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Probiotic *Lactiplantibacillus plantarum* KU210152 and its fermented soy milk attenuates oxidative stress in neuroblastoma cells

Hyun-Ji Bock, Hye-Won Lee, Na-Kyoung Lee, Hyun-Dong Paik *

Department of Food Science and Biotechnology of Animal Resources, Konkuk University, Seoul 05029, Republic of Korea

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ABSTRACT

We evaluated the probiotic properties and neuroprotective effects of *Lactiplantibacillus plantarum* KU210152 and its application in soy milk. *L. plantarum* KU210152 exhibited high tolerance to artificial gastrointestinal conditions, high adhesion to intestinal cells (HT-29), and safe enzyme production. Conditioned medium acquired from HT-29 cells treated with heat-killed lactic acid bacteria (LAB-CM) was used to evaluate the neuroprotective effects. The CM exhibited neuroprotective effects via cell viability assay, morphological observations, and suppression of ROS production. Heat-killed *L. plantarum* KU210152 increased brain-derived neurotrophic factor (*BDNF*) and tyrosine hydroxylase (*TH*) expression in HT-29 cells. In SH-SY5Y cells, pretreatment with *L. plantarum* KU210152 CM decreased *Bax/Bcl-2* ratio and upregulated *BDNF* and *TH* expression. The CM inhibited caspase-9 and caspase-3 activities. The neuroprotective effects of *L. plantarum* KU210152 were also confirmed in fermented soy milk. Therefore, both *L. plantarum* KU210152 and the fermented soy milk can be used as functional ingredients with neuroprotective effects against oxidative stress.

1. Introduction

Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, involve continuous and irreversible deterioration of the nervous system. An imbalance between reactive oxygen species (ROS) levels and the response of the antioxidant defense system causes oxidative stress (Dasuri et al., 2013). Cumulative oxidative stress triggered by ROS is related to apoptosis and plays a key role in the pathogenesis of neurodegenerative diseases (Patten et al., 2010). Hydrogen peroxide (H_2O_2) is a byproduct of normal and/or aberrant metabolic processes that use molecular oxygen. H_2O_2 causes mitochondrial dysfunction, lipid peroxidation, and DNA damage, and induces apoptosis in neuronal cells (Nissanka & Moraes, 2018).

The gut-brain axis (GBA) is a bidirectional signaling pathway between the gastrointestinal tract and the brain (Cryan & O'Mahony, 2011). The synergy between the host and the gut microbiota regulates the host brain and behavior via the GBA. Moreover, the GBA is a potential therapeutic target for diseases related to mental health and cognitive function (Cryan et al., 2019). Based on previous studies on the GBA, the gut microbiota can be modified by the administration of probiotics, prebiotics, and antibiotics, and fecal microbiota transplantation (Yeo, 2023). These approaches have been suggested to enhance mental health through their microbiota-modifying capabilities (Mörkl et al., 2020). Several studies have reported that probiotic strains are beneficial for the treatment of neurological diseases (Ishii et al., 2021; Sharma et al., 2023).

Probiotics are defined as "live microorganisms that confer health benefits to the host when administered in appropriate amounts". Some studies have reported that probiotics have several beneficial properties, such as antimicrobial, immunomodulatory, antioxidant, and anticancer properties (De Marco et al., 2018; Oh et al., 2018). Probiotics defend the host against several harmful physiological processes, such as oxidative stress, decrease in neurotransmitter levels, and chronic inflammation, which aggravate neurological disorders (Westfall et al., 2017). Additionally, probiotics exert beneficial modulatory effects on brain function, including alleviation of depression-like behavior and normalization of anxiety (Gazerani, 2019).

Probiotic bacteria are present in various fermented foods, and dairy products are the main carriers of probiotics (Pramanik et al., 2023). However, because of several reasons, such as the demand for vegetarian substitutes, allergenicity, and lactose intolerance, alternatives to cow milk are being investigated. Among the several alternatives, soymilk is an excellent substrate for probiotic strains (Farnworth et al., 2007). Soy products, especially soy yogurt, may act as a potential vehicle for

* Corresponding author.
E-mail address: hdpaik@konkuk.ac.kr (H.-D. Paik).

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Table 1
Probiotic properties of lactic acid bacteria used in this study.

Treatment	LAB strains	
	LGG	*L. plantarum* KU210152
Gastric acid tolerance (%) (0.3 % pepsin, pH 2.5, 3 h)	98.67 ± 1.82[b]	100.12 ± 1.30[a]
Bile salts tolerance (%) (0.3 % bile extract, 24 h)	94.99 ± 0.78[b]	96.68 ± 0.53[a]
Adhesion ability to HT-29 cells (%)	1.71 ± 0.26[b]	5.58 ± 0.12[a]

Data are indicated as mean ± standard deviation of triplicate experiments. For different strains in the same experiment, means with different superscript letters (a, b) were significantly different ($P < 0.05$, Student's *t*-test). LA, Lactic acid bacteria; LGG, *Lacticaseibacillus rhamnosus* GG; *L. plantarum*, *Lactiplantibacillus plantarum*.

probiotics, conferring health benefits to the host (Valero-Cases et al., 2020).

Soymilk contains bioactive compounds, including isoflavones and saponins, and is free of lactose and cholesterol (Rui et al., 2019). Isoflavones in soybeans have been investigated for their beneficial effects in the treatment of osteoporosis, menopausal symptoms, cardiovascular diseases, and cancer (Omoni & Aluko, 2005). Lactic acid bacteria (LAB) ferment the glucoside forms of isoflavones into isoflavone aglycones. These soy isoflavone aglycones can be absorbed more easily than their glucoside forms (Choi et al., 2022).

In the present study, we investigated the probiotic properties and neuroprotective effects of *Lactiplantibacillus plantarum* KU210152. The probiotic nature of the strains was assessed by investigating their tolerance to artificial digestive conditions, intestinal adhesion ability, and enzyme production. The neuroprotective effects were evaluated by measuring the levels of neuronal biomarkers and apoptotic factors. Additionally, the probiotic strain *L. plantarum* KU210152 was added to fermented soy milk, and the neuroprotective effects of the treated fermented soy milk were evaluated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactiplantibacillus plantarum KU210152 was isolated from kimchi samples. *Streptococcus thermophilus* P206 was isolated from an ABT-B commercial yogurt starter culture. *Lacticaseibacillus rhamnosus* GG (LGG) was obtained from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) and was used as the reference strain. *L. plantarum* KU210152 and LGG were cultured in de Man, Rogosa, and Sharpe broth (MRS; Difco Laboratories, Detroit, MI, USA) at 37 °C for 24 h. *Streptococcus thermophilus* P206 was isolated from commercial yogurt, this strain was cultured in MRS broth at 37 °C for 18 h.

The bacterial samples were prepared by centrifugation at 14,240 × *g* and 4 °C for 5 min; the pellet was washed thrice and resuspended in phosphate-buffered saline (PBS; HyClone Laboratories, Logan, UT, USA) (Cheon et al., 2020). Heat-killed cells were prepared by heating the bacterial cells at 80 °C for 30 min in water bath, and confirmed killed cell by plate counting methods.

2.2. Cell culture conditions

The HT-29 (human colon adenocarcinoma; KCLB 30038) cells were cultured in Roswell Park Memorial Institute (RPMI; HyClone) 1640 medium. The SH-SY5Y (human neuroblastoma; ATCC CRL-2266) cells were cultured in Dulbecco's Modified Eagle medium (DMEM; HyClone). Each medium contained 10 % fetal bovine saline (FBS; HyClone) and 1 % penicillin (HyClone) solution. The cells were incubated at 37 °C in 5 % CO_2 environment.

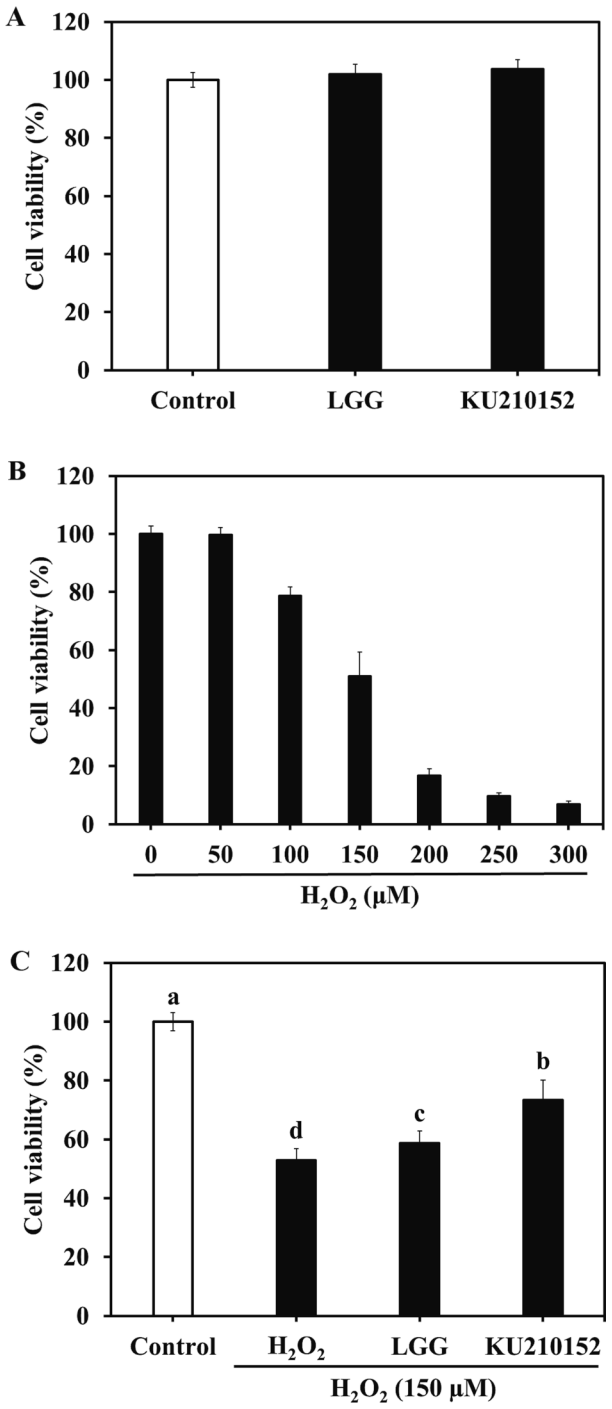

Fig. 1. Neuroprotective effect of lactic acid bacteria (LAB) conditioned medium (CM) on SH-SY5Y cells. (A) Effect of LAB CM on SH-SY5Y cells; (B) Effect of H_2O_2 on SH-SY5Y cells; (C) Effect of LAB CM on H_2O_2-treated SH-SY5Y cells. LGG, CM of heat-killed *Lacticaseibacillus rhamnosus* GG (LGG); KU210152, CM of heat-killed *Lactiplantibacillus plantarum* KU210152. Data are presented as mean ± standard deviation of triplicate experiments. Different letters on the error bars indicate significant differences ($P < 0.05$).

2.3. Tolerance to artificial gastric juice and bile salts

The tolerance of the bacterial strains to gastrointestinal conditions was evaluated as described by Song et al. (2020), with minor modifications. The artificial gastrointestinal conditions were simulated using 1) MRS broth (pH 2.5) containing 0.3 % pepsin (Sigma-Aldrich, St. Louis, MO, USA) (w/v) and 2) MRS broth containing 0.3 % bile extract

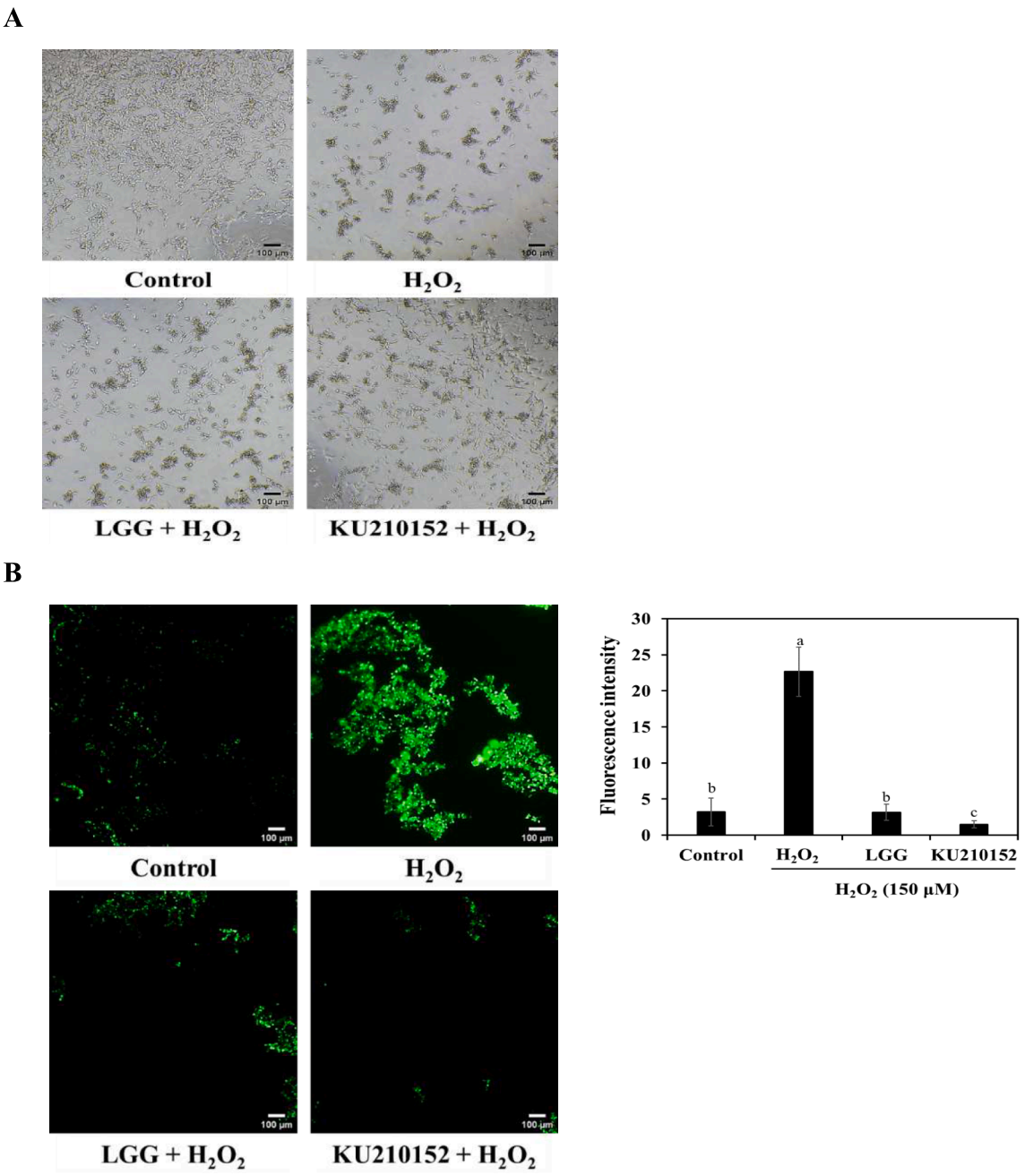

Fig. 2. Effect of lactic acid bacteria (LAB) conditioned medium (CM) on SH-SY5Y cells. (A) Morphological changes; (B) Reactive oxygen species (ROS) generation. LGG, CM of heat-killed *Lacticaseibacillus rhamnosus* GG (LGG); KU210152, CM of heat-killed *Lactiplantibacillus plantarum* KU210152. White and black scale bar indicate 100 μm at × 40 magnification. The graph is represented fluorescence intensity of ROS using imageJ. The data are presented as mean ± standard deviation.

porcine (Sigma-Aldrich) (w/v), respectively. Pre-cultured LAB strains (1×10^8 colony forming units (CFU)/mL) were inoculated in each type of MRS broth and incubated at 37 °C for 3 h and 24 h. Survival rates of the bacterial strains were determined using the following equation:

$$\text{Survival rate}\ (\%) = \frac{Nt}{Ni} \times 100$$

where N_t and N_i indicate the number of viable bacterial cells after treatment (log CFU/mL) and the initial cell number (log CFU/mL), respectively.

2.4. Adhesion ability to HT-29 cells

The adhesion ability of the LAB strains was assessed using intestinal cells as described by Son et al. (2018), with some modifications. The HT-29 cells were seeded in 24-well plates (1×10^5 cells/mL) and incubated for 24 h. Next, the medium was replaced with antibiotic-free medium, and 1×10^8 CFU/mL LAB was added to the cells. After incubation for 2 h, the non-attached bacterial cells were washed thrice with PBS, and the attached cells were detached from the HT-29 cells using 1 % Triton X-100 (Sigma-Aldrich). The bacteria were counted on MRS agar, and the adhesion ability was determined using the following equation:

$$\text{Adhesion ability}\ (\%) = \frac{Na}{Ni} \times 100$$

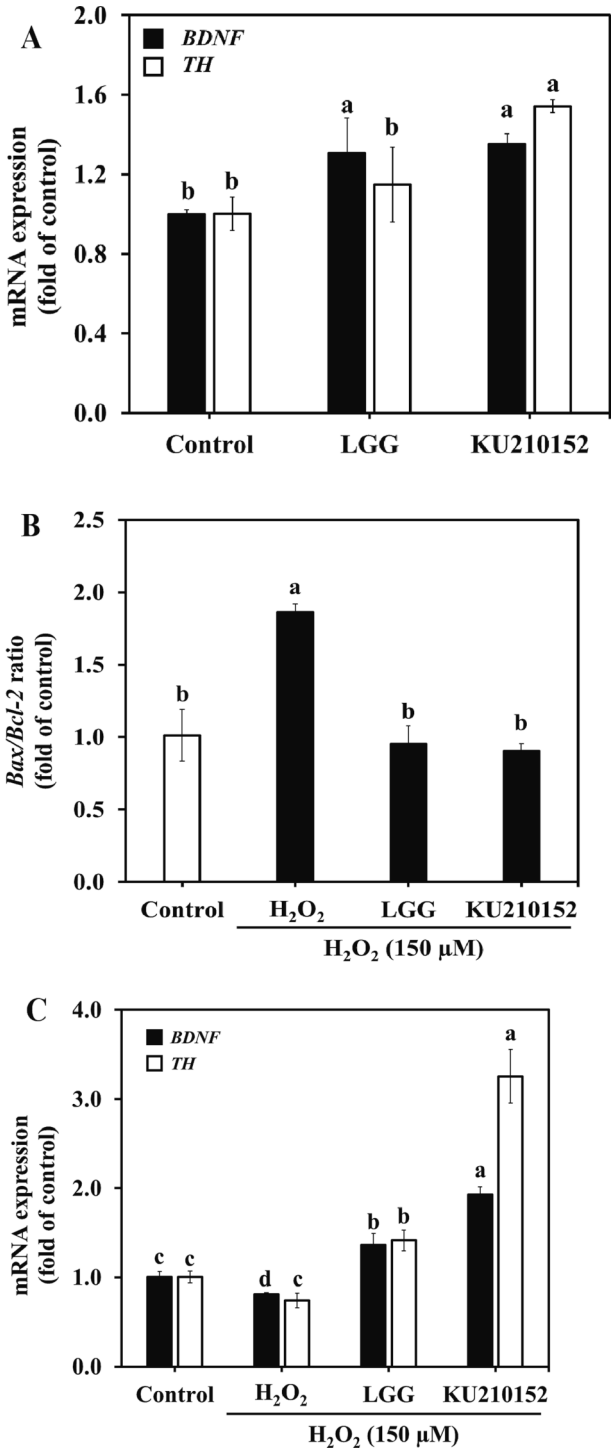

Fig. 3. mRNA expression of neuronal biomarkers and apoptosis-related genes in HT-29 cells and SH-SY5Y cells. (A) *BDNF* and *TH* expression in HT-29 cells; (B) *Bax/Bcl-2* ratio in SH-SY5Y cells; (C) *BDNF* and *TH* expression in SH-SY5Y cells. LGG, conditioned medium (CM) of heat-killed *Lacticaseibacillus rhamnosus* GG (LGG); KU210152, CM of heat-killed *Lactiplantibacillus plantarum* KU210152. Data are presented as mean ± standard deviation of triplicate experiments. Different letters on the error bars indicate significant differences ($P < 0.05$). *BDNF*, brain-derived neurotrophic factor; *TH*, tyrosine hydroxylase; *Bax*, Bcl-2 associated X protein; *Bcl-2*, B-cell lymphoma 2.

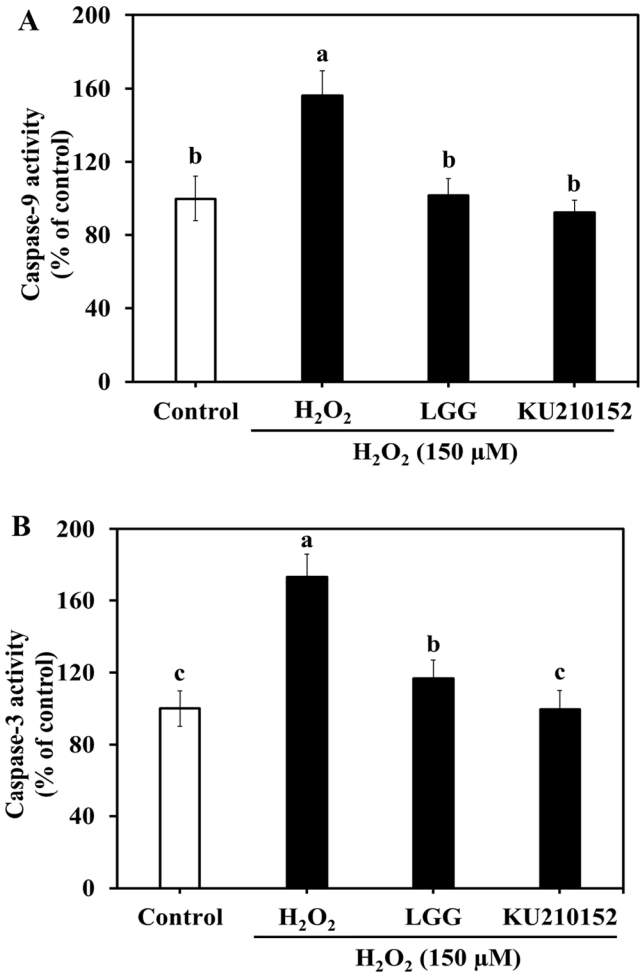

Fig. 4. Effect of lactic acid bacteria (LAB) conditioned medium (CM) on activity of caspase-9 and caspase-3 in SH-SY5Y cells. (A) Caspase-9 activity, and (B) caspase-3 activity. LGG, CM of heat-killed *Lacticaseibacillus rhamnosus* GG (LGG); KU210152, CM of heat-killed *Lactiplantibacillus plantarum* KU210152. Data are presented as mean ± standard deviation of triplicate experiments. Different letters on the error bars indicate significant differences ($P < 0.05$).

where N_a and N_i indicate the number of the attached bacterial cells (CFU/mL) and initial cell number (CFU/mL), respectively.

2.5. Enzyme production

Enzyme production by the LAB strains was evaluated using an API ZYM kit (bioMérieux, Marcy-l'Étoile, France). Live bacterial cells (65 μL) were added to cupules and incubated at 37 °C for 4 h. After incubation with the ZYM A and ZYM B reagents for 5 min, enzyme production was estimated based on color intensity.

2.6. Preparation of heat-killed LAB conditioned medium (CM)

To investigate neuroprotective effects, CM were manufactured as Cheon et al. (2020)'s method. The HT-29 cells were seeded in 6-well plates (5×10^5 cells/mL) and incubated until a monolayer was formed; heat-inactivated LAB (1×10^9 CFU/mL) was added to the monolayer to prepare LAB CM, and control CM was prepared by treatment of the cells with PBS. After treatment of the HT-29 cells for 24 h, the supernatant was collected by centrifugation at 14,000 × *g* and 4 °C for 10 min. Next, the supernatant was filtered using a 0.45-μm filter. Both control and LAB CM were stored at –80 °C for further experiments.

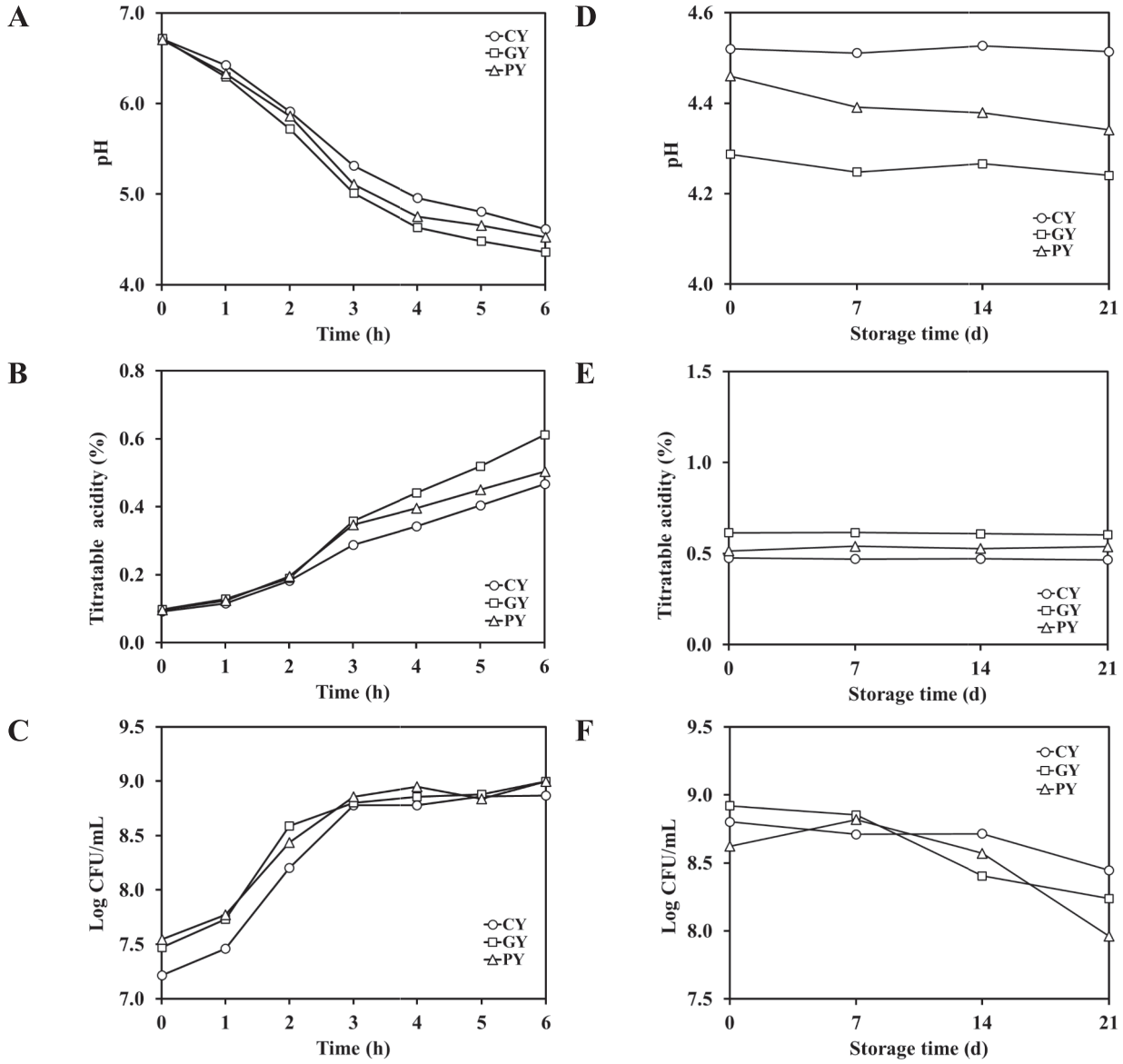

Fig. 5. Changes in pH, titratable acidity (TA), and viable cell counts of fermented soy milk samples during fermentation (A–C) and refrigerated storage (D–F). CY, soy milk fermented with *Streptococcus thermophilus* P206; GY, soy milk fermented with *Lacticaseibacillus rhamnosus* GG (LGG) and *S. thermophilus* P206; PY, soy milk fermented with *Lactiplantibacillus plantarum* KU210152 and *S. thermophilus* P206.

2.7. Preparation of fermented soy milk

Soymilk (Maeil Dairies Co., Seoul, Korea) was sterilized at 90 °C for 10 min and cooled to 42 °C. Next, the sterilized soymilk was divided into three equal parts and 1 % (v/v) of the precultured *S. thermophilus* P206 suspension was added to each sample. For the preparation of fermented soy milk sample, two of the three samples were inoculated with 1 % (v/v) LGG and *L. plantarum* KU210152 culture (separately), and the three soymilk samples were fermented at 42 °C until the pH reached 4.5. To measure the number of viable cells post acidification, the fermented soy milk samples were stored in a refrigerator at 4 °C for 21 d. Three different fermented soy milk samples were prepared and labeled as follows: CY, control fermented soy milk fermented with *S. thermophilus* P206; GY, fermented soy milk with *S. thermophilus* P206 and LGG; and PY, fermented soy milk with *S. thermophilus* P206 and *L. plantarum* KU210152.

2.8. Viable LAB count, pH, and titratable acidity (TA)

Viable cell count was measured by counting the number of colonies on MRS agar plates during soy milk fermentation and after storage for 21 d as described by Song et al. (2021). pH was measured using a pH meter (WTW, Weiheim, Germany). For the measurement of TA of the fermented soy milk samples, 10 g of each sample was mixed with 10 mL of distilled water and titrated with 0.1 N NaOH until the pH increased to 8.3. TA was calculated using the following equation:

$$\text{TA}\,(\%) = \frac{\text{N}}{\text{S}} \times 0.009 \times 100$$

where N, S, and 0.009 indicate the added amount of 0.1 N NaOH (mL), sample weight (g), and conversion factor for lactic acid, respectively.

2.9. Preparation of fermented soy milk supernatant

Supernatant of fermented soy milk was prepared according to the

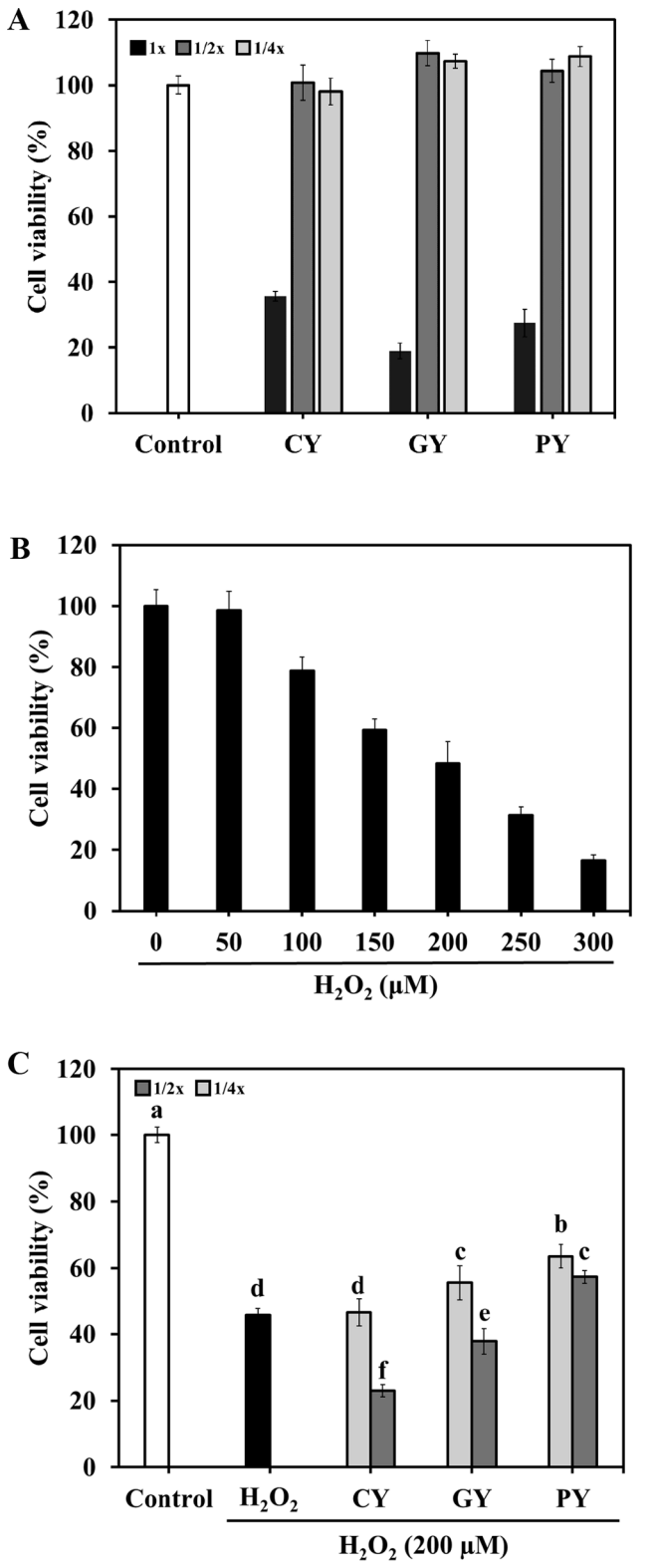

Fig. 6. Neuroprotective effect of fermented soy milk samples on SH-SY5Y cells. (A) Effect of fermented soy milk on SH-SY5Y cells; (B) Effect of H_2O_2 on SH-SY5Y cells; (C) Effect of fermented soy milk sample on H_2O_2-induced SH-SY5Y cells. CY, soy milk fermented with *Streptococcus thermophilus* P206; GY, soy milk fermented with *Lacticaseibacillus rhamnosus* GG (LGG) and *S. thermophilus* P206; PY, soy milk fermented with *Lactiplantibacillus plantarum* KU210152 and *S. thermophilus* P206. Data are presented as mean ± standard deviation of triplicate experiments. Different letters on the error bars indicate significant differences ($P < 0.05$).

method described by Song et al. (2021), with minor modifications. Samples of fermented soy milk (10 g) were mixed with sterile distilled water (2.5 mL) and homogenized. The mixture was centrifuged at 14,240 × *g* for 5 min at 4 °C. The pH of the supernatant was adjusted to 7.0 using 1 M NaOH and centrifuged under the same conditions as mentioned above. The collected supernatant was filtered using a 0.45-μm filter, and the supernatant of fermented soy milk were stored at –20 °C.

2.10. Effect of LAB CM or fermented soy milk supernatant on H_2O_2-induced SH-SY5Y cells

MTT assay was conducted to evaluate the effect of LAB CM or fermented soy milk supernatant according to the method of Cheon et al. (2020). The SH-SY5Y cells were plated in 96-well plates (1×10^6 cells/mL) and incubated for 24 h. After pre-treatment with the LAB CM or fermented soy milk supernatant for 4 h, the cells were incubated with H_2O_2 for 20 h. The medium was discarded, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added. Next, the reacted MTT solution was removed and dimethyl sulfoxide (DMSO) was added. Absorbance of the solution was measured at 570 nm. The cell viability was determined using the following equation:

$$\text{Cell viability}\ (\%) = \frac{\text{As}}{\text{Ac}} \times 100$$

where A_s and A_c represent the absorbance of the cells treated with the sample and control, respectively.

The effects of the LAB CM or fermented soy milk supernatant were also evaluated by observing morphological changes in the SH-SY5Y cells; the cells were treated with the LAB CM or fermented soy milk supernatant and H_2O_2 under the same conditions described above and observed using a Nikon Eclipse Ti2-U fluorescence microscope (Nikon Co., Ltd., Tokyo, Japan) and a DS-Ri2 digital camera (Nikon Co., Ltd).

2.11. ROS generation

SH-SY5Y cells were seeded in 12-well plates (1×10^6 cells/mL) and treated with the fermented soy milk supernatant for 4 h and with H_2O_2 for 20 h. The cells were washed with PBS and loaded with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich). After incubation at 37 °C for 30 min, the cells were washed with PBS again, examined using a Nikon Eclipse Ti2-U fluorescence microscope (Nikon Co., Ltd.), and images were captured using a DS-Ri2 digital camera (Nikon Co., Ltd.). This images were analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.12. Relative gene expression analysis using real-time PCR (RT-PCR)

To determine the expression of apoptosis-related genes and neuronal biomarkers, RT-PCR was performed using the RNA extracted from HT-29 and SH-SY5Y cells. The HT-29 cells were incubated in 6-well plates (5×10^5 cells/mL) until monolayer formation and treated with heat-killed LAB for 24 h. The SH-SY5Y cells were seeded in 6-well plates (1×10^6 cells/mL) and treated with the samples. After 4 h, the cells were treated with H_2O_2 for 3 h. Total RNA was extracted using the RNeasy® Mini kit (Qiagen, Hilden, Germany) and isolated RNA was reverse-transcribed to cDNA using a cDNA synthesis kit (Thermo-Fisher Scientific, Waltham, MA, USA). The expression of apoptosis-related genes (Bcl-2 associated X protein, *Bax*; B-cell lymphoma 2, *Bcl-2*) and neuronal biomarkers (brain-derived neurotrophic factor, *BDNF*; tyrosine hydroxylase, *TH*) was evaluated via RT-PCR using SYBR Green PCR Master Mix. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* was used as the housekeeping gene, and gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table S1 (Bock et al., 2023).

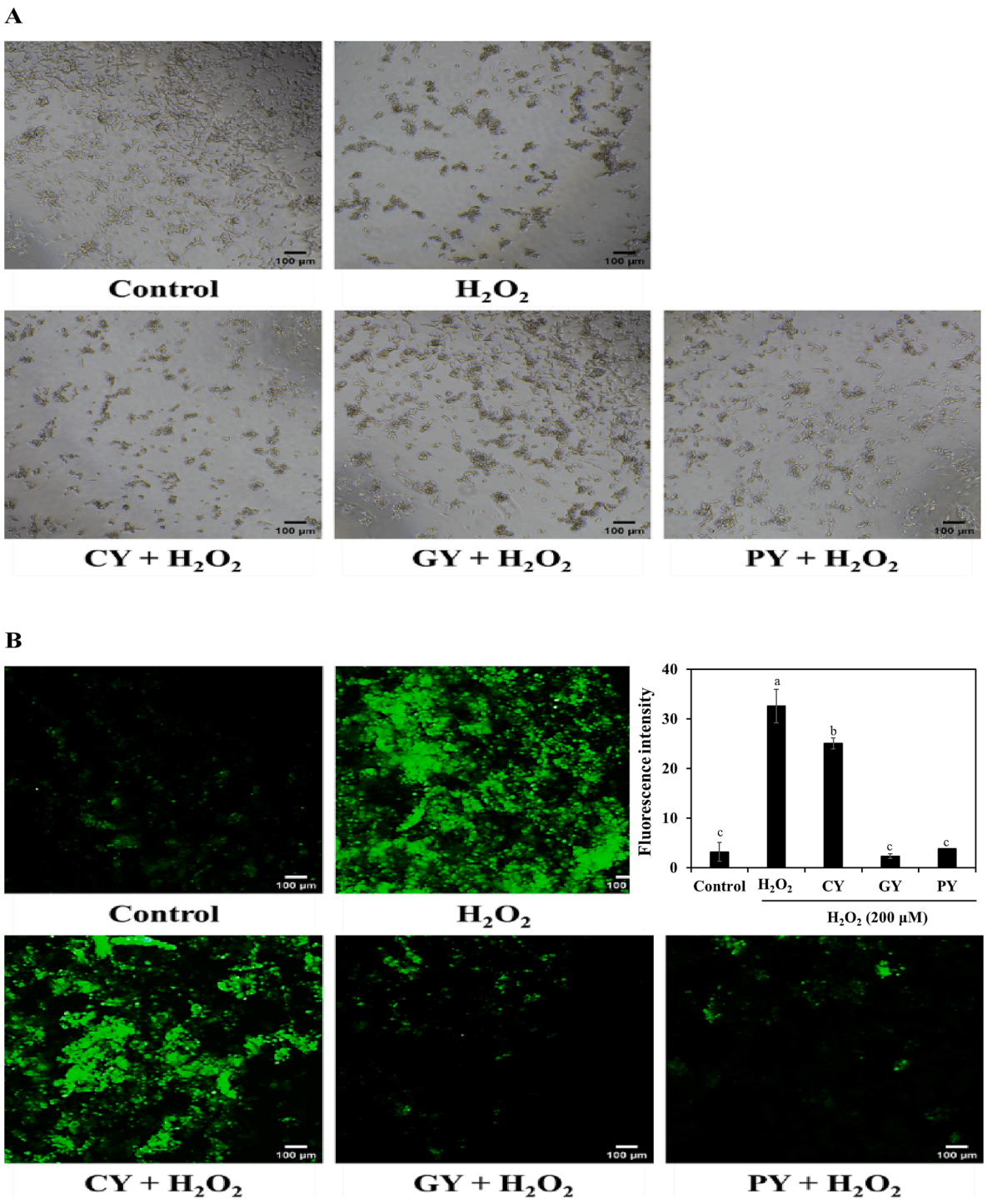

Fig. 7. Effect of fermented soy milk samples on SH-SY5Y cells. (A) Morphological changes; (B) Reactive oxygen species (ROS) generation. CY, soy milk fermented with *Streptococcus thermophilus* P206; GY, soy milk fermented with *Lacticaseibacillus rhamnosus* GG (LGG) and *S. thermophilus* P206; PY, soy milk fermented with *Lactiplantibacillus plantarum* KU210152 and *S. thermophilus* P206. White and black scale bar indicate 100 μm at × 40 magnification. The graph is represented fluorescence intensity of ROS using imageJ. The data are presented as mean ± standard deviation.

2.13. Caspase-9 and caspase-3 activity

The activities of caspase-9 and caspase-3 in the SH-SY5Y cells were determined using caspase-9 assay kit (colorimetric) and caspase-3 assay kit (colorimetric) (Abcam, Cambridge, UK), respectively. The cells were seeded in 6-well plates (1×10^6 cells/mL) and treated with the samples for 4 h and with H_2O_2 for 6 h. According to the manufacturer's instructions, the cell lysate containing 100 μg of protein was mixed in equal amounts with 50 μL of 2 × reaction buffer containing 10 mM DTT (dithiothreitol). Next, 5 μL of the LEHD-*ρ*-NA substrate (4 mM) was added to evaluate caspase-9 activity, and 5 μL of the DEVD-*ρ*-NA substrate (4 mM) was added to evaluate caspase-3 activity. After incubation at 37 °C for 2 h, the absorbance was measured at 400 nm.

2.14. Statistical analysis

All experimental results were acquired in triplicate and are presented as the mean ± standard deviation. One-way analysis of variance

(ANOVA) with Duncan's multiple range test and Student's *t*-test were used to verify significant differences. Values were considered significant at $P < 0.05$, and all data were analyzed using SPSS (IBM, Armonk, NY, USA).

3. Results and discussion

3.1. Tolerance of probiotic strains to simulated gastrointestinal conditions and adhesion ability to HT-29 cells

For application as a probiotics, LAB strains should be tolerant to gastric acid and bile salts (Son et al., 2018). Table 1 shows the gastric acid and bile salt tolerance abilities of the LAB strains. Under acidic gastric conditions, the survival rates of LGG and *L. plantarum* KU210152 cells were 98.67 % and 100.12 %, respectively. LGG and *L. plantarum* KU210152 demonstrated high bile salt tolerances of 94.99 % and 96.68 %, respectively. These results indicated that both LAB strains could survive under gastrointestinal conditions.

For probiotic microorganisms, adhesion ability to intestinal epithelial cells is an essential criterion (Alp & Kuleaşan, 2019). *L. plantarum* KU210152 exhibited higher adhesion to HT-29 cells (5.58 %) than to LGG cells (1.71 %) (Table 1). Therefore, *L. plantarum* KU210152 could be used as a potential probiotic strain.

3.2. Enzyme production

Enzyme production by LAB was investigated to elucidate their potential side-effects and benefits (Table S2). β-Glucuronidase is a harmful enzyme related to carcinogens (Śliżewska et al., 2020). However, LGG and *L. plantarum* KU210152 did not produce β-glucuronidase (Table S2). β-Glucosidase, which hydrolyzes glycosidic bonds, is important for the absorption and bioavailability enhancement of isoflavones during soymilk fermentation (Rekha & Vijayalakshmi, 2011). β-Galactosidase hydrolyzes lactose to glucose, and galactose and can alleviate the symptoms of lactose intolerance (Son et al., 2018). In the present study, both LAB strains produced high amounts of β-galactosidase and β-glucosidase. Thus, *L. plantarum* KU210152 can be safely used as a probiotic and its presence in fermented soymilk and dairy products will be beneficial for the consumers.

3.3. Effect of LAB CM on H_2O_2-induced SH-SY5Y cells

Live cells could not be used because of high cytotoxicity, therefore CM was used for evaluation of neuroprotective effects. The protective effects of LAB CM were evaluated using the MTT assay. Before identifying its neuroprotective effects, the cytotoxicity of LAB CM was evaluated. We observed that in both LGG CM and *L. plantarum* KU210152 CM groups, the cell viability was > 100 % (Fig. 1A). Cell viability was measured by exposing the SH-SY5Y cells to various concentrations of H_2O_2 (0–300 μM; Fig. 1B). At 150 μM H_2O_2, the cell viability was 50.92 %. Therefore, 150 μM H_2O_2 was used for the LAB CM experiments. Exposure to H_2O_2 (150 μM) significantly decreased cell viability to 52.85 %; however, pretreatment with LGG CM and *L. plantarum* KU210152 CM attenuated cell death by 58.75 % and 73.38 %, respectively (Fig. 1C). *L. plantarum* KU210152 CM notably increased cell viability compared to that in the H_2O_2-treated group.

3.4. Effect of LAB CM on morphological changes and ROS production in SH-SY5Y cells

Apoptotic cell death involves morphological changes, such as cell shrinkage, membrane blebbing, condensation of nuclei, and apoptotic cell bodies (Hollville & Martin, 2016). Fig. 2A shows the morphological changes in the SH-SY5Y cells after treatment with H_2O_2 and LAB-CM. The cells treated with H_2O_2 exhibited disappearance of neurites, cell shrinkage, and aggregation. However, *L. plantarum* KU210152 CM protected the cell damage and showed intact neurites in the cells compared with that in the group treated only with H_2O_2.

The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) method was used to measure ROS generation in H_2O_2-induced SH-SY5Y cells. DCFH-DA, a non-fluorescent compound, is hydrolyzed to non-fluorescent DCFH by intracellular esterases. DCFH is oxidized to fluorescent dichlorofluorescein (DCF) (Mehri et al., 2012). As shown in Fig. 2B, the exposure of the cells to H_2O_2 significantly induced ROS generation. However, pretreatment with *L. plantarum* KU210152 CM markedly inhibited ROS production. This suggests that *L. plantarum* KU210152 exhibits its neuroprotective effects by suppressing ROS generation.

3.5. Effect of heat-killed LAB and LAB CM on relative gene expression

BDNF, a member of the neurotrophin family of growth factors, regulates neuronal survival, differentiation, and synaptic plasticity (Binder & Scharfman, 2004). BDNF has therapeutic benefits for neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease (Cheon et al., 2020). TH is a key enzyme that catalyzes the hydroxylation of tyrosine to L-DOPA (Wang et al., 2002). TH suppresses the loss of dopaminergic neurons in the substantia nigra (Wang et al., 2002).

To investigate the effects of heat-killed bacterial cells, RT-PCR was performed using the RNA of treated and untreated HT-29 cells. Both LGG and *L. plantarum* KU210152 increased *BDNF* expression by 1.31- and 1.35-fold, respectively (Fig. 3A). Additionally, *L. plantarum* KU210152 significantly upregulated *TH* expression (1.54-fold). These results suggest that *L. plantarum* KU210152 exerts neuroprotective effects by improving the expression of neuronal biomarkers.

Bcl-2, an anti-apoptotic factor, is located in the outer mitochondrial membrane and inhibits the release of cytochrome *C* (Borner, 2003). Bax, a proapoptotic factor, is present in the cytosol. Translocation of Bax to the mitochondrial membrane increases mitochondrial membrane permeability. Finally, the secretion of cytochrome *C* and activation of the caspase cascade occur, which induce apoptosis (Leist & Jäättelä, 2001). The Bax/Bcl-2 ratio is more appropriate for evaluating apoptosis than comparing the expression of individual genes in evaluating apoptosis (Raisova et al., 2001).

We observed that the cells treated with only H_2O_2 exhibited increase in *Bax/Bcl-2* ratio by 1.83-fold compared to that in the untreated group (Fig. 3B). However, pretreatment with *L. plantarum* KU210152 CM decreased the ratio by 0.91-fold, which is similar to the effect observed by LGG CM (0.95-fold).

The expression of *BDNF* and *TH* decreased in the cells treated with H_2O_2 (Fig. 3C). Treatment with LGG CM or *L. plantarum* KU210152 CM increased *BDNF* expression by 1.36- and 1.93-fold, respectively. Moreover, LGG CM and *L. plantarum* KU210152 CM upregulated the expression of *TH* by 1.42- and 3.25-fold, respectively. The increase in *BDNF* and *TH* expression was higher by *L. plantarum* KU210152 CM than by LGG CM. *L. plantarum* KU210152 exerts notable neuroprotective effects by decreasing the expression of apoptosis-related genes and increasing the expression of neuronal biomarkers.

3.6. Effect of LAB CM on caspase-9 and caspase-3 activity

The release of cytochrome *C* induces the formation of an apoptosome complex by binding to apoptotic protease activation factor-1, which activates caspase-9. Activated caspase-9 activates caspase-3 and caspase-3, which causes DNA fragmentation, degradation, and apoptosis (Vodovotz et al., 2004).

In Fig. 4A, exposure to H_2O_2 increased caspase-9 activity by 156.26 % compared with that in the control group. However, LGG CM and *L. plantarum* KU210152 CM ameliorated the enzyme activity by 101.74 % and 92.37 %, respectively. Caspase-3 activity also decreased following *L. plantarum* KU210152 CM treatment. Enzyme activity

increased in the cells treated with H_2O_2 by 173.37 % (Fig. 4B). After pretreatment with LGG CM or *L. plantarum* KU210152 CM, enzyme activity decreased by 116.86 % and 99.55 %, respectively. These results demonstrate that *L. plantarum* KU210152 CM can prevent the apoptosis of SH-SY5Y cells by inhibiting caspase-9 and caspase-3 activities.

3.7. Changes in pH, TA, and viable LAB during fermentation of soy milk and refrigerated storage

The changes in pH, TA, and viable bacteria count in the three different fermented soy milk samples during fermentation are presented in Fig. 5A–C. The initial pH of the three samples was approximately 6.7. The pH gradually decreased during 6 h of fermentation in all three samples. The final pH values for the CY, GY, and PY samples were 4.6, 4.4, and 4.5, respectively. Moreover, the TA value gradually increased in all three samples; the GY sample exhibited the highest change, from 0.10 % to 0.60 %; this was followed by the PY sample (0.10 %–0.50 %), whereas the CY sample exhibited the lowest increase of 0.10 %–0.47 %. The total number of viable bacterial cells increased gradually. In the three samples, the initial viable cell count was 7.22–7.54 log CFU/mL and the final cell count was 8.87–9.00 log CFU/mL.

During 21 d of refrigerated storage, pH, TA, and viable LAB were measured weekly (Fig. 5D–F). The CY, GY, and PY samples exhibited constant pH and TA values after 21 d. However, the total number of live bacterial cells slightly decreased in all three fermented soy milk samples. After 21 d of storage, the number of viable LAB in the CY sample decreased from 8.80 log CFU/mL to 8.45 log CFU/mL and that in the GY sample decreased from 8.92 log CFU/mL to 8.24 log CFU/mL. The change in the number of viable LAB in the PY sample ranged from 8.62 log CFU/mL to 7.96 log CFU/mL. Among the three types, the CY type exhibited the lowest decrease. The decrease in the GY and PY samples was similar during storage. The decrease in the number of viable LAB depends on nutrient concentration in fermented soy milk (Choi et al., 2022).

3.8. Effect of fermented soy milk on SH-SY5Y cells

The cytotoxicity of fermented soy milk supernatant was measured at three different concentrations (1, 1/2, and 1/4-diluted). The MTT assay revealed that the cell viability of 1-diluted samples of the three fermented soy milk supernatant was < 40 % (Fig. 6A). The three types of 1/2-and 1/4-diluted fermented soy milk supernatant exhibited survival rates of approximately 100 %. The cell viability gradually decreased after exposure to various concentrations of H_2O_2 (0 to 300 μM) in a dose-dependent manner (Fig. 6B). Treatment with 200 μM H_2O_2 resulted in cell viability of 48.32 %. Therefore, 1/2-and 1/4-diluted fermented soy milk supernatant and 200 μM H_2O_2 were used for subsequent fermented soy milk experiments. The cells treated with H_2O_2 exhibited 45.68 % viability (Fig. 6C). Both 1/2-and 1/4-diluted PY samples ameliorated cell viability by 57.38 % and 63.57 %, respectively. The GY sample also exhibited higher cell viability at 1/4-diluted concentration (55.59 %) than that of the H_2O_2 treated group, but its value was lower than that of PY.

3.9. Effect of fermented soy milk on morphological changes and ROS production in SH-SY5Y cells

The protective effects of the PY sample were identified based on the morphological changes in SH-SY5Y cells (Fig. 7A). Treatment with H_2O_2 led to the disappearance of neurites and cell shrinkage. Pretreatment with PY (1/4-diluted) ameliorated the cell damage. The cells treated with PY (1/4-diluted) indicated normal neurites and diminished shrinkage. These results suggested that soy milk fermented with the probiotic *L. plantarum* KU210152 may be an effective functional food against neurodegenerative diseases.

Fig. 7B shows ROS production after the treatment of the cells with fermented soy milk supernatant and H_2O_2. Exposure to only H_2O_2 significantly induced ROS generation compared to that in the control group. Of the three fermented soy milk supernatant, 1/4-diluted PY sample notably attenuated ROS production. In this study, soy milk fermented with *L. plantarum* KU210152 exerted neuroprotective effects by inhibiting ROS generation.

4. Conclusions

In the present study, we evaluated the probiotic properties and neuroprotective effects of *L. plantarum* KU210152. *L. plantarum* KU210152 exhibited high tolerance to artificial gastrointestinal conditions, high adhesion to HT-29 cells, and production of beneficial and safe enzymes. Pre-treatment with *L. plantarum* KU210152 CM attenuated H_2O_2-induced decrease in viability and morphological damage of SH-SY5Y cells. *L. plantarum* KU210152 CM suppressed ROS generation in neurons Moreover, *L. plantarum* KU210152 decreased the expression of apoptosis-related genes and increased the levels of neuronal biomarkers. Caspase-9 and caspase-3 activities were inhibited by pretreatment with *L. plantarum* KU210152 CM. Additionally, the soy milk fermented with *L. plantarum* KU210152 and *S. thermophilus* P206 (PY) sample attenuated the viability and morphological changes of H_2O_2-treated SH-SY5Y cells. The PY sample also suppressed ROS generation. Therefore, *L. plantarum* KU210152 has probiotic, neuroprotective, and antioxidant properties and can be used as a prophylactic ingredient in fermented foods to prevent neurodegenerative diseases.

CRediT authorship contribution statement

Hyun-Ji Bock: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Hye-Won Lee:** Investigation, Methodology. **Na-Kyoung Lee:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Hyun-Dong Paik:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113868.

References

Alp, D., & Kuleaşan, H. (2019). Adhesion mechanisms of lactic acid bacteria: Conventional and novel approaches for testing. *World Journal of Microbiology and Biotechnology, 35*, 1–9.

Binder, D. K., & Scharfman, H. E. (2004). Brain-derived neurotrophic factor. *Growth Factors, 22*, 123.

Bock, H. J., Lee, N. K., & Paik, H. D. (2023). Neuroprotective effects of heat-killed *Levilactobacillus brevis* KU15152 on H_2O_2-induced oxidative stress. *Journal of Microbiology and Biotechnology, 33*, 1–8.
Borner, C. (2003). The Bcl-2 protein family: Sensors and checkpoints for life-or-death decisions. *Molecular Immunology, 39*, 615–647.
Cheon, M. J., Lim, S. M., Lee, N. K., & Paik, H. D. (2020). Probiotic properties and neuroprotective effects of *Lactobacillus buchneri* KU200793 isolated from Korean fermented foods. *International Journal of Molecular Science, 21*, 1227.
Choi, G. H., Bock, H. K., Lee, N. K., & Paik, H. D. (2022). Soy yogurt using *Lactobacillus plantarum* 200655 and fructooligosaccharides: Neuroprotective effects against oxidative stress. *Journal of Food Science and Technology, 59*, 4870–4879.
Cryan, J. F., & O'Mahony, S. M. (2011). The microbiome-gut-brain axis: From bowel to behavior. *Neurogastroenterology & Motility, 23*, 187–192.
Cryan, J. F., O'Riordan, K. J., Cowan, C. S. M., Sandhu, K. V., Bastiaanssen, T. F. S., Boehme, M., & Dinan, T. G. (2019). The microbiota-gut-brain axis. *Physiological Reviews, 99*, 1877–2013.
Dasuri, K., Zhang, L., & Keller, J. N. (2013). Oxidative stress, neurodegeneration, and the balance of protein degradation and protein synthesis. *Free Radical Biology and Medicine, 62*, 170–185.
De Marco, S., Sichetti, M., Muradyan, D., Piccioni, M., Traina, G., Pagiotti, R., & Pietrella, D. (2018). Probiotic cell-free supernatants exhibited anti-inflammatory and antioxidant activity on human gut epithelial cells and macrophages stimulated with LPS. *Evidence-Based Complementary and Alternative Medicine, 2018*, 1756308.
Farnworth, E. R., Mainville, I., Desjardins, M. P., Gardner, N., Fliss, I., & Champagne, C. (2007). Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. *International Journal of Food Microbiology, 116*, 174–181.
Gazerani, P. (2019). Probiotics for Parkinson's disease. *International Journal of Molecular Sciences, 20*, 4121.
Hollville, E., & Martin, S. J. (2016). Measuring apoptosis by microscopy and flow cytometry. *Current Protocols in Immunology, 112*, 14–38.
Ishii, T., Furuoka, H., Kaya, M., & Kuhara, T. (2021). Oral administration of probiotic *Bifidobacterium breve* improves facilitation of hippocampal memory extinction via restoration of aberrant higher induction of neuropsin in an MPTP-induced mouse model of Parkinson's disease. *Biomedicines, 9*, 167.
Leist, M., & Jäättelä, M. (2001). Four deaths and a funeral: From caspases to alternative mechanisms. *Nature Reviews Molecular Cell Biology, 2*, 589–598.
Mehri, S., Abnous, K., Mousavi, S. H., Shariaty, V. M., & Hosseinzadeh, H. (2012). Neuroprotective effect of crocin on acrylamide-induced cytotoxicity in PC12 cells. *Cellular and Molecular Neurobiology, 32*, 227–235.
Mörkl, S., Butler, M. I., Holl, A., Cryan, J. F., & Dinan, T. G. (2020). Probiotics and the microbiota-gut-brain axis: Focus on psychiatry. *Current Nutrition Reports, 9*, 171–182.
Nissanka, N., & Moraes, C. T. (2018). Mitochondrial DNA damage and reactive oxygen species in neurodegenerative disease. *FEBS Letters, 592*, 728–742.
Oh, N. S., Joung, J. Y., Lee, J. Y., & Kim, Y. (2018). Probiotic and anti-inflammatory potential of *Lactobacillus rhamnosus* 4B15 and *Lactobacillus gasseri* 4M13 isolated from infant feces. *PLoS One, 13*, e0192021.
Omoni, A. O., & Aluko, R. E. (2005). Soybean foods and their benefits: Potential mechanisms of action. *Nutrition Reviews, 63*, 272–283.
Patten, D. A., Germain, M., Kelly, M. A., & Slack, R. S. (2010). Reactive oxygen species: Stuck in the middle of neurodegeneration. *Journal of Alzheimer's Disease, 20*, S357–S367.
Pramanik, S., Venkatraman, S., Karthik, P. A., & Vaidyanathan, V. K. (2023). Systematic review on selection characterization and implementation of probiotics in human health. *Food Science and Biotechnology, 32*, 423–440.
Raisova, M., Hossini, A. M., Eberle, J., Riebeling, C., Orfanos, C. E., Geilen, C. C., & Daniel, P. T. (2001). The Bax/Bcl-2 ratio determines the susceptibility of human melanoma cells to CD95/Fas-mediated apoptosis. *Journal of Investigative Dermatology, 117*, 333–340.
Rekha, C. R., & Vijayalakshmi, G. (2011). Isoflavone phytoestrogens in soymilk fermented with β-glucosidase producing probiotic lactic acid bacteria. *International Journal of Food Sciences and Nutrition, 62*, 111–120.
Rui, X., Zhang, Q., Huang, J., Li, W., Chen, X., Jiang, M., & Dong, M. (2019). Does lactic fermentation influence soy yogurt protein digestibility: A comparative study between soymilk and soy yogurt at different pH. *Journal of the Science of Food and Agriculture, 99*, 861–867.
Sharma, N., Kang, D. K., Paik, H. D., & Park, Y. S. (2023). Beyond probiotics: A narrative review on an era of revolution. *Food Science and Biotechnology, 32*, 413–421.
Śliżewska, K., Markowiak-Kopeć, P., & Śliżewska, W. (2020). The role of probiotics in cancer prevention. *Cancers, 13*, 20.
Son, S. H., Jeon, H. L., Yang, S. J., Sim, M. H., Kim, Y. J., Lee, N. K., & Paik, H. D. (2018). Probiotic lactic acid bacteria isolated from traditional Korean fermented foods based on β-glucosidase activity. *Food Science and Biotechnology, 27*, 123–129.
Song, M. W., Chung, Y., Kim, K. T., Hong, W. S., Chang, H. J., & Paik, H. D. (2020). Probiotic characteristics of Lactobacillus brevis B13–2 isolated from kimchi and investigation of antioxidant and immune-modulating abilities of its heat-killed cells. *LWT-Food Science and Technology, 128*, Article 109452.
Song, M. W., Park, J. Y., Lee, H. S., Kim, K. T., & Paik, H. D. (2021). Co-fermentation by *Lactobacillus brevis* B7 improves the antioxidant and immunomodulatory activities of hydroponic ginseng-fortified yogurt. *Antioxidants, 10*, 1447.
Valero-Cases, E., Cerdá-Bernad, D., Pastor, J. J., & Frutos, M. J. (2020). Non-dairy fermented beverages as potential carriers to ensure probiotics, prebiotics, and bioactive compounds arrival to the gut and their health benefits. *Nutrients, 12*, 1666.
Vodovotz, Y., Kim, P. K., Bagci, E. Z., Ermentrout, G. B., Chow, C. C., Bahar, I., & Billiar, T. R. (2004). Inflammatory modulation of hepatocyte apoptosis by nitric oxide: *In vivo*, *in vitro*, and *in silico* studies. *Current Molecular Medicine, 4*, 753–762.
Wang, Z. H., Ji, Y., Shan, W., Zeng, B., Raksadawan, N., Pastores, G. M., & Kolodny, E. H. (2002). Therapeutic effects of astrocytes expressing both tyrosine hydroxylase and brain-derived neurotrophic factor on a rat model of Parkinson's disease. *Neuroscience, 113*, 629–640.
Westfall, S., Lomis, N., Kahouli, I., Dia, S. Y., Singh, S. P., & Prakash, S. (2017). Microbiome, probiotics and neurodegenerative diseases: Deciphering the gut brain axis. *Cellular and Molecular Life Sciences, 74*, 3769–3787.
Yeo, J. D. (2023). Influence of food-derived bioactives on gut microbiota compositions and their metabolites by focusing on neurotransmitters. *Food Science and Biotechnology, 32*, 1019–1027.