bacterial isolates cultured in MRS medium with 1% MSG.

Table 2. Production of GABA in MRS media containing 500 mM MSG.

No.	Groups	Isolate names	GABA Production (mM)	Source isolated (sample maker)
1	**A**	LA43	454.18	Salted shrimp sauce (OS, Korea)
2		LA51	434.75	Sand lance sauce (CH, Korea)
3		LA49	421.59	Salted shrimp sauce (OS, Korea)
4		LA45	405.54	Salted shrimp sauce (OS, Korea)
5		LA47	336.32	Salted shrimp sauce (OS, Korea)
6		LA44	285.55	Salted shrimp sauce (OS, Korea)
7		LA46	256.22	Salted shrimp sauce (OS, Korea)
8		LA52	245.19	Sand lance sauce (CH, Korea)
9		LA42	196.42	Salted shrimp sauce (OS, Korea)
10		LA410	44.95	Salted shrimp sauce (OS, Korea)
11	**B**	LB48	224.18	Salted shrimp sauce (OS, Korea)
12		LB41	141.56	Salted shrimp sauce (OS, Korea)
13		LB18	136.94	Fish sauce (SM, Vietnam)
14	**C**	LC129	180.97	Fish sauce (SM, Vietnam)
15	**F**	LF27	101.58	Fish sauce (NP, Vietnam)
16	**G**	LG46	89.96	Fish sauce (NN, Vietnam)
17		LG532	64.82	Fish paste (MNX, Vietnam)
18		LG238	40.57	Fish paste (MNX, Vietnam)
19		LG230	18.48	Fish sauce (NN, Vietnam)
20	**H**	LH2134	366.02	Fish sauce (Barona, Vietnam)
21		LH2241	92.87	Fish sauce (KH, Vietnam)
22		LH139	46.36	Fish sauce (NP, Vietnam)
23		LH510	27.23	Fish sauce (CM, Vietnam)
24	**I**	LI813	49.83	Anchovy paste (TP, Vietnam)
25		LI915	34.77	Sour shrimp paste (NL, Vietnam)
26		LI140	10.62	Fish sauce (NP, Vietnam)
27		LI324	5.4	Sour shrimp paste (SM, Vietnam)

gene sequencing of all 27 isolates were identified from 99 to 100% identity. LAB, as the dominant species, included 14 isolates: 12 *Lactococcus lactis*, one *E. faecium*, and one *Lactococcus pentosus*. The second group identified belonged to the *B. cereus* group with eight strains. Seven strains were identified as *B. thuringiensis* and one strain as *B. cereus*. Lastly, gene sequencing of five isolates identified as *Staphylococcus* spp. included two isolates identified as *Staphylococcus pasteuri* and *Staphylococcus piscifermentans*, respectively. All the 27 isolates that were identified are shown with their sequence identity in Table 3. LAB was the dominant species as indicated by the 14 isolates that were identified, in which 12 strains belonged to *Lactococcus lactis* spp. with 100% sequence identity to type strains. The remaining isolates of LAB belonged to *E. faecium* and another one was identified as *L. pentosus*. The second dominant species belonged to the *B. cereus* group with 99–100% sequence identity to type strains. Gene sequencing of the five isolates identified them as *Staphylococcus* spp. and included two *S. hominis*, one *S. carnosus*, one *S. pasteuri*, and one *S. piscifermentans*.

Moreover, PCR using the species-specific primer pair LlaR and LlaF was carried out to confirm the 12 strains of LAB. The result revealed that all 12 strains belonged to the genus *Lactococcus*, and the phylogenic analyses of *L. lactis* LA43 and *B. thuringiensis* LH2134 are shown in Fig. 2. Identification by an API kit also obtained the accurate identification result of 99.9% for *B. thuringiensis* LH2134 and 99.1% for *Lactococcus lactis* LA43 (data not shown).

Identification of *Bacillus* spp.

Among the eight strains of the *B. cereus* group, seven isolates were *B. thuringiensis* strains and one isolate was identified as *B. cereus*. These *Bacillus* strains were confirmed by PCR using the primer pairs following BCrevnew/BCFW1, BT1/BT2r, and K3/K5 targeting the *cry* gene because the crystal toxin is only expressed by *B. thuringiensis*. The results showed that LH2134, LH2241, LH139, LI140, LI915, LI324, and LI813 harbor the *cry* gene, except LH510, which was identified as a *B. cereus* strain without the *cry* gene (Fig. 3). Among them, strains LI324 and LH510 contained the *bceT* gene, which is expressed for enterotoxin production and were recognized by the ETR/ETF primer pair (Table 4). Results of the PCR using species-specific primers are presented in Table 6. Finally, seven selected strains of *B. thuringiensis* were identified based on the presence of the crystal toxin protein, and these results are presented in Fig. 4.

The results obtained from Vietnamese fish sauces that revealed mostly species of *B. cereus* group in this study were in accordance with results from a previous study of nam pla (traditional Thai fish sauce) which showed all isolates belonged to *Bacillus* species such as *Bacillus cereus*, *B. circulans*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, and *B. subtilis* [43]. Furthermore, the samples of patis liquid collected after one month of fermentation from the Rufina Co., Malabon, the Philippines, showed that they contained single strains of *B. pumilus* and another strains of *Micrococcus colpogenes*, *M. varians*, and *Candida clausenii*. The patis residue contained single strains of *B. coagulans*, *B. licheniformis*, and *Achromobacter thalassius* [43]. Similarly, the samples of

Table 3. Identification of the bacterial 16S rRNA gene sequences obtained from fermented fish samples.

No.	Group	Isolate name	Phylotype	Closest sequence	Identity %	Accession Number
1	A	**LA42**	*Lactococcus lactis* sp.	*Lactococcus lactis* subsp. *lactis* KLab14	100	KM485587
2		**LA43**	*Lactococcus lactis* sp.	*Lactococcus lactis* subsp. *lactis* RCP438	100	KT260650
3		**LA44**	*Lactococcus lactis* sp.	*Lactococcus lactis* RCB1015	100	KT261227
4		**LA45**	*Lactococcus lactis* sp.	*Lactococcus lactis* HP16	100	KX586694
5		**LA46**	*Lactococcus lactis* sp.	*Lactococcus lactis* subsp. *lactis* JC10	100	GU936959
6		**LA47**	*Lactococcus lactis* sp.	*Lactococcus lactis* Lc1	100	MG825732
7		**LA49**	*Lactococcus lactis* sp.	*Lactococcus lactis Sourdough C6*	100	MG754583
8		**LA410**	*Lactococcus lactis* sp.	*Lactococcus lactis* subsp. *lactis* Lc2	100	MG825730
9		**LA51**	*Lactococcus lactis* sp.	*Lactococcus lactis* subsp. *lactis* Lc5	100	MG825736
10		**LA52**	*Lactococcus lactis* sp.	*Lactococcus lactis* subsp. *cremoris* Lc4	100	MG825739
11	B	**LB41**	*Lactococcus lactis* sp.	*Lactococcus lactis* BGAL3-40	100	HE646382
12		**LB48**	*Lactococcus lactis* sp.	*Lactococcus lactis* subsp. *lactis* G50	100	CP025500
13		**LB18**	*Enterococcus* sp.	*Enterococcus faecium* PH-1	99	AY723748
14	C	**LC129**	*Lactobacillus* sp.	*Lactobacillus pentosus* SY124	100	LC041124
15	F	**LF27**	*Staphylococcus* sp.	*Staphylococcus piscifermentans* NCTC13836	99	LT906447
16	G	**LG230**	*Staphylococcus* sp.	*Staphylococcus pasteuri* H23	100	KU922334
17		**LG238**	*Staphylococcus* sp.	*Staphylococcus carnosus* HSP-S16	99	MG669651
18		**LG46**	*Staphylococcus* sp.	*Staphylococcus hominis* BC26	99	KF254627
19		**LG532**	*Staphylococcus* sp.	*Staphylococcus hominis* SubaKolSh24	99	JX625996
20	H	**LH510**	*Bacillus cereus* group	*Bacillus cereus* M13	99	CP016360
21		**LH139**	*Bacillus cereus* group	*Bacillus thuringiensis* YJB4	99	KU291379
22		**LH2134**	*Bacillus cereus* group	*Bacillus thuringiensis* DItb1006-3	100	KT835652
23		**LH2241**	*Bacillus cereus* group	*Bacillus thuringiensis* SDY-3	99	JX015365
24	I	**LI140**	*Bacillus cereus* group	*Bacillus thuringiensis* serovar *galleriae* HD-29	99	CP010089
25		**LI324**	*Bacillus cereus* group	*Bacillus thuringiensis* YBT-1518	99	CP005935
26		**LI915**	*Bacillus cereus* group	*Bacillus thuringiensis*VKK-LO	100	KT714050
27		**LI813**	*Bacillus cereus* group	*Bacillus thuringiensis* c25	100	CP022345

koami and ounago were prepared from shrimp (*Mysis* spp.) and a small unidentified fish in Japan contained one strain for *B. cereus* and *B. sphaericus*, four strains for *B. megaterium*, and one strain for *Penicillium notatum*. Particularly, *B. thuringiensis* was not detected, neither were yeasts, fungi, and obligate anaerobic bacteria [43]. However, in this study, the isolates that were identified were mainly strains of *B. thuringiensis*; therefore, our study is the first to report that strains of *B. thuringiensis* are distributed in Vietnamese fermented fish products.

The genetic analysis revealed 14 LAB, seven *Bacillus thuringiensis*, one *B. cereus*, and five *Staphylococcus* spp. strains. The results indicated that species of LAB predominated as the microflora for fermenting raw fish under high salt conditions. This could explain why LAB play an important role in preserving and processing a variety of fermented foods. Lactic acid fermentation would help to prevent the growth of foodborne microorganisms. Acid produced by the fermentation could also change the flavor of the raw materials and improve the nutritional values [44].

The presence of *Bacillus* species, spore-forming bacteria in the completely fermented products, reflected the strong resistant nature of these bacterial species. *Bacillus* spp. might be detected only in fermented fish products of Vietnam as a tropical country. While *Bacillus* are warmth-loving bacteria with an optimum growth temperature of 30–45°C, some species can even grow at temperatures of 65°C [45]. Moreover, some previous studies revealed that

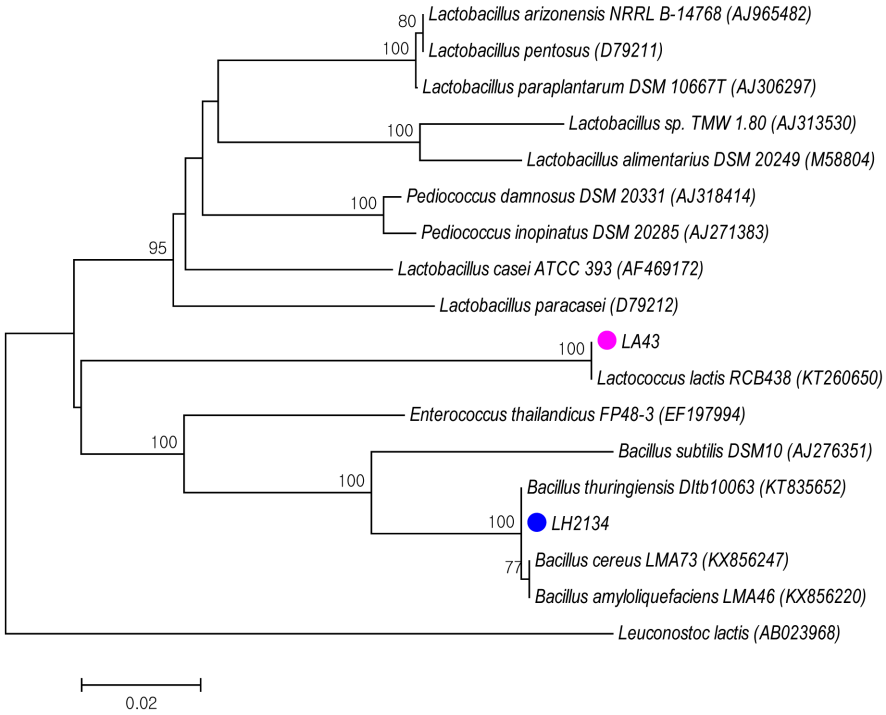

Fig. 2. Neighbor-joining tree based on 16S rRNA gene sequences of representative strains and the reference strains. Bootstrap values based on 100 replications are given at the nodes.

fermented fish supernatants often contained large amounts of microorganisms, where millions of cells could be found in 1 ml of the sauce extracts. The bacteria found in a nine-month-old fish sauce included LAB, *Micrococcus*, *Spirilum*, *Proteus*, *Leuconostoc*, *Clostridium*, and mold, and approximately 70% of the bacterial isolates were halophiles of *Bacillus* types [46].

Despite *B. thuringiensis* being a unique bacterium in that it shared common traits with a number of chemical compounds that were used commercially to control insects, it is important for agriculture and public health. Importantly, *B. thuringiensis* is safe for humans and is the most widely used environmentally compatible biopesticide worldwide [47]. Therefore, the discovery of *B. thuringiensis* strains in this study may be of interest for isolating GABA-producing strains. The appearance of *Staphylococcus* spp. was accordant with the finding of Tanasupawat and ten new coagulase-negative *Staphylococci* strains isolated from fermented fish in Thailand [48]. However, the presence of LAB strains, *B. thuringiensis* and *L. pentosus*, which could be tolerant at high-salt environments (15%) in the complete fermentation of fish sauces, suggests that these strains could play an active role in the fermentation process of fish sauces in Vietnam. Our result was also strongly supported by a previous study that reported *L. pentosus* strain S1 isolated from a Malaysian fermented fish product known as pekasam, which possessed probiotic potential and was safe for human consumption [49].

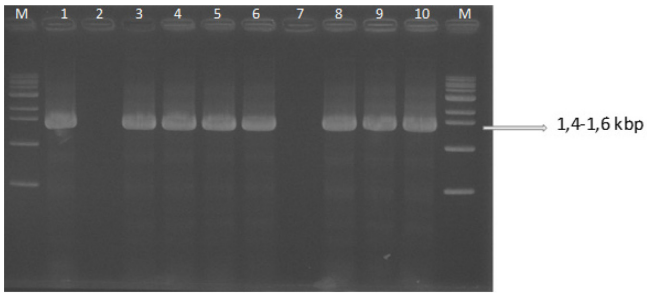

Fig. 3. PCR amplification of the *cry* gene from isolates performed with the primer pair K3/K5.
Lane 1: (+) control *Bacillus thuringiensis* KCTC 1094; lane 2: (-) control *Bacillus cereus* KCCM 1034; Lane 3–10: isolates D2134, E2241, J139, A140, G510, C915, B324, and C813.

As shown in Table 5, it was interesting that the GABA yields by strains isolated from Korean fermented fish products were higher than those of strains from Vietnam. The best producer of GABA was found in shrimp paste from Korea, but not from fish sauce, which has been the

Table 4. Differentiation of *B. cereus* and *B. thuringiensis* strains using 16S rRNA gene and specific-gene targeted PCR primers.

Isolate name	16s rRNA gene	*gyrB* gene			*cry* gene	*entFM* gene		*bceT* gene
	27F/1492R	BC1/BC2r	BT1/BT2r	BCRevnew/BCFw1	K3/K5	ENTA/ENTB	EntFM-F/EntFM-R	ETR/ETF
LH2134	*Bacilllus* sp.	+	+	+	+	-	-	-
LH2241	*Bacilllus* sp.	+	+	+	+	-	-	+
LH139	*Bacilllus* sp.	+	+	+	+	-	-	-
LI140	*Bacilllus* sp.	+	+	+	+	-	-	-
LH510	*Bacilllus* sp.	+	-	-	-	-	-	+
LI915	*Bacilllus* sp.	+	+	+	+	-	-	-
LI324	*Bacilllus* sp.	+	+	+	+	-	-	-
LI813	*Bacilllus* sp.	+	+	+	+	-	-	-

more popular food item in Korea. This study was the first to reveal that the strain *L. lactis* LA43 produced GABA at the highest amount (454 mM) among those strains isolated from the shrimp paste (jeotgal) from Korea.

The fact that fermented fish products in Asian countries may contain 20 and up to 30% NaCl suggests that they could contain a distinct halophilic microflora. The detection of *L. pentosus* with a high tolerance to NaCl (15%) in this study suggested it was a new moderate halophilic LAB, which could not be isolated from the population of halophilic microorganisms in ikashiokara, a fermented fish product containing 20% NaCl [43]. Furthermore, in this study, we have found that among the GABA-producing strains, the strain *B. thuringiensis* LH2134 isolated from *nuoc mam* of the Barona brand from Vietnam produced GABA at a concentration of 366 mM, which was higher than the amount of GABA produced by *Weissella hellenica* SB105 strain isolated from ika-kurozukuri salted squid with ink and liver in Japan in a previous study [42]. The *W. hellenica* SB105 strain produced GABA at the highest concentration of 7.69 mg/ml (approximately 74.57 mM) among the LAB strains isolated from traditional fermented foods of Ishikawa Prefecture, Japan. However, in a previous study, the authors were unable to detect any strains that

Table 5. Comparison between GABA-producing strains isolated from the fermented fish products in Vietnam and Korea.

Fermented fish products	Ingredients	Number of samples	Species identification (number of isolates)	GABA production yield (mM)	
				Vietnam samples	Korea samples
Fish sauces	Anchovy, sand lance (75–85%), salt, water, with or without preservatives, stabilizers, flavor, degrees of protein (10–40%)	29	*Bacillus thuringiensis* (4) *Lactococcus Lactis* (2) *Enterococcus faecium* (1) *Lactobacillus pentosus* (1) *Staphylococcus pasteuri* (1) *Staphylococcus piscifermentans* (1) *Staphylococcus hominis* (1) *Bacillus cereus* (1)	(10.62–366.02)	(245.19–434.75)
Shrimp pastes	Small shrimp, salt, spices	7	*Bacillus thuringiensis* (1) *Bacillus thuringiensis* (1) *Lactococcus Lactis* (10)	(5.4–34.77)	(44.95–454.18)
Anchovy pastes	Anchovy , small mackerel, salt, garlic, chili, sugar	5	*Bacillus thuringiensis* (1) *Staphylococcus hominis* (1) *Staphylococcus carnosus* (1)	(18.48–64.82)	ND
	Total	41	27	(5.4–366.02)	(44.95–454.18)

ND; not detected

Table 6. GABA production under glutamate-reinforced condition by *Lactococcus lactis* LA43.

Medium	Nutrients	pH	Temperature (°C)	Incubation time (h)	Condition	References	Log CFU/ml	GABA production mM	GABA conversion yield (%)
A	MRS + 500 mM MSG	6.8	30	72	Anearobic	This study	12.6 ± 0.13	454.17	90.83
B	MRS + 700 mM MSG	5	30	72	Anearobic	This study	10.6 ± 0.04	684.54	97.79
C	MRS + 700 mM MSG + 2% Maltose + 3% Tryptone	5	30	72	Anearobic	This study	12.2 ± 0.15	695.97	99.42
D	MRS + 400 mM MSG + 3.5% Glucose	5	32	48	Anearobic	[18]	9.4 ± 0.13	372.38	93.1

produced GABA from ishiru (fish sauce) although it was sampled for experiments. GABA production from fermented shrimp paste and fish sauce was also previously reported [50] for some of the LAB species (not shown in detail) with maximum GABA levels of 0.011 and 0.106 mM, respectively. These amounts were very small in comparison with our study. In comparison with the GABA produced by the best producer *L. paracasei* NFRI 7415, which was isolated from a Japanese traditional fermented fish (funa-sushi), at a concentration of 302 mM, the strain *L. lactis* LA43 produced GABA at 454 mM higher concentration, although it was cultured under non-optimal cultivation conditions, hence *L. lactis* LA43 could be considered a potential GABA producer under optimal conditions.

GABA Production on Glutamate-Reinforced Medium

After the identification and quantitative assay for the highest GABA producer, *L. lactis* LA43 was further examined for optimum GABA production under glutamate-reinforced conditions. GABA yields are shown in the Table 6. The optimal cultivation for GABA accumulation of *L. lactis* LA43 was determined in MRS supplemented with 700 mM MSG, 2% maltose, and 3% tryptone at an initial pH of 5.0 and incubation temperature of 30°C for 72 h, resulting in the production of 696 mM GABA. It revealed also that supplements of carbon sources by maltose and tryptone contributed to cell growth, and the GABA production increased from 685 to 696 mM. Conditions such as medium composition and additives might significantly affect microbial GABA production. Most previous studies on the optimization of GABA production by LAB reported yeast extract as an optimal nitrogen source. However, it should be noted that the mixed nitrogen sources of tryptone and yeast extract have occasionally been reported to be optimal nitrogen sources for GABA production by LAB [51]. Diverse carbon sources have been previously optimized according to GABA-producing LAB strains, such as 1% glucose for *L. buchneri* MS, 4% sucrose for *L. sakei* B2-16, and 3% sucrose for *L. brevis* 340G [19]. Maltose, as an optimal carbon source, and tryptone, as an ideal nitrogen source, in our study were determined to be the best nutrient supply for GABA production by *L. lactis* LA43,

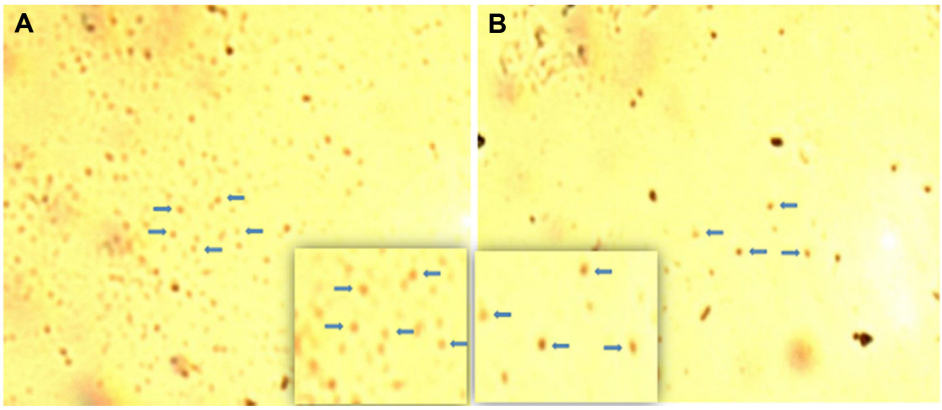

Fig. 4. Microscopy photographs of crystal proteins (δ-endotoxin): *B. thuringiensis* KCTC 1094 as a positive control (**A**), and *B. thuringiensis* LH2134 from fish sauce Barona of Vietnam (**B**).
The cells with δ-endotoxin stained were shown clearly at the small boxes.

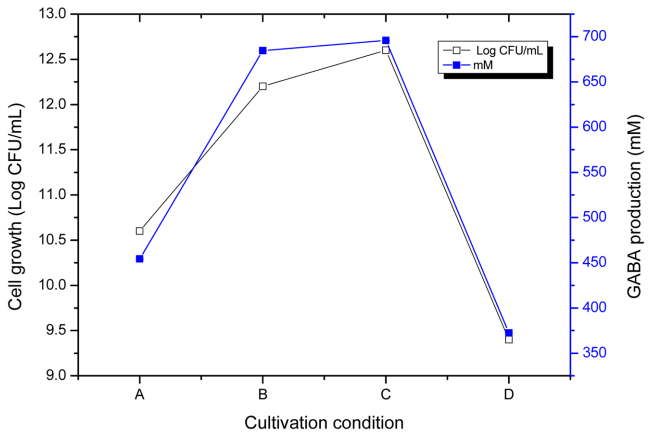

Fig. 5. Growth and GABA production of *L. lactis* LA43 in optimum culture conditions.
(**A**) MRS with 500 mM MSG; (**B**) MRS with 700 mM MSG; (**C**) MRS with 700 mM MRS, 2% maltose, and 3% tryptone; (**D**) MRS with 400 mM MSG and 3.5% glucose.

which was in agreement with a previous study reporting 2% maltose and 3% tryptone for the optimum GABA production by *L. brevis* HYE1 [51].

Furthermore, another previous study reported that when the glutamate concentration in the culture medium was in excess of 500 mM, glutamate would inhibited the GABA production of the strain *L. paracasei* NFRI 7415, which was isolated from a Japanese traditional fermented fish (funa-sushi) at a maximum concentration of 302 mM [15]. However, in our study, when the medium contained 700 mM MSG, there was a higher accumulation of GABA at 685 mM in comparison with medium that contained 500 mM resulting in GABA yields of 454 mM, suggesting that the glutamate concentration in the medium was essential for GABA accumulation. Additionally, the optimum culture conditions for GABA production by *L. buchneri* MS from kimchi has been previously reported to be MRS broth containing 5% MSG, 1% NaCl, and 1% glucose, at an initial pH of 5.0 and incubation temperature of 30°C for 36 h. Under these conditions, *L. buchneri* MS produced GABA at a concentration of 251 mM with a 94% GABA conversion rate. In this study, *L. lactis* LA43 produced GABA at a concentration of 454 mM, and reached a conversion rate up to 99% in the optimum medium containing 700 mM MSG, 2% maltose, and 3% tryptone. As the result, the strain *L. lactis* LA43 could be considered as a potential candidate for the optimum production of GABA under the optimal conditions.

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Conflict of Interest

The authors have no financial conflict of interest to declare.

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