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# Potential of γ-Aminobutyric Acid-Producing *Leuconostoc mesenteroides* Strains Isolated from Kimchi as a Starter for High-γ-Aminobutyric Acid Kimchi Fermentation

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**ABSTRACT:** γ-Aminobutyric acid (GABA)-producing *Leuconostoc mesenteroides* K1501 and K1627, isolated from kimchi, exhibited the highest GABA production in 1% monosodium glutamic acid. Both strains showed high survival rates of approximately 87% in artificial gastric juice (pH 3.0) and >80% in 0.1% artificial bile salt fluid. The survival rate was approximately 28% in 0.3% artificial bile salt fluid and 0% in 0.5% artificial bile salts. Both strains showed excellent adhesion to intestinal epithelial cells (>99%). Furthermore, it was observed that growth was not inhibited at 2% salt concentration; however, it was slightly retarded at salt concentrations of 3% and 4%. Moreover, *L. mesenteroides* K1501 and K1627 inhibited the growth of certain species of *Lactobacillus*, whose presence in kimchi fermentation is undesirable. Therefore, *L. mesenteroides* K1501 and K1627 have the potential to be used as starter organisms for functional GABA-rich kimchi.

**Keywords:** gamma-aminobutyric acid, kimchi, *Leuconostoc mesenteroides*

## INTRODUCTION

Kimchi is a traditional Korean fermented food. It contains several functional ingredients such as vitamins, minerals, and dietary fiber; it is also rich in lactic acid bacteria (LAB) (Park et al., 2014a). LAB produce lactic acid by fermentation of carbohydrates. The main anaerobic bacteria involved in kimchi fermentation are *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, and *Lactobacillus brevis* (Cheigh and Park, 1994). Among these, *L. mesenteroides* is an essential heterofermentative LAB that determines the flavor of kimchi during fermentation; several attempts have been made to develop kimchi of consistent quality using these strains as starter organisms (Jung et al., 2012; Lee et al., 2018).

γ-Aminobutyric acid (GABA) is a nonprotein amino acid that is widely distributed in nature. It exhibits physiological functions such as neurotransmission, hypotension induction, and diuretic and sedative effects (Manyam et al., 1981; Siragusa et al., 2007). In particular, GABA increases oxygenation in the brain and cerebral blood and promotes brain cell metabolism (Sarasa et al., 2020). It has been reported that some strains of LAB use glutamic acid decarboxylase to catalyze the α-decarboxylation of L-glutamic acid, releasing the end products GABA and $CO_2$ (Higuchi et al., 1997; Ueno, 2000). GABA-producing LAB have been isolated from various types of fermented foods; research into the development of functional fermented foods using GABA-producing LAB is actively being conducted (Diez-Gutiérrez et al., 2020). Examples of functional foods using GABA are soy yogurt using *Lb. brevis*, which has high GABA production (Park and Oh, 2007), and tomato juice using GABA-producing LAB (Nakatani et al., 2022). Recent studies reported that consuming fermented foods or probiotics containing GABA-producing LAB may improve mental health (Gao et al., 2023); mice fed black soy milk with a high GABA content produced using *Lb. brevis* FPA 3709 isolated from fish guts, demonstrated a reduction in depression (Ko et al., 2013). In another study, high GABA content produced by *Lb. brevis* SBC8803 improved chronic sleep disorders induced by psychophysiological stress in mice (Higo-Yamamoto et al., 2019).

Fermented foods can be effective delivery vehicles for functional probiotics (Thomas and Versalovic, 2010; Tamang et al., 2020). Because kimchi is often consumed during daily meals, it can be a useful probiotic delivery vehicle. Kimchi is considered to prevent human diseases owing to the development of functional LAB (Seo et al., 2021).

Herein, we examined the GABA production activity of

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*L. mesenteroides* strains isolated from kimchi to develop a new GABA-producing kimchi starter. Furthermore, we analyzed whether the isolated strains have the potential to cause health hazards when added to kimchi as a starter, and whether they can be delivered to the intestines and function as probiotics.

## MATERIALS AND METHODS

### Isolation of the LAB from kimchi

In total, 350 LAB were isolated from home-prepared kimchi. Kimchi broth was serially diluted using phosphate-buffered saline (PBS) buffer (pH 7.4), plated onto bromocreol purple glucose agar (2% tryptone, 0.5% glucose, 0.5% yeast extract, 0.4% NaCl, 0.15% sodium acetate, and 40 mg/L bromocresol purple), and incubated at 30°C for 24 h. Following incubation, the colonies showing yellow rings were randomly selected and isolated. An 80% glycerol stock was prepared and stored at −70°C for use in subsequent experiments.

### Screening the GABA-producing LAB

Lactobacilli isolated from kimchi were incubated in de Man-Rogosa-Sharpe (MRS) broth at 30°C for 24 h. Next, the culture supernatants were obtained by removing the bacteria via a 0.2 μm syringe filter and centrifugation (25,000 *g*, 4°C, 15 min). The culture supernatant was used to measure the GABA concentration using the microtiter plate assay described previously (Tsukatani et al., 2005). In brief, 80 mM Tris-HCL buffer (pH 9.0) containing 1.4 mM $NADP^+$, 10 mM dithiothreitol, 750 mM sodium sulfate, 2 mM α-ketoglutarate, and 30 μg of GABase was dispensed in 90-μL increments onto a microtiter plate. Next, 10 μL of the previously prepared culture supernatant was added, the sample was incubated at 30°C for 60 min, and the absorbance was measured at 340 nm. MRS broth was used as blank was, and the standard blank was distilled water without GABase. LAB were preselected based on the GABA concentration and were incubated in MRS supplemented with 5% monosodium glutamic acid (MSG) for 24∼48 h. Culture supernatants were obtained, and the GABA concentration was measured to determine the strain with the highest GABA concentration.

### Identification through sequencing of the 16S rRNA genes

Chromosomal DNA was extracted from the selected strains, and the 16S rRNA gene was sequenced. The 16S rRNA gene was amplified using polymerase chain reaction with primers 27f (5'-AGAGTTTGATCMTGGCTC AG-3') and 1525r (5'-AAGGAGGTGWTCCARCC-3'). Sequence analysis was performed using Cosmogenetech; sequences were identified with the help of Basic Local Alignment Search Tool of National Center for Biotechnology Information.

### Growth and GABA production in the LAB determined through MSG concentration

The strains that were finally selected were inoculated with 1% of MRS medium containing 0∼5% MSG and incubated at 30°C for 48 h. Growth curves were generated by measuring the absorbance at 600 nm every 4 h (UVmini-1240, Shimadzu). The culture supernatant was collected using a 0.20 μm syringe filter and centrifugation every hour at 4-h intervals during the construction of the growth curve. The GABA concentration was measured at each incubation time. The method used to measure the GABA concentration was the same as that used for screening.

### *In vitro* hemolysis assay

Hemolytic activity was assessed by modifying the method described by Argyri et al. (2013). In brief, the LAB strains were grown in triplicate on MRS medium to increase their activity. Then, they were streaked onto tryptic soy agar plates containing 5% (w/v) sheep blood and incubated at 37°C for 48 h. Hemolytic activity was determined by visually evaluating α-hemolysis (green ring around colonies), β-hemolysis (yellow clear ring around colonies), and γ-hemolysis (no ring around colonies). *Bacillus cereus* ATCC 14579 was used as a positive control for β hemolysis and *Salmonella* Typhimurium TA98 was used as a positive control for γ-hemolysis.

### Biogenic amine (BA) assay

BA production was tested in decarboxylase medium (DCM) supplemented with amino acids using a modified version of the method described by Bover-Cid and Holzapfel (1999). To promote the production of related enzymes, the LAB were incubated in MRS medium supplemented with 0.5% of each precursor amino acid (L-histidine monohydrochloride monohydrate, L-lysine monohydrochloride, and L-ornithine monohydrochloride) and 0.005% pyridoxal 5-phosphate for 24 h at 30°C to induce the related enzyme activity. This step was replicated five times. Then, the LAB were streak-inoculated onto DCM supplemented with the respective precursor amino acids and incubated under anaerobic conditions at 37°C for 96 h. In case LAB produced enzymes that degraded that amino acids, the medium would turn purple; this color change was considered as a positive result. As a negative control, the LAB were incubated in DCM without amino acids. *Enterococcus faecalis* KACC13807 was used as the positive control.

### Determination of antimicrobial activity

The antimicrobial activity assay was performed as described by Ji et al. (2015) with some modifications; cul-

ture supernatants were used to determine the potential inhibitory effects on foodborne pathogens using the paper disk method (8 mm, Toyo Roshi Kaisha). The pathogenic bacteria and *Lactobacilli* used as indicators were *Escherichia coli* O157:H7, *Klebsiella pneumoniae* subsp. *pneumoniae* KCTC 1560, *Staphylococcus aureus* subsp. *aureus* KCTC 3881, *Listeria monocytogenes* ATCC 1911, *S*. Typhimurium TA98, *B. cereus* ATCC 14579, *Lactobacillus sakei* P3-1, *Lb. plantarum* subsp. *plantarum* KCTC 1048, *Lb. plantarum* JP0059, *Lb. brevis* 2.14, and *Lactobacillus pentosus* ATCC 8041. Following incubation of the LAB strains in MRS broth at 30°C for 18 h, culture supernatants were obtained through centrifugation and a 0.2-μm syringe filter to remove the bacteria. The pH of the filtered culture supernatants was adjusted to 6.5. Following 18 h of incubation, the pathogenic bacteria were smeared on each medium, and 50 μL of each culture supernatant was prepared on a paper disk. The plates were incubated at 37°C for 18 h and frequently observed for clear inhibition rings. Each assay was performed in triplicate.

**Gastric juice tolerance test**

Gastric juice tolerance tests were performed to determine the ability of the isolates to tolerate gastric juices under conditions similar to those in the human digestive tract. The artificial gastric juice tolerance test was performed using a modified version of the method described by Kobayashi et al. (1974). The artificial gastric juice was prepared by adding 1% pepsin filtered with a 0.45-μm syringe to MRS medium (Difco) adjusted to pH 2 and 3 with 1 N HCl. After incubating the LAB strains at 30°C for 18 h, 1 mL of the culture [$1.0\times10^9$ colony-forming units (CFU)/mL] was added to 50 mL of the MRS broth and the samples were incubated at 37°C for 2 h. Viable counts were calculated and expressed as viability (%) using the following formula:

$$\text{Viability (\%)} = \frac{\text{log index value of experimental group}}{\text{log index value of control group}} \times 100$$

for the control group, MRS broth without pepsin and pH adjustment was used.

**Bile fluid tolerance test**

Tolerance to artificial bile fluid was analyzed by modifying the method described by Paik et al. (2002). Artificial bile broth was prepared by adding 0.1, 0.3, or 0.5% bile salts (Difco) to MRS medium, and the medium was adjusted to pH 8.0 using 1 N NaOH. Following incubation of the LAB strains at 30°C for 18 h, 1 mL of culture ($1.0\times10^9$ CFU/mL) was added to 50 mL of artificial bile broth, and the samples were incubated at 37°C for 6 h. Then, the viable bacteria were counted and expressed as viability (%) using the formula described in the "gastric juice tolerance test" section of the Materials and Methods. MRS medium without bile salts was used as a control.

**Measurement of adhesion and cell surface hydrophobicity on HT-29 intestinal epithelial cells**

Adhesion to HT-29 intestinal epithelial cells was analyzed using the methods of Tuomola and Salminen (1998) and Fernández et al. (2003) with some modifications. HT-29 cells were inoculated in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine, 100 U/mL penicillin, 1 mM sodium pyruvate, and 0.1 mg/mL streptomycin. Next, cells were heat-treated at 56°C for 30 min and cultured at 37°C under 5% $CO_2$ conditions. The HT-29 cells that formed monolayers were adjusted to a concentration of $1.0\times10^5$ cells/mL and inoculated into a 6-well culture plate dispensed with DMEM medium without FBS and antibiotics and then pre-incubated for 2 h at 37°C under 5% $CO_2$. The LAB strains cultured in MRS medium for 18 h at 30°C were centrifuged (7,000 *g*, 10 min, 4°C) to collect only the cells, washed with PBS (pH 7.0), and the concentration of the LAB strains in DMEM medium was adjusted to $1.0\times10^8$ CFU/mL. The plate wells precultured with HT-29 cells were inoculated with the LAB strain suspension and incubated at 37°C for 2 h. Following removal of unattached cells, the attached cells were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) solution, washed with PBS (pH 7.0), and cultured on an MRS agar plate to determine the live bacterial count. The methods described by Donohue and Salminen (1996) and Kos et al. (2003) were used to measure the hydrophobicity of the LAB strain cell surfaces. The LAB strains were inoculated into MRS medium and incubated at 30°C for 18 h. After harvesting the bacteria from the cultures, they were washed twice with PBS and suspended in the same solution. Chloroform was added to the suspension at a ratio of one:one. The suspensions were vortexed for 2 min and then left at 20°C for 30 min. The absorbance of the aqueous solution layer (OD 580 nm reading 2) in the layer separated from the suspension (OD 580 nm reading 1) was measured at 580 nm, and the hydrophobicity (%) was calculated using the following formula:

$$\text{Hydrophobicity (\%)} = \frac{OD_{580\,nm}\ \text{reading 1} - OD_{580\,nm}\ \text{reading 2}}{OD_{580\,nm}\ \text{reading 1}} \times 100$$

**Growth of the LAB through NaCl concentration**

The final selected strains were inoculated with 1% of MRS medium containing 0~4% NaCl and incubated at 30°C for 24 h. Absorbance was measured at 600 nm every

4 h and a growth curve was generated (UVmini-1240).

### Statistical analysis

Data analysis was performed using the IBM SPSS Statistics 19 (IBM Corp.). Data were expressed as the mean and standard deviation (SD). Duncan's multiple range test was used to compare the data, and statistical significance was set at a $P<0.05$.

## RESULTS AND DISCUSSION

### Screening and identification of the GABA-producing LAB

In total, 350 LAB strains were isolated from kimchi. The supernatant was obtained by culturing the 350 strains in MRS medium, and the GABA concentration was measured. The two strains with the highest GABA production among all samples were selected. K1501 and K1627 were identified as *L. mesenteroides* using 16S rRNA gene sequencing, with 99.88% and 100.00% identity, respectively. K1501 was named *L. mesenteroides* K1501 and K1627 was named *L. mesenteroides* K1627. From Table 1, we observe that K1501 displays 99.88% similarity to *L. mesenteroides* CJNU 0705 and *L. mesenteroides* strain 4490; K1627 displays 100.00% similarity to *L. mesenteroides* subsp. *jonggajibkimchii* and 99.62% similarity to *L. mesenteroides* CJNU 0705. *L. mesenteroides* CJNU 0705, which is similar to both the isolated strains, is derived from breast milk. It adsorbs heavy metals and exhibits antibacterial activity against organic acids (Han and Moon, 2020). *L. mesenteroides* subsp. *jonggajibkimchii*, which displayed similarities to K1627, was approved as a new subspecies in 2017 after complete genome sequencing; it is known to synthesize mannitol, which enhances the flavor of kimchi and is commercially used as a kimchi starter (Jeon et al., 2017).

**Table 1.** Identification of the isolated lactic acid bacteria (LAB) strains using 16S rRNA gene sequencing

| LAB | 16S rRNA sequencing | |
|---|---|---|
| | Related strain from NCBI | Identity (%)[1)] |
| K1501 | *Leuconostoc mesenteroides* strain CJNU 0705 | 99.88 |
| | *L. mesenteroides* strain 4490 | 99.88 |
| K1627 | *L. mesenteroides* subsp. *jonggajibkimchii* | 100 |
| | *L. mesenteroides* strain CJNU 0705 | 99.62 |

[1)]These results were obtained using Basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from National Center for Biotechnology information.

### GABA production of *L. mesenteroides* K1501 and K1627 through MSG concentration

*L. mesenteroides* K1501 and K1627 were incubated in an MRS broth containing 0, 1, 3, or 5% MSG for 48 h at 30°C, and the growth and GABA production was measured every 4 h as a function of MSG concentration. For *L. mesenteroides* K1501, the growth showed similar trend irrespective of MSG concentration (Fig. 1). The cells entered the logarithmic phase after 4 h of incubation and reached the stationary phase after 8 h. Although the strains showed identical growth trends, their ability to produce GABA varied according to the MSG concentration. When cultured in MRS broth containing 0% MSG, GABA production increased rapidly after 24 h of incubation and remained moderate after 36 h. When cultured in the MRS broth containing 3% MSG, GABA production increased rapidly after 40 h, whereas when cultured in the MRS me-

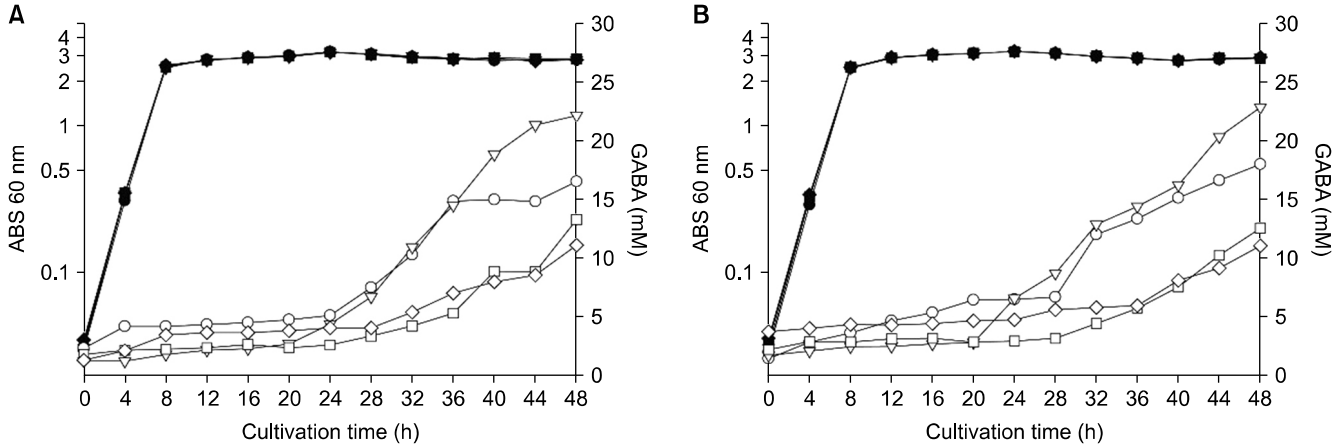


**Fig. 1.** Growth and γ-aminobutyric acid (GABA) production of *Leuconostoc mesenteroides* K1501 (A) and K1627 (B) in Man-Rogosa-Sharpe (MRS) broth containing various concentrations of monosodium glutamic acid (MSG). *L. mesenteroides* K1501 and K1627 were inoculated into MRS broth containing 0, 1, 3, or 5% MSG for 48 h to measure changes in GABA production. In the figure, black circles indicate growth in MRS medium containing 0% MSG, black triangles indicate growth in MRS medium containing 1% MSG, black squares indicate growth in MRS medium containing 3% MSG, and black diamonds indicate growth in MRS medium containing 5% MSG. Furthermore, open circles indicate GABA production in MRS medium containing 0% MSG, open triangles indicate GABA production in MRS medium containing 1% MSG, open squares indicate GABA production in MRS medium containing 3% MSG, and open diamonds indicate GABA production in MRS medium containing 5% MSG.

dium containing 5% MSG, GABA production increased rapidly after 32 h. In contrast, in MRS medium containing 1% MSG, GABA production started to increase after 28 h and increased rapidly after 40 h, reaching a peak at 48 h. At this time, GABA production was 22.13 mM, which was 2-fold higher than the 11.10 mM produced at the same time in the MRS containing 5% MSG and >0.5-fold higher than the 16.52 and 13.28 mM produced simultaneously in the MRS containing 0% MSG and 3% MSG (Fig. 1A). For *L. mesenteroides* K1627, similar to *L. mesenteroides* K1501, the log phase began after 4 h; the cells entered the stationary phase after 8 h, irrespective of the MSG concentration. When cultured in MRS medium with 0% MSG, GABA production increased rapidly after 28 h. However, when cultured in MRS medium with 3% or 5% MSG, GABA production increased moderately over time. In contrast, in MRS broth containing 1% MSG, GABA production increased rapidly after 24 h, reaching a peak at 48 h. MRS containing 1% MSG produced the highest amount of GABA (22.81 mM), which was higher than that produced simultaneously in MRS containing 0% MSG (17.98 mM). This was approximately twice as high as the amount produced simultaneously in MRS containing 3% or 5% MSG (12.54 and 11.05 mM, respectively) (Fig. 1B). Overall, GABA production by *L. mesenteroides* K1501 and K1627 began to increase gradually after the stationary phase, and production was highest when 1% MSG was included. GABA production increased rapidly with the onset of logarithmic proliferation, and peaked in the stationary phase, in contrast to *Lb. brevis* (Wang et al., 2018). Furthermore, compared to the results of *Lb. plantarum*, *Lb. plantarum* K154 showed the highest GABA production in MRS broth containing 3% MSG though its growth characteristics were similar in MRS broth with different MSG concentrations (Park et al., 2014b).

### Hemolysis of *L. mesenteroides* K1501 and K1627

The hemolytic activity was measured to evaluate the safety of *L. mesenteroides* K1501 and K1627 (Table 2). The hemolytic activity of probiotics is divided into α-hemolysis, which is the incomplete lysis of red blood cells and produces a green ring around the colony, β-hemolysis, which is the complete lysis of red blood cells and produces a white or yellow circle, and γ-hemolysis, which is the absence of hemolytic activity and does not produce a ring (Halder et al., 2017). The hemolytic activities of *L. mesenteroides* K1501 and K1627 were tested and both strains were determined to be γ-hemolytic, with no hemolysis in agar medium containing sheep blood. As a positive control, *S. aureus* KCTC 3881 exhibited β-hemolysis in agar medium containing sheep blood. These results indicate that the strain does not have hemolytic activity; therefore, it is safe for food fermentation. It has been reported that four *L. mesenteroides* strains isolated from aguamiel, a sap obtained from *Agave salmiana* in Mexico (Diana et al., 2015), and nine *Leuconostoc* strains isolated from kimchi (Jeong and Lee, 2015) exhibited γ-type hemolytic activity.

**Table 2.** Hemolytic activity of *Leuconostoc mesenteroides* K1501 and K1627

| Content | Strain | |
|---|---|---|
| | *L. mesenteroides* K1501 | *L. mesenteroides* K1627 |
| Hemolysis | | |
| α[1)] | – | – |
| β | – | – |
| γ | + | + |

[1)]The hemolytic activity of probiotics is divided into α-hemolysis, which is the incomplete lysis of red blood cells and produces a green ring around the colony, β-hemolysis, which is the complete lysis of red blood cells and produces a white or yellow circle, and γ-hemolysis, which has no hemolytic activity and does not produce a ring.
+, positive activity; –, negative activity.

### BA production of *L. mesenteroides* K1501 and K1627

BAs are low-molecular-weight organic compounds that can be produced by LAB that contain the gene that encodes amino acid decarboxylase (Barbieri et al., 2019). The BA production by *L. mesenteroides* K1501 and K1627 is shown in Table 3. *L. mesenteroides* K1501 and K1627 did not produce BAs from histamine, cadaverine, or putrescine by decarboxylating histidine, lysine, or ornithine precursors. Similarly, it has been reported that some LAB species, such as *Enterococcus lactis* and *Lb. plantarum* isolated from camel milk do not produce BAs; thus they can be safely be used as starter organisms (Sharma et al., 2021). The results showed that both *L. mesenteroides* K1501 and K1627 are safe from BAs that may be harmful to humans when consumed.

### Antimicrobial activities of *L. mesenteroides* K1501 and K1627

The antimicrobial activities of LAB inhibit spoilage and pathogenic bacteria by producing short-chain fatty acids, organic acids, bacteriocins, and antibiotics (Tulumoglu et al., 2013). In addition to extending the shelf life of food and inhibiting the growth of pathogens, these metabolites improve the odor, taste, and color of fermented

**Table 3.** Biogenic amine (BA) production by *Leuconostoc mesenteroides* K1501 and K1627

| Content | Strain | |
|---|---|---|
| | *L. mesenteroides* K1501 | *L. mesenteroides* K1627 |
| BA production | | |
| Histamine | – | – |
| Cadaverine | – | – |
| Putrescine | – | – |

–, negative activity.

**Table 4.** Antimicrobial activity of *Leuconostoc mesenteroides* K1501 and K1627

| Foodborne pathogen | Strain | |
|---|---|---|
| | *L. mesenteroides* K1501 | *L. mesenteroides* K1627 |
| *Escherichia coli* O157:H7 | − | − |
| *Klebsiella pneumoniae* subsp. *pneumoniae* KCTC1560 | + | + |
| *Staphylococcus aureus* subsp. *aureus* KCTC 3881 | − | − |
| *Listeria monocytogenes* ATCC 19111 | − | − |
| *Salmonella* Typhimurium TA98 | − | − |
| *Bacillus cereus* ATCC 14579 | − | − |
| *Lactobacillus* sakei P3-1 | + | + |
| *Lactobacillus plantarum* subsp. *plantarum* KCTC 1048 | − | − |
| *Lb. plantarum* JP0059 | + | + |
| *Lactobacillus brevis* 2.14 | + | + |
| *Lactobacillus pentosus* ATCC 8041 | + | + |

+, the presence of an inhibition zone surrounding the *Leuconostoc* colony; −, no inhibition.

foods (Zarour et al., 2017). Table 4 shows that *L. mesenteroides* K1501 and K1627 inhibited *K. pneumoniae* subsp. *pneumoniae* KCTC1560. *K. pneumoniae* is a gram-negative bacterium that primarily causes urinary tract infections, pneumonia, and intra-abdominal infections, and has also been reported to be responsible for bacteremia (Meatherall et al., 2009).

*L. mesenteroides* K1501 and K1627 were tested for antibacterial activity against *Lactobacillus* strains that are thought to adversely affect kimchi quality and were found to inhibit all strains except *Lb. plantarum* subsp. *plantarum* KCTC 1048. Previous studies have reported that the genus *Leuconostoc* inhibited *Lactobacilli*; for example, *L. mesenteroides* subsp. *dextranicum* isolated from Boza, a traditional Bulgarian grain-fermented beverage, inhibited *Lactobacillus casei* and *Lb. plantarum* (Todorov and Dicks, 2004), and *Leuconostoc citreum* GJ7 isolated from kimchi inhibited *Lb. plantarum* KFRI 464 (Chang and Chang, 2010). It has been reported that *Lb. plantarum*, *Lb. brevis*, and *Pediococcus* spp. cause overacidification of kimchi, with *Lb. plantarum* accounting for >80% of LAB isolated from overacidified kimchi (Lee et al., 1992). *L. mesenteroides* K1501 and K1627 were relatively potent in inhibiting *Lactobacilli* in this study; therefore, they may be able to effectively prevent the overacidification of kimchi. Collectively, both the strains are expected to be able to inhibit harmful bacteria and *Lactobacilli* that can cause overacidification of kimchi.

### Artificial gastric fluid tolerance of *L. mesenteroides* K1501 and K1627

The pH of the human stomach (1.5～2.5) varies depending on food intake, diet, age, and medical conditions (Evans et al., 1988). Survival under gastrointestinal conditions, including a low pH, is an essential characteristic of effective probiotics. From Table 5, we observe that *L. mesenteroides* K1501 demonstrated 88.69±0.27% survival at pH 3.0 (8.29±0.01 log CFU/mL) and 27.86±0.33% survival at pH 2.0 (2.60±0.03 log CFU/mL). *L. mesenteroides* K1627 demonstrated 86.85±0.51% survival at pH 3.0 (8.13±0.02 log CFU/mL) and 38.05±0.46% survival at pH 2.0 (3.56±0.05 log CFU/mL). At pH 2.0, *L. mesenteroides* K1501 and K1627 demonstrated lower survival rates of approximately 35%; however, they showed higher survival rates of approximately 85% at pH 3.0. Lim et al. (2016) reported that *L. mesenteroides* AJ13 isolated from anchovy fillets survived at approximately 6 log CFU/mL at pH 3.0. Therefore, *L. mesenteroides* K1501 and K1627 showed low survival at pH 2.0 but high survival at pH 3.0, respectively, suggested that they have a high probability of reaching the intestine and exhibiting health benefits when consumed orally.

### Artificial bile tolerance of *L. mesenteroides* K1501 and K1627

Bile salt tolerance is essential for LAB survival in the small

**Table 5.** Survival of *Leuconostoc mesenteroides* K1501 and K1627 after incubation in artificial gastric juice

| Strain | Survival cell counts (log CFU/mL) | | |
|---|---|---|---|
| | pH of gastric juice (survival rate %) | | |
| | Control | 3.0 | 2.0 |
| *L. mesenteroides* K1501 | 9.35±0.02$^{a}$ | 8.29±0.01$^{b}$ (88.69±0.27) | 2.60±0.03$^{c}$ (27.86±0.33) |
| *L. mesenteroides* K1627 | 9.37±0.03$^{a}$ | 8.13±0.02$^{b}$ (86.85±0.51) | 3.56±0.05$^{c}$ (38.05±0.46) |

Values are presented as mean±SD of triplicate analyses.
Different letters (a-c) in the same row indicate statistically significant differences at $P<0.05$ by Duncan's multiple range test.

**Table 6.** Survival of *Leuconostoc mesenteroides* K1501 and K1627 after incubation in bile salts

| Strain | Survival cell counts (log CFU/mL) | | |
|---|---|---|---|
| | Concentration of bile salts (%) [survival rate (%)] | | |
| | 0.1 | 0.3 | 0.5 |
| *L. mesenteroides* K1501 | 6.58±0.03[a] (79.09±0.02) | 2.40±0.09[b] (28.95±1.28) | − |
| *L. mesenteroides* K1627 | 6.78±0.09[a] (82.21±1.39) | 2.34±0.04[b] (28.39±0.38) | − |

Values are presented as mean±SD of triplicate analyses.
Different letters (a,b) in the same row indicate statistically significant differences at $P<0.05$ by Duncan's multiple range test.
−, no growth.

intestine (Singh et al., 2012). Physiologically, the concentration of bile salts in the small intestine ranges from 0.3% to 0.5% (Prasad et al., 1998). As shown in Table 6, *L. mesenteroides* K1501 showed 79.09±0.02% survival (6.58±0.03 log CFU/mL) at a 0.1% bile salt concentration, and 28.95±1.28% survival (2.40±0.09 log CFU/mL) at a 0.3% bile salt concentration. *L. mesenteroides* K1627 showed 82.21±1.39% survival (6.78±0.09 log CFU/mL) at a 0.1% bile salt concentration, and 28.39±0.38% survival (2.34±0.04 log CFU/mL) at a 0.3% bile salt concentration. *Lactobacillus rhamnosus* isolated from kefir was reported to have a 40% survival rate in 0.1% bile salt and only 2% in 0.5% bile salt (You et al., 2005), whereas *Streptococcus salivarius* and *Lactococcus lactis* strains isolated from goat milk have been reported to have survival rates of 22∼29% in 0.3% bile salts (Lim et al., 2008). Although *Lactobacillus* sp. FF-3, isolated from Dongchimi, showed high resistance to artificial gastric juice, it showed a low survival rate of 6% in bile salts (Chung et al., 2003). *L. mesenteroides* C4 and C10, isolated from kimchi, showed survival rates of 30.41% and 42.82%, respectively, in 0.3% bile salts (Lee et al., 2016). The survival rates of over 70% in 0.1% bile salts and approximately 28% in 0.3% bile salts for *L. mesenteroides* K1501 and K1627 were comparable to those of other previously reported probiotic strains; however, both these strains were completely inhibited by 0.5% bile salts, suggesting that they may have difficulty in effectively reaching the colon if they were to be administered as probiotics.

### Adhesion and cell surface hydrophobicity of *L. mesenteroides* K1501 and K1627 to intestinal epithelial cells (HT-29 cells)

To be characterized as a probiotic, LAB must pass through the stomach and duodenum to reach their final destination, the intestine, where they typically attach to intestinal epithelial cells to function (Kim et al., 2019). Polysaccharides, proteins, fatty acids, and teichoic acid can also covalently bind to the epithelial surface of the intestinal tract. Probiotic strains and intestinal cells can attach to each other because the hydrophobic residues on the cell surface recognize carbohydrate residues such as fructose, glycoproteins, mannose, and galactose (Falah et al., 2019).

The adherence of *L. mesenteroides* K1501 and K1627 to HT-29 cells, and the cell surface hydrophobicity that may in turn affect adherence of *L. mesenteroides* K1501 and K1627, was measured (Table 7). *L. mesenteroides* K1501 had an adherence rate of 99.97±0.31% (5.00±0.02 log CFU/mL), and *L. mesenteroides* K1627 had an adherence rate of 99.97±0.31% (5.00±0.01 log CFU/mL); both strains had an adherence rate of >95%. The adhesion rate of *Limosilactobacillus fermentum* KGC1601, isolated from ginseng, to HT 29 cells has been reported to be 19.77% (Kim et al., 2022). *L. reuteri* strains K7 and K14, isolated from infant stool samples, showed intestinal adhesion rates of 37.2% and 35.5%, respectively (Krausova et al., 2019).

The cell surface hydrophobicity of *L. mesenteroides* K1501 and K1627 was 84.41±0.72% and 81.20±0.72%, respectively. The surface hydrophobicity of *L. mesenteroides* SD1 and SD23 isolated from aguamiel (the sap of the Mexican maguey plant) was reported to be 52.23±3.72% and 63.38±2.17%, respectively (Diana et al., 2015), and *Lactobacillus fermentum* strain 4-17 isolated from kashkineh, an Iranian grain-fermented food, was reported to exhibit 43% surface hydrophobicity (Falah et al., 2019). Cell surface hydrophobicity plays an essential role in the protection and colonization of the human gut (Yasmin et al.,

**Table 7.** Surface adhesion and hydrophobicity of *Leuconostoc mesenteroides* K1501 and K1627 to intestinal epithelial HT-29 cells

| Stain | Adhered cell count (log CFU/mL) | Adhesion (%) | Hydrophobicity (%) |
|---|---|---|---|
| *L. mesenteroides* K1501 | 5.00±0.02 | 99.97±0.31 | 84.41±0.72 |
| *L. mesenteroides* K1627 | 5.00±0.01 | 99.97±0.31 | 81.20±0.72 |
| *t*-value[1)] | − | 0.000 | 6.621 |

Values are presented as mean±SD obtained from triplicate measurements.
[1)] *t*-values were obtained from independent sample *t*-tests.

2020), and probiotics that adhere to the gut provide practical functions, such as preventing the adhesion of intestinal pathogens and modulating the immune function of the host (Saraniya and Jeevaratnam, 2015).

*L. mesenteroides* K1501 and K1627 exhibited higher cell surface hydrophobicity than other previously reported probiotics as well as a high degree of adhesion to intestinal epithelial cells. These findings suggested that they have a high probability of colonizing epithelial cells, provided that they reach the intestine viably.

**Salt tolerance of *L. mesenteroides* K1501 and K1627**

The salinity of long-fermented kimchi ranges from 2% to 4%, whereas regular cabbage kimchi has a salinity of 2.5%. Traditionally, higher salinity of long-fermented kimchi permitted it to be stored for extended periods, even in winter (Nam et al., 2007). *L. mesenteroides* K1501 and K1627 were incubated in MRS broth containing 0, 1, 2, 3, or 4% NaCl for 24 h at 30°C, and growth was measured every 4 h as a function of NaCl concentration. When *L. mesenteroides* K1501 and *L. mesenteroides* K1627 were cultured in MRS broth containing 0, 1, or 2% NaCl, they entered the log phase after 4 h of incubation. The cells reached the stationary phase after 8 h. When cultured in MRS broth containing 3% and 4% NaCl, the absorbance increased rapidly after 4 h of incubation, indicating that the cells entered the log phase after 4 h; the stationary phase was reached after 12 h. We observed that the absorbance was lower when cells were cultured in MRS broth containing 3% and 4% NaCl than when cultured in MRS broth containing 0, 1, and 2% NaCl, and that both strains reached higher populations at 0, 1, and 2% NaCl (Fig. 2). Therefore, it is estimated that the growth of both strains is not inhibited in low-Na kimchi, highlighting its potential to be developed as a starter for low-Na kimchi with high GABA content.

In conclusion, GABA-producing *L. mesenteroides* K1501 and K1627 isolated from kimchi showed the highest GABA production at 1% MSG. Both strains showed high survival rates of approximately 87% in artificial gastric juice at pH 3.0 and >80% in 0.1% artificial bile salt fluid. However, they showed approximately 28% survival in 0.3% artificial bile salt fluid and no survival in 0.5% artificial bile salt. Excellent adhesion (>99%) to intestinal epithelial cells was observed. Furthermore, there was no change in growth at the 2% salt concentration; however, growth was slightly delayed at 3% and 4% salt concentrations. Based on these results, we suggest that *L. mesenteroides* K1501 and K1627 isolated from kimchi have the potential to be used as starters for the production of GABA-functional kimchi. However, further studies are required to determine whether GABA can be produced in low-temperature environments or whether more extreme conditions through direct inoculation during kimchi production are required.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Concept and design: JYP. Analysis and interpretation: JA.

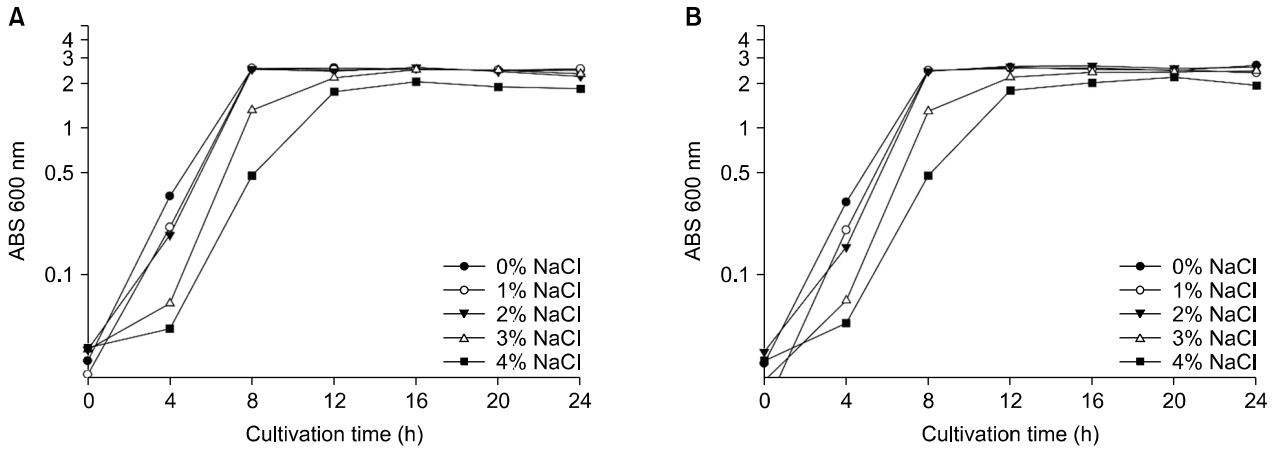


**Fig. 2.** Growth curves of *Leuconostoc mesenteroides* K1501 (A) and K1627 (B) in Man-Rogosa-Sharpe (MRS) broth containing 0~4% NaCl. *L. mesenteroides* K1501 and K1627 were inoculated in MRS containing 0, 1, 2, 3, or 4% NaCl for 24 h and the absorbance at 600 nm was measured. In the figure, black circles indicate growth in MRS medium containing 0% NaCl, open circles indicate growth in MRS medium containing 1% NaCl, black triangles indicate growth in MRS medium containing 2% NaCl, open triangles indicate growth in MRS medium containing 3% NaCl, and black squares indicate growth in MRS medium containing 4% NaCl.

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