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ORIGINAL ARTICLE

Isolation and Identification of γ-Aminobutyric acid (GABA)-producing Lactic Acid Bacteria from Kimchi

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Abstract Presumptive lactic acid bacteria (LAB) were isolated from 20 kimchi samples (total of 230 isolates) and screened for their capacity to synthesize γ-aminobutyric acid (GABA). Only 68 isolates (ca. 30%) showed this activity and were identified by a polyphasic approach consisting of morphological characteristics, catalase and biochemical tests, and species-specific polymerase chain reaction and 16S rRNA gene sequence analyses. Five species were found, including *Lactobacillus plantarum* (55 isolates), *Lactobacillus brevis* (six), *Leuconostoc mesenteroides* (four), *Leuconostoc lactis* (one), and *Weissella viridescens* (two). The 68 GABA-producing LAB isolates were isolated from only 11 among 20 kimchi samples indicating that they were not evenly distributed. This is the first report on the isolation of two species of *Leuconostoc* (*Le. mesenteroides* and *Le. lactis*) and one species of *Weissella* (*Ws. viridescens*) from kimchi with the capacity to synthesize GABA under *in vitro* conditions. Additionally, in previous screening results, *Le. lactis* and *Ws. viridescens* with the capacity to synthesize GABA isolated and identified from fermented food source were not observed.

Keywords γ-aminobutyric acid · kimchi · lactic acid bacteria · *Leuconostoc lactis* · *Weissella viridescens*

Introduction

Kimchi is a traditional Korean fermented vegetable food made of baechu (Chinese cabbage), radish, green onion, red pepper powder, garlic, ginger, and fermented seafood (jeotgal) (Kwon and Kim, 2007). Kimchi is traditionally made at home and served as a side dish. More than 1.5×10^6 tons of kimchi is consumed each year in South Korea. Kimchi was introduced to the global community during the Seoul Olympics in 1988, resulting in a sharp increase in kimchi export (Kim and Jhon, 2001). Kimchi has been added to the Codex Alimentarius in ref. (Codex, 2001), and the kimchi market continues to expand worldwide. Commercially produced kimchi is generally made through lactic acid fermentation of Korean napa cabbage at low temperatures (2–5°C), which ensures proper ripening and preservation. Generally, the process of kimchi fermentation is divided into four stages based on acidity; initial stage (acidity <0.2), immature stage (acidity 0.2–0.4), optimum-ripening stage (acidity 0.4–0.9), and over-ripening or rancid stage (acidity >0.9) (Codex, 2001; Cho et al., 2009).

Kimchi is low in calories, carbohydrates, and fat but contains various health-promoting components such as β-carotene, chlorophyll, vitamin C, and dietary fiber (Park et al., 1995). Additionally, kimchi has anti-mutagen (Oh et al., 2005), anti-oxidation (Yoo et al., 2005), and anti-diabetic effects (Shahidul and Choi, 2009), as well as inhibition of angiotensin-converting enzyme (Yoo et al., 2004) activities, which are thought to protect against disease. Furthermore, a high number of lactic acid bacteria (LAB) are produced in kimchi during lactic acid fermentation. Bacteria isolated from kimchi produce beneficial enzymes such as dextransucrase and alcohol/acetaldehyde dehydrogenase. Because of these beneficial biochemical and microbiological properties, kimchi was nominated as one of the world's healthiest foods in the 2006 issue of Health Magazine (http://www.health.com/).

γ-Aminobutyric acid (GABA) is synthesized by glutamate decarboxylase (GAD), a pyridoxal 5'phosphate-dependent enzyme that catalyzes the irreversible α-decarboxylation of L-glutamate into GABA. GAD is widely distributed among eukaryotes and prokaryotes (Thwe et al., 2011). GABA is a nonprotein amino acid that possesses well-known physiological functions such as neurotransmission, induction of hypotension,

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and diuretic and tranquilizing effects (Jakobs et al., 1993). Stimulation of immune cells (Oh and Oh, 2003) and treatments for sleeplessness, depression, autonomic disorders (Okada et al., 2000), and chronic alcohol-related symptoms (Oh et al., 2003) were also found to be related to GABA administration. Lastly, GABA is a strong secretagogue of insulin from the pancreas (Adeghate and Ponery, 2002) and may prevent diabetic conditions (Hagiwara et al., 2004). These various value-added benefits of GABA on human health have recently attracted increased attention in the food industry. Several GABA-enriched functional foods are currently manufactured such as tea leaves treated anaerobically, rice germ soaked in water, red mold rice, tempeh-like fermented soybeans, and dairy products such as yogurt, fermented milk products, and cheese (Thwe et al., 2011). Therefore, great efforts are focused on mass production of GABA and its utilization as a bioactive food ingredient in the modern food industry.

LAB are widely used in a variety of fermented foods, particularly for the manufacture of dairy and vegetable products with functional and probiotic properties (Leroy and de Vuyst, 2004). Screening of LAB based on their capacity to synthesize GABA may open new perspectives on the production of GABA-enriched dairy and vegetable products. Screening various types of LAB with GABA-producing ability from various fermented food sources is important for the food industry, because individual LAB have specific fermentation profiles, such as acid production and flavor formation ability. The quality of fermented foods, such as taste and flavor, depends on LAB fermentation profiles; thus consideration of such profiles is an important factor when LAB are used as starters during fermented food production (Komatsuzaki et al., 2005). More importantly, GABA-producing ability varies among LAB, although GAD is widely distributed in them, because the capacity to synthesize the highest levels of GABA is markedly strain-dependent (Siragusa et al., 2007). Therefore, more extensive screening of GABA-producing LAB isolates from various fermented food sources may yield higher probabilities to isolate individual LAB with higher GABA-producing ability and specific fermentation profiles. Up until now, GABA-producing LAB isolates have been screened less extensively from kimchi than from dairy fermented products.

The objectives of the present study were to extensively screen and identify GABA-producing LAB isolates from kimchi. GABA-producing LAB isolates have potential for commercial use as possible production starters for fermented foods, such as pickled vegetables and fermented meats and fish. Such isolates are also expected to enhance the development of functional fermented foods containing GABA.

Materials and Methods

Strains and growth conditions. A total of 1,100 bacterial colonies were isolated from kimchi. Among the isolates, 230 were randomly selected and screened representing each of different morphological groups. *Lactobacillus brevis* ATCC 8287, *Lactobacillus curvatus* ATCC 25601, *Lactobacillus pentosus* IFO 12011, *Lactobacillus plantarum* NCIMB 6105, *Lb. plantarum* ATCC 10012, *Lactobacillus sakei* subsp. *sakei* ATCC 15521, *Lactococcus lactis* subsp. *lactis* ATCC 19435, *Leuconostoc carnosum* ATCC 49367, *Leuconostoc lactis* ATCC 19256, *Leuconostoc mesenteroides* ATCC 10830, *Pediococcus pentosaceus* ATCC 33316, *Weissella confusa* ATCC 10881, *Weissella koreensis* KCCM 41516, *and Weissella viridescens* ATCC 12706 were used as reference strains. *Bacillus cereus* ATCC 13061 was used as a negative control strain. The LAB strains were grown in de Man Rogosa Sharpe (MRS) broth (Difco, Becton Dickinson Co., USA), and the non-LAB strain was grown in nutrient broth (Difco) at 37°C for 24 h.

Isolation of LAB from kimchi. Various types of homemade or commercially sold kimchi were collected from various sources. Kimchi samples KC-A–KC-L, KC-Q, and KC-T were collected from 14 different households, and kimchi samples KC-M–KC-P and KC-R and S were purchased at six different local markets in Ansung, Korea.

The LAB strains were isolated from kimchi samples by homogenization, ten-fold serial dilutions with saline solution, and plating on MRS agar containing 2% (w/v) $CaCO_3$. After a 2–3 day incubation at 37°C, 50–100 colonies were randomly picked from each sample and transferred to new MRS agar plates. After a 24 h incubation at 37°C, they were differentiated and counted based on morphology. At least 10 colonies from each kimchi sample, possibly with different morphologies, were isolated from the highest plate dilution. All isolates considered for further analyses were able to acidify the culture medium. Presumptive LAB colonies were selected, and each colony was purified by three consecutive single colony isolations (Lee and Lee, 2006; Shin et al., 2008). Purified colonies were grown in 5 mL MRS broth at 37°C for 24 h. Bacterial pellets were harvested and kept at −70°C after resuspension in 80% glycerol.

Preparation of cell extracts. A total of 230 selected isolates and 14 reference strains were grown in 25 mL MRS broth at 37°C for 48 h. After cultivation, the cells were centrifuged at 6,500 rpm for 5 min and washed three times with Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8). The cells were suspended in 0.5 mL TE buffer, and 1 g undersized (0.1 mm diameter) glass beads (Biospec Products Inc., USA) were added to the cell suspension. Cell extracts were obtained after the cells were disrupted three times at 42 rpm for 1 min using a Bead-beater (Biospec Products) (Hurley et al., 1987).

Screening of GABA-producing strains using thin layer chromatography (TLC). GABA-producing strains were screened by TLC (Cho et al., 2007). Cell extracts were incubated with 10 mg monosodium glutamate (MSG) at 37°C for 4 h. After incubation, culture supernatants were obtained by centrifuging at 10,000 rpm for 5 min and subjected to TLC and high performance liquid chromatography (HPLC) analyses. Four microliters of

supernatant was spotted on a silica TLC plate (Aluminum Sheets Silica gel 60 F_{254}, Merck, Germany). GABA separation by TLC was conducted using a solvent mixture (1-butanol:acetic acid: distilled water (3:2:1 v/v/v/)). GABA spots were detected after spraying 0.5% (w/v) ninhydrin and exposing the plates to a heat source for several minutes (Thwe et al., 2011).

Determination of GABA-producing ability using HPLC. The GABA produced by the GABA-producing LAB isolates was quantified by HPLC. Culture supernatants and GABA standard solutions were derivatized with phenylisothiocyanate (PITC) (Rossetti and Lombard, 1996). Aliquots of 500 μL of culture supernatant or GABA standard solution were dried under a vacuum using a speed-vacuum concentrator (RC 10.22, Jouan, France). The residue was dissolved in 100 μL ethanol-water-triethylamine (2:2:1 v/v/v/) and evaporated to dryness under vacuum. An aliquot of 150 μL ethanol-water-triethylamine-PITC (7:1:1:1 v/v/v/v/) was added to the residue and allowed to react for 20 min at room temperature to form phenylthiocarbamyl-GABA. Excess reagents were removed under vacuum. The dry residue was dissolved in 500 μL of mobile phase consisting of 80% solution A (1.4 mM sodium acetate, 0.1% triethylamine, and 6% acetonitrile) and 20% solution B (60% acetonitrile), filtered though a 0.45-μm filter, and subjected to HPLC analysis. The GABA analysis was performed using an HPLC system equipped with a Cosmosil 5C18-AR-II column (5 μm, 4.6 mm × 250 mm, Nacalai Tesque, Japan), a Waters 600E multisolvent delivery system (Waters, USA), and a UV-VIS Waters 486 tunable absorbance detector (Waters). 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. The retention time of GABA was 25.3 min. A standard curve was created using known concentrations (0.5, 1, 5, and 10%) of GABA after PITC derivatization as described above.

Identification of GABA-producing LAB isolates. A polyphasic approach consisting of morphological, biochemical, and molecular characterizations was applied to the GABA-producing bacterial isolates. Cell morphology was observed after Gram staining using an optical microscope (CHK2-F3-100, Olympus Co., Japan). Catalase activity was tested by placing a few drops of 3% H_2O_2 onto the colonies and observing formation of oxygen bubbles (Foster et al., 1997). The capacity to acidify the culture medium was tested using MRS agar containing $CaCO_3$.

Species-specific polymerase chain reaction (PCR) and 16S rRNA gene sequence analyses were performed to identify the GABA-producing bacterial isolates. Total DNA was extracted from the isolates using the Qiagen DNeasy Mini Kit (Valencia, USA) according to the manufacturer's instructions. The presence, concentration, and purity of total DNA in the prepared samples were detected by measuring absorbance at 260 and 280 nm using an UV-VIS spectrophotometer (Specord 200, Analytik Jena AG, Germany). Thirteen species-specific primer sets were used to distinguish 13 species of LAB in the PCR analysis (Table 1). A universal primer set was used for the 16S rRNA gene sequence analysis (Table 1). PCR was performed in 20 μL reaction mixtures containing 3 μL template DNA, 10× PCR buffer, 10 mM dNTPs, 5 μM each primer, and 2 U *Taq* DNA polymerase (Solgent Co., Korea). PCR amplification reactions were conducted using a PTC-100TM Programmable Thermal Controller (MJ Research Inc., USA). All PCR amplifications were performed a minimum of three times to validate the results. Amplification products were analyzed by separating 10 μL reaction mixtures on 1.5% (w/v) agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]), followed by staining and examination under UV light. Suitable molecular size markers were included on each gel. The quality of any DNA extract that failed to amplify in a specific reaction was examined by attempting amplification with a pair of universal primers designed to amplify a 16S rRNA gene region. Negative controls (without DNA) were included in all amplifications. Suitable controls such as buffer, media, PCR mixtures, and template DNA were employed to check any false-positive or false-negative reactions.

The consensus sequences of each PCR amplicon from the target genes were determined by bidirectional sequencing as described previously (Song et al., 2003). Sequencing data were analyzed by comparing the consensus sequences with GenBank sequences using the basic local alignment search tool (BLAST) (Benson et al., 2002). The isolates were analyzed by comparing the sequences against the relevant sequences of type strains retrieved from GenBank.

Results

Isolation of LAB from kimchi. Colonies were isolated from different kimchi samples after they were grown and formed a clear zone on MRS agar plates containing 2% (w/v) $CaCO_3$. The colonies were presumptively considered to be LAB according to their characteristics on MRS agar plates. Among them, 1,100 LAB colonies were selected from 20 kimchi samples and were subjected to three consecutive steps to obtain pure colonies. Total viable counts and numbers of colonies selected for each kimchi sample are listed in Table 2.

Screening of GABA-producing strains using TLC. Among the pure 1,100 LAB isolates, 230 isolates were randomly tested for GABA-producing ability on TLC plates. All positive control strains produced GABA when MSG was present, whereas no GABA was detected in the absence of MSG (Fig. 1). A negative control strain did not produce GABA even when MSG was present (Fig. 1). Some LAB isolates showed various degrees of GABA production ability (Fig. 1). GABA-producing LAB isolates for each kimchi sample are listed in Table 2. Among the 230 LAB isolates tested, 68 (30%) produced GABA spots on TLC plates.

Determination of GABA-producing ability using HPLC. GABA production of the 68 GABA-producing LAB isolates was

Table 1 Polymerase chain reaction (PCR) primers and amplification conditions used in the species-specific PCR and 16s rRNA gene analyses

Species	Genes	Primer names	Oligonucleotide sequences (5'→3')	Amplification conditions[a]			Products (bp)	References
				D	A	E		
Lactobacillus brevis	16S ribosomal RNA	LbrevF LbrevR	CCT GCA CTG ATT TTA ACA GGG CGG TGT GTA CAA GGC	94°C, 30 s	60°C, 1 m	72°C, 2 m	1400	This study
Lactobacillus curvatus	RNA polymerase, beta subunit	Lcur_rpoB2F Lcur_rpoB2R	GTA ACG TGC TAA AAC AGT ATC TGG CTA GTT ACG CTG TTT GTT	94°C, 30 s	60°C, 1 m	72°C, 2 m	197	This study
Lactobacillus pentosus	Recombinase A	LpeF LpeR	TCC GGT TTA CGC GGA ACA TTT CCT TGA TTT GTT CAG CAC GAC G	94°C, 30 s	60°C, 1 m	72°C, 2 m	398	Cho et al., 2009
Lactobacillus plantarum	Cadmium-manganese transport ATPase	LplF LplR	AAG GCC GTA GTC AGT CGT CTA TGG TCA ACA CAC GAA TAT CAG CCG G	94°C, 30 s	60°C, 1 m	72°C, 2 m	313	Cho et al., 2009
Lactobacillus sakei	Putative lipase-esterase	LsaF LsaR	TTA AAG GCA TTA GCT GAA GGC TGT CGC CAT GGT GGC CAC GAT	94°C, 30 s	60°C, 1 m	72°C, 2 m	274	Cho et al., 2009
Lactococcus lactis	Tributyrin esterase	LlaF LlaR	TTG CAT GGA ATG AGC GGA AAC TAT CCT CCC ATT GAT AAA CCA GCG	94°C, 30 s	60°C, 1 m	72°C, 2 m	248	Cho et al., 2009
Leuconostoc carnosum	DNA-dependant RNA polymerase	LcaF LcaR	GAT TGT TGC TGC AGG TAT CGA GAA G TCC AAC GTA TCT GTG ACA GAC AAT AGC	94°C, 30 s	61°C, 1 m	72°C, 2 m	506	Cho et al., 2009
Leuconostoc lactis	RNA polymerase alpha chain	Lac_rpoAF Lac_rpoAR	TGC AGA CGT TGA AGT TTT GAA C CAC CTA GGC TCA AAG CAT CAC TA	95°C, 30 s	60°C, 1 m	72°C, 2 m	302	This study
Leuconostoc mesenteroides	Alcohol-acetaldehyde dehydrogenase	LmeF LmeR	GAG CCG TTA TTC AAG CAC CAA TC CCT GCG CCT TGA TAG TTT AAC AAG	94°C, 30 s	60°C, 1 m	72°C, 2 m	358	Cho et al., 2009
Pediococcus pentosaceus	Esterase-lipase	PpeF PpeR	CTT TGT GCC CGG TGG ATC CT AAA GGC TGC AAT GTA GTT GAT GCT A	94°C, 30 s	60°C, 1 m	72°C, 2 m	330	Cho et al., 2009
Weissella confusa	D-alanine: D-alanine ligase	WcoF WcoR	AAG CGT ATC TTG AAC CAA GCT GG GCA AAG CGT CCT TAA CAC CAG C	94°C, 30 s	60°C, 1 m	72°C, 2 m	208	Torriani et al., 2001
Weissella koreensis	Maltose phosphorylase	WkoR WkoF	TCT GCC GAA GCT TGA CCG G GCC GAA TTA AGT AGT GTA AAG TCA AAT G	94°C, 30 s	60°C, 1 m	72°C, 2 m	154	Cho et al., 2009
Weissella viridescens	DNA gyrase subunit B	Vir_gyrBF Vir_gyrBR	GAT AGT ATC CAT GAA GTG AGC TTA CGA GCT TTA TTC GCC AAC	95°C, 30 s	61°C, 1 m	72°C, 2 m	710	This study
universal primer	16S ribosomal RNA	27F 1492R	AGA GTT TGA TCM TGG CTC AG GGT TAC CTT GTT ACG ACT T	95°C, 30 s	55°C, 1 m	72°C, 2 m	1466	Thwe et al., 2011

[a] All PCR protocols used an initial denaturation step at 95 ℃ for 10 min, followed by 35 cycles at the temperatures and times indicated (D, denaturation; A, annealing; E, extension). A final extension step (10 min at 72 ℃) was also included.

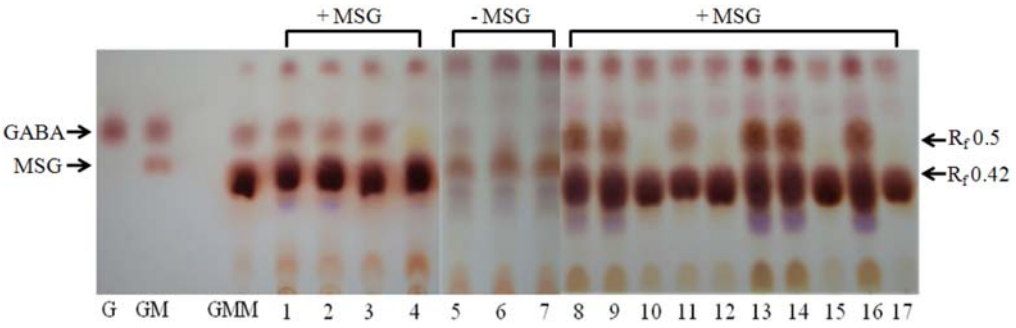

Fig. 1 Verification of γ-aminobutyric acid (GABA) on a thin layer chromatography (TLC) plate produced after decarboxylation reactions of the tested strains with or without MSG added as a substrate. Lane G, GABA standard; lane GM, GABA and MSG standards; lane GMM, GABA and MSG standards in MRS broth; lane 1, *Lb. plantarum* NCIMB 6105 (positive control) with MSG; lane 2, *Lb. plantarum* ATCC 10012 (positive control) with MSG; lane 3, *Lc. lactis* subsp. *lactis* ATCC 19435 (positive control) with MSG; lane 4, *Ba. cereus* ATCC 13061 (negative control) with MSG; lane 5, *Lb. plantarum* NCIMB 6105 without MSG; lane 6, *Lb. plantarum* ATCC 10012 without MSG; lane 7, *Lc. lactis* subsp. *lactis* ATCC 19435 without MSG; lanes 8–17, LAB isolates with MSG.

Table 2 Screening of γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB) isolates from kimchi

Sample numbers	Sources	Total viable counts (CFU/g sample)	Numbers of tested isolates/ numbers of selected isolates	Numbers of GABA-producing isolates (%)[a]
KC-R	Market E	2.9×10^7	28/40	27 (96)
KC-Q	House M	1.9×10^6	10/48	10 (100)
KC-S	Market F	2.1×10^7	11/38	10 (91)
KC-L	House L	5.5×10^6	10/34	8 (80)
KC-I	House I	6.4×10^7	10/71	6 (60)
KC-K	House K	6.8×10^7	7/79	2 (29)
KC-D	House D	3.8×10^5	15/83	1 (7)
KC-F	House F	6.2×10^5	10/70	1 (10)
KC-H	House H	7.2×10^6	20/93	1 (5)
KC-J	House J	4.3×10^6	10/70	1 (10)
KC-M	Market A	6.2×10^5	6/11	1 (17)
KC-A	House A	3.4×10^5	10/89	0 (0)
KC-B	House B	4.2×10^5	11/88	0 (0)
KC-C	House C	3.0×10^4	17/48	0 (0)
KC-E	House E	4.5×10^5	8/33	0 (0)
KC-G	House G	5.6×10^5	12/50	0 (0)
KC-N	Market B	5.9×10^5	10/34	0 (0)
KC-O	Market C	3.4×10^7	8/50	0 (0)
KC-P	Market D	5.8×10^7	6/49	0 (0)
KC-T	House N	1.4×10^6	11/22	0 (0)
Total			230/1,100	68 (30)

[a] (number of GABA-producing isolates/number of tested isolates) × 100.

screened on TLC plates and quantitatively determined using HPLC. The GABA standard curves showed a linear relationship ($R^2 = 0.9979$) between peak areas and GABA concentrations range from 0.5 to 10% GABA. HPLC chromatograms of the GABA standard solution and GABA produced after decarboxylation reactions by all the tested strains were obtained (data not shown). All 68 GABA-producing LAB isolates were divided into four groups based on their GABA producing ability (Table 3). The average GABA concentrations produced in each group were 44.67, 35.24, 25.36, and 17.78 mg/g cell extract. All four GABA-producing LAB isolates (isolates KC-D13, KC-Q1, KC-R1, and KC-R36) within group 1 produced higher concentrations of GABA (43.01–48.65 mg/g cell extract) than that of the reference strain (41.70 mg/g cell extract). However, 64 other GABA-producing LAB isolates showed GABA concentrations lower than that of the reference strain.

Identification of GABA-producing LAB isolates. All 68 GABA-producing isolates were Gram-positive, catalase-negative, acidifying, and rod-shaped bacteria. They were presumptively considered to be LAB based on their morphological and biochemical characteristics. Species-specific PCR and 16S rRNA gene sequence analyses were performed to identify all 68 isolates

Table 3 Grouping of γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB) isolates from kimchi based on concentrations of GABA produced by each isolate

Groups	Isolate names	Numbers of GABA-producing isolates within each group	Concentration ranges of GABA produced for each group (mg GABA/g cell extract)	Average concentrations of GABA produced within each group (mg GABA/g cell extract)
reference strain		*Lb. plantarum* NCIMB 6105	41.70	-
group 1	KC-Q1, KC-R1, 9, 36	4	40.0–49.9	44.67±2.67[a]
group 2	KC-D13, KC-I19, 39, 55, 60 KC-J23, KC-L10, 30, 37,40 KC-Q3, 8, 10 KC-R12, 16, 23, 24, 33, 34 KC-S1, 6, 7, 13, 22, 25, 33, 38	27	30.0–39.9	35.24±2.73
group 3	KC-F76, KC-I10, KC-K71 KC-L2, 5, 18, 26, KC-M3 KC-Q13, 16, 22, 27, 34, 37, KC-R2, 4, 5, 6, 7, 8, 10, 11, 13, 14, 25, 20, 26, 27, 29, 32, KC-S4, 11	32	20.0–29.9	25.36±2.63
group 4	KC-H23, KC-I67, KC-K6, KC-R22, 28	5	10.0–19.9	17.78±2.20

[a] Mean ± standard deviation among isolates within a group.

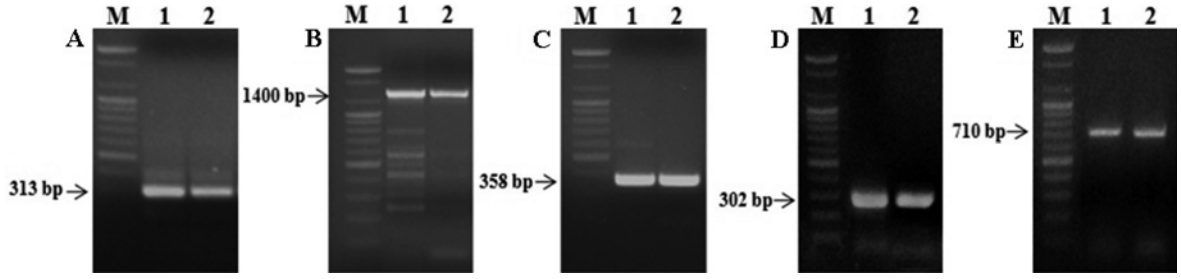

Fig. 2 Representative banding patterns of species-specific polymerase chain reaction (PCR) reactions for *Lb. plantarum, Lb. brevis, Le. mesenteroides, Le. lactis,* and *Ws. viridescens*. (A) A 313 bp PCR fragment was amplified using the primer pair LplF/LplR from *Lb. plantarum* NCIMB 6105 (lane 1) and lactic acid bacteria (LAB) isolate KC-H23 (lane 2). (B) A 1400 bp PCR fragment was amplified using the primer pair LbreF/LbreR from *Lb. brevis* ATCC 8287 (lane 1) and LAB isolate KC-D13 (lane 2). (C) A 358 bp PCR fragment was amplified using the primer pair LmeF/LmeR from *Le. mesenteroides* ATCC 10830 (lane 1) and LAB isolate KC-I39 (lane 2). (D) A 302 bp PCR fragment was amplified using the primer pair Lac_rpoAF/Lac_rpoAR from *Le. lactis* ATCC 19256 (lane 1) and LAB isolate KC-J6 (lane 2). (E) A 710 bp PCR fragment was amplified using the primer pair Vir_gyrBF/Vir_gyrBR from *Ws. viridescens* ATCC 12706 (lane 1) and LAB isolate KC-J13 (lane 2). A 100 bp DNA ladder was used as the DNA molecular weight marker (lane M for (A–E).

at the species level. Using three DNA templates independently prepared from each tested strain, PCR amplification results of triplicate experiments were 100% reproducible for each target gene (data not shown).

The first molecular approach used relied on PCR amplification of the 13 species-specific target genes. To identify all 68 isolates at the species level, each isolate was amplified using the 13 species-specific PCR primer sets. Only one species-specific PCR amplicon was generated from each isolate, and five species-specific PCR primer sets were able to species-specifically amplify each target gene from all 68 isolates. Representative PCR amplicons are shown in Fig. 2. All five species-specific PCR primer sets amplified the expected PCR amplicon sizes from their respective target genes of the five species: 313 bp amplicon for *Lb. plantarum*, 1,400 bp amplicon for *Lb. brevis*, 358 bp amplicon for *Le. mesenteroides*, 302 bp amplicon for *Le. lactis*, and 710 bp amplicon for *Ws. viridescens*. All 68 isolates identified by species-specific PCR assays were confirmed by sequencing of the species-specific PCR amplicons to provide for sequence-specific identification of the amplified genes. Matching the sequences amplified from each isolate available on GenBank showed that all were identical to those of the five respective species-specific target genes. All 68 isolates were identified by the species-specific PCR results (Table 4). Five species isolated were: *Lb. plantarum* (55 isolates), *Lb. brevis* (six), *Le. mesenteroides* (four), *Le. lactis* (one), and *Ws. viridescens* (two).

The second molecular approach used relied on PCR amplification of the 16S rRNA genes to verify the species-specific PCR results described above. All 68 isolates were further identified by partial 16S rRNA gene sequencing (Table 4). PCR fragments of 1,466 bp were obtained for all 68 isolates, and the fragments represented 98.8% of the 16S rRNA gene sequences. All 68 isolates were

Table 4 Identification of γ-aminobutyric acid (GABA)-producing lactic acid bacteria (LAB) isolates from kimchi

Species names	Isolate names	Numbers of isolates identified by	
		Species-specific PCR	16S rRNA gene sequencing
Lactobacillus plantarum	KC-F76, KC-H23, KC-J23, KC-K71 KC-L2, 5, 10, 18, 26, 30, 37, 40 KC-Q1, 3, 8, 10, 13, 16, 22, 27, 34, 37 KC-R1-2, 4-16, 20, 22-24, 26-29, 32-34, 36 KC-S1, 4, 11, 22, 33, 38	55	55
Lactobacillus brevis	KC-D13, KC-I 10, 19, 55, 60, 67	6	6
Leuconostoc mesenteroides	KC-I39, KC-K6, KC-M3, KC-S25	4	4
Leuconostoc lactis	KC-S6	1	1
Weissella viridescens	KC-S7, 13	2	2
Total	68	68	68

subjected to comparative 16S rRNA gene sequencing analysis using the BLAST search program. The 16S rRNA gene sequencing of 55 isolates identified *Lb. plantarum* as the first choice with 99% sequence identities to the type strain (*Lb. plantarum* AF1) sequence (accession no. FJ386491.1). The 16S rRNA gene sequencing of six isolates identified *Lb. brevis* as the first choice with 99% sequence identities to the type strain (*Lb. brevis* NRIC 0138) sequence (accession no. AB362619.1). The 16S rRNA gene sequencing of four isolates identified *Le. mesenteroides* as the first choice with 99% sequence identity to the type strain (*Le. mesenteroides* B8) sequence (accession no. HQ450738.1). The 16S rRNA gene sequencing of one isolate identified as *Le. lactis* was the first choice with 99% sequence identity to the type strain (*Le. lactis* LMG 22650) sequence (accession no. AJ970316.1). The 16S rRNA gene sequencing of two isolates identified as *Ws. viridescens* was the first choice with 100% sequence identities to the type strain (*Ws. viridescens* NBRC 3949) sequence (accession no. AB680180.1).

The two different molecular approaches used in this study resulted in identical identification patterns (Table 4). Among the 68 GABA-producing LAB isolates from kimchi, 55 isolates (80%) were identified as *Lb. plantarum*, six isolates (9%) as *Lb. brevis*, four isolates (6%) as *Le. mesenteroides*, one isolate (2%) as *Le. lactis*, and two isolates (3%) as *Ws. viridescens*.

Discussion

The screening of LAB with the capacity to synthesize GABA has been previously conducted for fermented dairy products and primary starters. Previous reports have shown GABA synthesis by primary starters such as *Lc. lactis*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* for commercial yogurt or cheese (Hayakawa et al., 1997; Nomura et al., 1998). *Lactobacillus buchneri* OPM-1 was more recently isolated from naturally aged cheese and also showed high GABA-producing capacity (Park and Oh, 2006). Most recently, an extensive screening experiment for a set of LAB isolates from 22 Italian cheese varieties identified 12 species of 61 GABA-producing LAB isolates: *Lb. plantarum* (17 isolates), *Lactobacillus paracasei* (16), *Lactobacillus casei* (five), *Lb. brevis* (three), *Lb. delbrueckii* subsp. *bulgaricus* (two), *Lactobacillus pseudomesenteroides* (two), *Lactobacillus rhamnosus* (two), *St. thermophilus* (six), *Lc. lactis* (two), *Le. mesenteroides* (two), *Enterococcus durans* (one), and *Weissella cibaria* (one) (Siragusa et al., 2007). Among the 12 species of LAB isolates, *Lb. brevis* PM17, *Lb. delbrueckii* subsp. *bulgaricus* PR1, *Lb. paracasei* PF6, *Lb. plantarum* C48, and *Lc. lactis* PU1, were the best GABA-producing strains during fermentation of reconstituted skimmed milk. By screening 13 species of previously reported GABA-producing LAB isolates were identified from dairy sources. Most of the GABA-producing LAB species isolated from dairy sources were lactobacilli.

LAB with the capacity to produce GABA have been previously screened from kimchi. The following seven GABA-producing LAB species have been isolated from kimchi. All GABA-producing LAB species except one belonged to lactobacilli. *Lb. brevis* IFO 12005 showing GABA-producing capacity was originally isolated from kimchi (Ueno et al., 1997). This strain was later used to produce GABA from *kome shochu* (rice alcohol) distillery lees (Yokoyama et al., 2002). *Lb. brevis* OPK-3, with GABA-producing capacity, has been isolated from kimchi by Park and Oh (2007). GABA-producing *Lb. brevis* GABA 100 was isolated from kimchi and used to ferment black raspberry juice by Kim et al. (2009). Cho et al. (2007) reported that the GABA-producing ability of *Lb. buchneri* MS isolated from kimchi was higher than that of *Lb. brevis* IFO 12005, and that culture extracts of *Lb. buchneri* MS protect neurons against neurotoxicant-induced cell death. *Lb. brevis*, *Lb. buchneri*, *Lb. curvatus*, and *Lb. sakei* with GABA-producing ability were recently isolated from commercial sour kimchi by Cho et al. (2011). *Lb. buchneri* has been selected as a starter for producing sour kimchi with enriched GABA content, because it showed the highest GABA production. Liu et al. (2011) recently reported that *Lb. plantarum* NTU 102, isolated from homemade Korean-style cabbage pickles, has GABA and angiotensin I-converting enzyme inhibitory activities. Hayakawa et al.(1997)isolated *Lactobacillus hilgardii* K-3 from

kimchi, and this strain has been used to manufacture commercial natural GABA formulations. Kook (2010) also isolated *Lb. sakei* B2-16 from kimchi and obtained an optimal medium composition for the mass production of GABA. GABA-producing *Lc. lactis* subsp. *Lactis* has been isolated from kimchi, and its GABA-producing ability was optimized using brown rice juice, germinated soybean juice, and enzymolyzed skim milk (33:58:9 v/v/v) as medium compositions (Lu et al., 2008).

In the present study, an extensive screening experiment for a set of 230 LAB isolates randomly selected from 20 kimchi samples identified five species of 68 GABA-producing LAB isolates: *Lb. plantarum* (55 isolates), *Lb. brevis* (six), *Le. mesenteroides* (four), *Le. lactis* (one), and *Ws. viridescens* (two). A small number (ca. 30%) of the total 230 isolates showed the capacity to synthesize GABA under *in vitro* conditions. The 68 GABA-producing LAB isolates were not evenly distributed among the 20 tested kimchi samples, because they were isolated from only 11 kimchi samples. Among the 68 GABA-producing LAB isolates, *Lb. brevis* KC-D13, *Lb. plantarum* KC-Q1, KC-R1, and KC-R36 were the best GABA-producing strains.

This is the first report on the isolation of two species of *Leuconostoc* (*Le. mesenteroides* and *Le. lactis*) and one species of *Weissella* (*Ws. viridescens*) with GABA-synthesizing capacity from kimchi under *in vitro* conditions. Additionally, previous screening results did not show *Le. lactis* and *Ws. viridescens* with the capacity to synthesize GABA that were isolated and identified from fermented food sources. Although many GABA-producing LAB strains have been isolated and identified, further isolation and characterization research is needed, because screening various types of GABA-producing LAB is important for the food industry. In further screenings, the isolation sources should be expanded to as many fermented foods as possible to obtain GABA-producing LAB strains. This will lead to wider applications and higher starter culture flexibility.

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