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Probiotic and Antioxidant Properties of Novel *Lactobacillus brevis* KCCM 12203P Isolated from Kimchi and Evaluation of Immune-Stimulating Activities of Its Heat-Killed Cells in RAW 264.7 Cells S

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The purpose of this study was to determine the probiotic properties of *Lactobacillus brevis* KCCM 12203P isolated from the Korean traditional food kimchi and to evaluate the antioxidative activity and immune-stimulating potential of its heat-killed cells to improve their bio-functional activities. *Lactobacillus rhamnosus* GG, which is a representative commercial probiotic, was used as a comparative sample. Regarding probiotic properties, *L. brevis* KCCM 12203P was resistant to 0.3% pepsin with a pH of 2.5 for 3 h and 0.3% oxgall solution for 24 h, having approximately a 99% survival rate. It also showed strong adhesion activity (6.84%) onto HT-29 cells and did not produce β-glucuronidase but produced high quantities of leucine arylamidase, valine arylamidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase. For antioxidant activity, it appeared that viable cells had higher radical scavenging activity in the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay, while in the 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, heat-killed cells had higher antioxidant activity. Additionally, *L. brevis* KCCM 12203P showed higher lipid oxidation inhibition ability than *L. rhamnosus* GG; however, there was no significant difference ($p < 0.05$) between heat-killed cells and control cells. Furthermore, heat-killed *L. brevis* KCCM 12203P activated RAW 264.7 macrophage cells without cytotoxicity at a concentration lower than 10^8 CFU/ml and promoted higher gene expression levels of inducible nitric oxide synthase, interleukin-1β, and interleukin-6 than *L. rhamnosus* GG. These results suggest that novel *L. brevis* KCCM 12203P could be used as a probiotic or applied to functional food processing and pharmaceutical fields for immunocompromised people.

Keywords: *Lactobacillus brevis*, probiotics, heat-killed bacteria, antioxidant activity, immune-stimulating activity

Introduction

The human life span is being prolonged by rapid advancements in medicine and food technology, and the average life expectancy has increased by 2.5 years every 10 years since 1850 [1]. However, the western diet has increased the risk of several diseases such as hypertension, obesity, diabetes, and hyperlipidemia [2]. In addition, these modern lifestyles could also affect dysbiosis of the microbiome in the human intestine. Microbial dysbiosis, which is a functional and compositional alteration of the intestinal microbial ecosystem, allows pathogens to invade the host immune system more easily. Although the specific mechanisms for how gut homeostasis increases the defense system of the host and which microorganisms improve health conditions of human-beings have not reported clearly, many previous studies have indicated that intestinal homeostasis plays significant roles in maintaining immunity and susceptibility against internal or external pathogens in human and animal models [3].

Consumption of probiotics could be one of the therapeutic strategies to improve the homeostasis of intestinal microbiota to ameliorate gastrointestinal diseases caused by gut dysbiosis. The role of probiotics in the alteration of the gut microbiome has been verified clinically in several gastrointestinal diseases such as inflammatory bowel syndrome [4]. For example, it was reported that *Lactobacillus rhamnosus*, a typical probiotic strain, could reinforce the diversity of microbial composition by modulating the colon microecology and preventing aberrant crypt foci formation in a rat model [4]. Lactic acid bacteria (LAB) are major strains of microflora in Asian fermented foods such as Japanese natto, Korean makgeolli, cheonggukjang, and kimchi. They are crucially important for the unique taste and flavor of each food product [5], and fermentation by LAB offers various health benefits through formation of antioxidant, antihypertensive, anti-hyperglycemic, and antimicrobial substances [6–9]. The survival ability of probiotics in the human digestive track is one of the most important factors for the fermented food benefits. Although the inherent gastrointestinal acid tolerance of probiotics is remarkable, it was reported that the viability of probiotics could be affected depending on the food matrix [10]. Therefore, consistent intake of probiotics with other food products is recommended to improve health.

Many studies presented various biological activities of dead (usually heat-killed) probiotic cells or their cellular components. For instance, heat-killed LAB have been known to have a variety of advantages including immunomodulatory activity, extension of shelf-life of food products, convenient transportation, and stable preservation [11]. In in vivo testing, heat-killed *Enterococcus faecalis* FK-23 treatment was reported to increase the phagocytic ability of neutrophils by nearly 1.4-fold in healthy dog models [12]. Chick feed supplemented with EC-12, which is a commercial heat-killed *Enterococcus faecalis* product, was reported to enhance gastrointestinal immunity by increasing the serum IgA and IgG levels in hatched chicks [13]. Sashihara *et al*. [14] also showed that administration of *Lactobacillus gasseri* to ovalbumin-sensitized BALB/c mice elevated IL-12 gene expression and suppressed serum antigen-specific IgE levels. It was also reported that heat-killed or γ-irradiated *Lactobacillus reuteri* had a pain relief effect in model rats with colorectal distension [15].

Therefore, the purposes of this study were to investigate the probiotic properties of *Lactobacillus brevis* KCCM 12203P isolated from kimchi as a functional food material and to evaluate the antioxidant and immune stimulating activity of heat-killed cells to improve their bio-functional activity in heat-processed food products.

Materials and Methods

Bacterial Strain Isolated from Kimchi, Culture Condition and Heat Treatment

Lactobacillus brevis KCCM 12203P was isolated from the Korean traditional food kimchi and cultured in lactobacilli Man, Rogosa, and Sharpe (MRS) broth (Difco, BD Biosciences, USA) at 37°C for

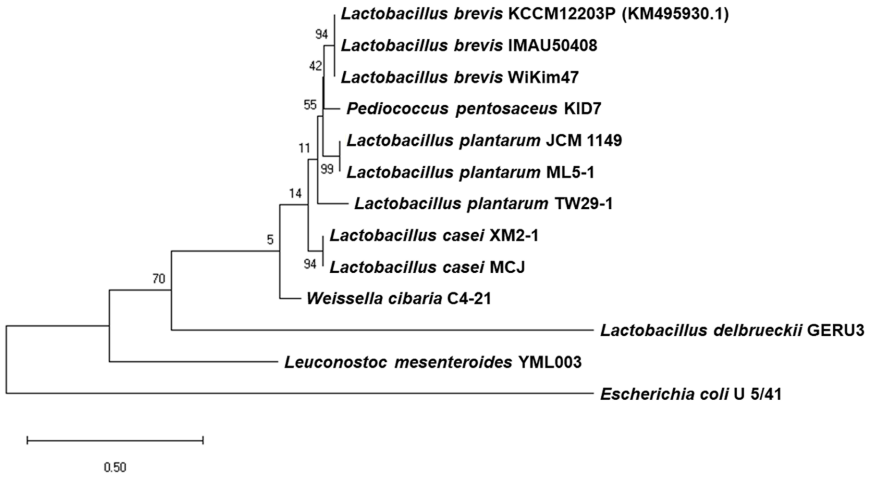

Fig. 1. Phylogenetic tree of *L. brevis* KCCM 12203P with other lactic acid bacteria and *Escherichia coli* based on partial 16S rRNA gene sequence.

Phylogenetic relation of *L. brevis* KCCM12203P with other strain was built by the neighbor-joining method with 1,000 bootstrap using MEGA-X software and its accession number was noted beside the strain number. The scale bar and number at the bottom indicated the 0.5 substitutions per nucleotide.

15 h. For identification of isolate, the 16S rRNA gene sequencing method was used (Table S1), and the result was described as a phylogenetic tree using MEGA-X software (Fig. 1). *Lactobacillus rhamnosus* GG as a reference strain for probiotic properties was obtained from the Korean Collection for Type Cultures (KCTC, Korea). Each cultured strain was centrifuged at 14,000 ×*g* at 4°C for 5 min. Each harvested cell was washed twice and re-suspended in phosphate-buffered saline (PBS; Hyclone, USA). Heat-killed samples were prepared by heating the harvested cells in a water bath at 85°C for 30 min.

Tolerance of Artificial Digestive Tract Conditions

The tolerances of strains against artificial gastric and enteric conditions were examined according to the method of Lee *et al*. [16]. To simulate the gastric and enteric phase, the cultured strains at a cell concentration of 1 × 10^8 CFU/ml were inoculated in MRS broth (pH 2.5) with 0.3% (w/v) pepsin (Sigma-Aldrich, USA) at 37°C for 3 h and normal MRS broth supplemented with 0.3% (w/v) oxgall (Difco, BD Biosciences) at 37°C for 24 h, respectively. After incubation, cells were enumerated by the standard plate count (SPC) method.

The survival rate of each LAB strain was calculated as follows:

$$\text{Survival rate (\%)} = \frac{A}{B} \times 100$$

where A is a number of bacterial cells after incubation in gastric conditions (log CFU/ml) and B is the initial number of bacterial cells (log CFU/ml)

Adhesion Assay in HT-29 Cells

HT-29 cells (human colon adenocarcinoma) were obtained from the Korean Cell Line Bank (KCLB, Korea) for the adhesion assay. The cell line was incubated in Roswell Park Memorial Institute 1640 medium (RPMI; Hyclone, Logan) with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (P/S) at 37°C in a 5% CO_2 incubator (MCO-18AIC, SANYO Co., Japan)

To evaluate adhesion ability of the LAB strains in HT-29 cells, the cells were cultured at a concentration of 10^5 cells/well in 24-well culture plates and incubated for 24 h. Thereafter, 100 µl of washed bacterial samples (1 × 10^9 CFU/well) were added to adhered cells and incubated for 2 h. The wells were washed three times with PBS and treated with Triton X-100 (Sigma-Aldrich) solution to remove the bacterial cells. The adhesion ability of the strains to the HT-29 cells was measured by a plate counting method on MRS agar and calculated as follows:

$$\text{Adhesion ability (\%)} = \frac{\text{The number of bacteria after incubation (CFU/ml)}}{\text{The number of bacteria before incubation (CFU/ml)}} \times 100$$

API ZYM Kit Assay for Enzyme Production

The production of various enzymes of *L. brevis* KCCM 12203P was determined by using the API ZYM kit (BioMerieux, France). The cells were washed with PBS and then diluted to 10^7 CFU/ml. A sample (75 µl) was inoculated into each cupule and incubated at 37°C for 4 h. Subsequently, ZYM A and ZYM B were added to each cupule. The level of enzyme production was measured through the degree of color change.

2,2-Diphenyl-1-Picryl-hydrazyl (DPPH) Radical Scavenging Assay

Antioxidant activity of LAB samples was measured by the DPPH assay as described by Yang *et al*. [17] with minor modifications. Five hundred microliters of DPPH solution (0.4 mM) in ethyl alcohol was added to 500 µl of viable and heat-killed bacteria samples (10^9 CFU/ml). The mixtures were stirred at 25°C for 30 min in dark conditions and centrifuged at 14,000 ×*g* for 1 min. The absorbance of supernatants was measured at 517 nm, and radical scavenging activity was determined as follows:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_s}{A_c}\right) \times 100$$

where A_s and A_c are the absorbance value of the sample and control, respectively.

2-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) Radical Scavenging Assay

The ABTS assay for antioxidant activity of LAB was carried out as described by Jang *et al*. [18] with minor modifications. The ABTS solution was prepared by mixing 14 mM ABTS and 5 mM potassium persulfate in 0.1 M potassium phosphate buffer (pH 7.4) and incubated at 25°C for 12–16 h under dark conditions. Reacted ABTS solution was diluted with 0.1 M potassium phosphate buffer (pH 7.4) until the final absorbance reached 0.7 at 734 nm. Subsequently, a mixture of ABTS solution and samples (1:1 v/v) were incubated at 25°C for 15 min. After centrifugation at 14,000 ×*g* for 1 min, the absorbance of the supernatant was measured at 734 nm, and radical scavenging activity was determined as follows:

$$\text{ABTS radical scavenging activity (\%)} = \left(1 - \frac{A_s}{A_c}\right) \times 100$$

where A_s and A_c are absorbance values of the sample and control, respectively.

Lipid Oxidation Inhibitory Assay

The β-carotene bleaching inhibition assay was performed according to the method of Kachouri *et al*. [19]. β-Carotene solution was composed of 2 mg of β-carotene, 44 µl of linoleic acid, and 200 µl of Tween 80 dissolved in 10 ml of chloroform. To eliminate chloroform, the mixed solution was evaporated using a rotary evaporator at 40°C, and the final absorbance at 470 nm of the solution was adjusted to 1.8. A mixture of the samples and solution above (1:9) was incubated at 50°C in a water bath for 2 h. After centrifugation, the absorbance was measured at 470 nm, and β-carotene bleaching inhibitory activity was measured as follows:

$$\text{β-Carotene bleaching inhibitory activity (\%)} = \frac{A_{\text{sample, 2 h}} - A_{\text{control, 2 h}}}{A_{\text{control, 0 h}} - A_{\text{control, 2 h}}}$$

Cytotoxicity and Nitric Oxide (NO) Production Ability of LAB in RAW 264.7 Cells

A murine macrophage RAW 264.7 cell line (procured from KCLB, Korea) was used to assess cytotoxicity and immune-stimulating potential. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone) with 10% (v/v) FBS and 1% (v/v) P/S at 37°C in a 5% CO_2 incubator. The amount of NO produced and MTT assay were evaluated using the method described by Jeon *et al*. [20] and Lee *et al*. [16], respectively.

The cultured medium (100 µl) of RAW 264.7 cells was added at a density of 2 × 10^6 cells/well and incubated for 2 h, and 50 µl of bacterial sample was added to each well. The treatments with and without 50 µl of lipopolysaccharide solution (LPS, 1.0 ng/ml) were used as a positive and negative control, respectively. After 24 h incubation, 100 µl of Griess reagent was mixed with a supernatant (100 µl) of the RAW 264.7 cells media described above and then absorbance was measured at 540 nm with a microplate reader (Molecular Devices, USA). The produced NO concentration was evaluated by comparison with a standard curve of sodium nitrite.

MTT assay was performed to determine cytotoxicity of strains using RAW 264.7 cells. RAW 264.7 cells were washed twice with PBS and 100 µl of MTT reagent (0.5 mg/ml) dissolved with Dulbecco's PBS was added to each wells. After 1 h incubation, MTT reagent then was discarded and 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve formed formazan as a reactant between MTT reagent and metabolite of live cells. The absorbance (A) was measured at 570 nm and cytotoxicity was calculated as contrasted with the result of a negative control group as follows.

$$\text{Cytotoxicity (\%)} = \frac{A_{\text{sample}}}{A_{\text{negative control}}} \times 100$$

RNA Extraction and Real-Time PCR Analysis

To investigate immune-stimulating abilities, the RNA of RAW 264.7 cells was extracted as described by Chang *et al*. [21]. The cells were seeded into a 6-well plate (1 × 10^6 cells/well) and incubated for 24 h. Subsequently, 1 ng/ml of LPS and heat-killed samples (10^8 cells/ml) were transferred into each well and incubated for an additional 24 h. The RNA was extracted using the RNeasy Mini Kit (QIAGEN, Germany), and the cDNA synthesis Kit (Thermo Fisher Scientific, USA) was used for cDNA synthesis, following the manufacturer's instructions. Three kinds of immune-stimulating factors (TNF-α, IL-1β, and IL-6) and inducible nitric oxide synthase were used to evaluate immune-stimulating activity, and β-actin was measured as a reference gene. The gene expression levels of each factor were determined by real-time PCR (PikoReal 96, Scientific Pierce, USA) with SYBR green fluorescence. The primer sequences of different cytokines are listed in Table 1. The relative gene expression level was calculated by $2^{-\Delta\Delta Cq}$ against the endogenous gene β-actin. Each stimulated sample was compared to RAW cells without LPS stimulation. All cell-related assays and the RT-PCR assay were conducted in triplicate. The reaction specificity of the RT-PCR results was verified by melting curve analysis.

Table 1. Primer sequences of immune-modulating mediators for real-time PCR.

Primer[a]		Sequence (5′ to 3′)
β-Actin	(Forward)	GTGGGCCGCCCTAGGCACCAG
	(Reverse)	GGAGGAAGAGGATGCGGCAGT
iNOS	(Forward)	CCCTTCCGAAGTTTCTGGCAGC
	(Reverse)	GGCTGTCAGAGCCTCGTGGCTTTGG
TNF-α	(Forward)	TTGACCTCAGCGCTGAGTTG
	(Reverse)	CCTGTAGCCCACGTCGTAGC
IL-1β	(Forward)	CAGGATGAGGACATGAGCACC
	(Reverse)	CTCTGCAGACTCAAACTCCAC
IL-6	(Forward)	GTACTCCAGAAGACCAGAGG
	(Reverse)	TGCTGGTGACAACCACGGCC

[a]iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1 β; IL-6, interleukin-6.

Statistical Analysis

The results for each experiment were obtained in at least triplicate and expressed as the means ± standard deviations. The mean values of two different species were analyzed by an independent sample *t*-test. One-way analysis of variance (ANOVA) followed by Duncan's multiple range test were carried out to determine the degree of significant differences. Differences of means with $p < 0.05$ were considered significant, and all analyses were conducted using SPSS software.

Results and Discussion

Artificial Gastric Acid and Bile Salt Tolerance

Probiotic bacteria pass through the gastrointestinal tract, which has an acidic environment (pH 1.5–3.5) and bile salt (pH 5.0–6.0), and should be able to tolerate these circumstances [22]. Table 2 shows the tolerances of *L. rhamnosus* GG and *L. brevis* KCCM 12203P, and the survival rate of *L. rhamnosus* GG was 101.31% and 102.51% in gastric acid and bile salt conditions, respectively. Those of *L. brevis* KCCM 12203P were 99.71% and 107.04%, respectively. In previous studies, *L. plantarum* Lb41 isolated from kimchi decreased by 0.06 log CFU/ml and 1.36 log CFU/ml under gastric and bile acid conditions, respectively [20]. Son *et al*. [22] reported that *L. brevis* FI10700 decreased 0.04 log CFU/ml at pH 1.5 and Park *et al*. [23] reported that *Pediococcus strains* isolated from *makgeolli* had a high acid tolerance (more than 80%). According to these results, a tolerance ability of *L. brevis* KCCM 12203P indicates a higher survival rate in the human digestive tract.

Table 2. Tolerance ability against artificial gastric acid and bile salt and adhesion ability to HT-29 cells of *L. rhamnosus* GG and *L. brevis* KCCM 12203P.

Treatment	Cell number (Log CFU/ml)	
	L. rhamnosus GG	*L. brevis* KCCM 12203P
Tolerance to artificial gastric acid and bile salts		
Initial cell number	8.62 ± 0.05	8.08 ± 0.08
pH 2.5, 0.3% (w/v) pepsin, 3 h	8.73 ± 0.01 (101.31 ± 0.14[a])[1]	8.08 ± 0.02 (99.71 ± 0.27[b])
0.3% (w/v) oxgall, 24 h	8.83 ± 0.02 (102.51 ± 0.23[b])	8.66 ± 0.01 (107.04 ± 0.24[a])
Adhesion ability to HT-29 cells		
Initial cell number	8.67 ± 0.04	8.72 ± 0.04
Adherent cell number	7.46 ± 0.07 (6.21 ± 0.93[a])[2]	7.55 ± 0.04 (6.84 ± 0.58[a])

[a,b]Values are means ± standard deviation of triplicate measurement. For different lactic acid bacteria in same experiment, means with different superscript letters (a, b) were significantly different ($p < 0.05$, Student's *t*-test).

[1]Survival rate from acid and bile salt tolerance assay (%).

[2]Adhesion ratio to HT-29 cells (%).

Ability to Adhere to HT-29 Cells

The adherence ability of LAB is one of its probiotic potential indicators and is a strain-specific property. A wide range of health benefits of probiotics are related to composition of gut microflora through the attachment onto epithelial cells and mucosal surfaces, and thus colonization capacity in the intestine is a crucial parameter indicating functional properties [24]. The adhesion process involves a complex interaction between the bacterial cell membrane and host cell surfaces. The adhesion degree of bacteria is positively correlated with the saturated number of bacterial cells on binding sites on epithelial cells. It was suggested that surface-layer binding of proteins such as mucin-binding protein in beneficial bacterial could promote colonization on gut epithelial cells [25]. Another important factor for effective adhesion is cell surface hydrophobicity and electrostatic forces of bacteria, although the process and pathway of interaction are still unknown [26].

As shown in Table 2, *L. rhamnosus* GG and *L. brevis* KCCM 12203P had 6.21% and 6.84% adhesion ability on HT-29 cells after 2 h incubation, respectively. Generally, it is known that members of the *Lactobacillus* genus possess adherent ability ranging from 2% to 10% [17, 27, 28]. *L. brevis* KCCM 12203P had high adhesion ability to intestinal epithelial cells and this result was little higher than that of *L. rhamnosus* GG.

Enzyme Production Assay Using the API ZYM Kit

To measure enzyme production ability, the API ZYM kit, which is a rapid tool for the detection of bacterial enzymes, was used. This assay serves as a crucial indicator for detection of carcinogenic enzymes such as β-glucuronidase [30]. In the lumen of the intestine, β-glucuronidase hydrolyze glucuronides to glucuronic acid and aglycone, which produce harmful and carcinogenic substances. Generally, glucuronide is removed with bile in the liver; however, bacterial β-glucuronidase regenerates these toxic aglycones in the bowel. Some microorganisms such as *Escherichia coli*, *Clostridium perfringens*, *Bacteroides vulgatus*, and *Ruminococcus gnavus* were detected in colorectal cancer patients [29].

The *L. brevis* KCCM 12203P strain showed weak production ability of almost all enzymes tested with the API ZYM kit (Table 3). However, these strains did not show the productive ability of β-glucuronidase, which is a tumorigenic enzyme.

Table 3. Enzyme production of *L. brevis* KCCM 12203P strain determined using the API ZYM kit.

Enzymes	Enzyme activity[1)]	Enzymes	Enzyme activity
	L. brevis KCCM 12203P		*L. brevis* KCCM 12203P
Control	0	Acid phosphatase	1
Alkaline phosphate	0	Naphthol-AS-BI-phosphohydrolase	1
Esterase	0	α-Galactosidase	1
Esterase lipase	0	β-Galactosidase	5
Lipase	0	β-Glucuronidase	0
Leucine arylamidase	5	α-Glucosidase	1
Valine arylamidase	4	β-Glucosidase	1
Cystine arylamidase	0	*N*-Acetyl-β-glucosaminidase	3
Trypsin	0	α-Mannosidase	0
α-Chymotrypsin	0	α-Fucosidase	0

[1]0, 0 nM; 1, 5 nM; 2, 10 nM; 3, 20 nM; 4, 30 nM; 5, ≥40 nM.

Antioxidant Activity of Live and Heat-Killed LAB

Oxidative stress results from an imbalance between reactive oxygen species (ROS) and antioxidant activity. If the accumulated ROS overrun the alleviating capacity of intrinsic antioxidant scavengers, the components of cells or tissues are damaged by uncontrollable oxidation. External supplements or treatments with antioxidant ability are required to reduce oxidative stress because the individual's antioxidant capacity (AOC) is limited.

Several antioxidant assays, which are related to stable non-biological radicals, superoxide anion ($\bullet O_2^-$), and hydrogen peroxide (H_2O_2), were performed to measure the antioxidative radical scavenging activity of probiotics or their products [30]. In this study, the DPPH and ABTS radical scavenging assay and lipid peroxidation inhibition assay were performed to determine the antioxidant capacity of viable and heat-killed *L. brevis* KCCM 12203P (Table 4). The DPPH radical scavenging activity of viable *L. brevis* KCCM 12203P was 25.66%, a slightly higher result than that of the reference strain. However, heat-killed bacteria showed a 3% decrease in DPPH radical scavenging ability. For ABTS, both heat-killed *L. rhamnosus* GG (37.10%) and *L. brevis* KCCM 12203P (22.07%) revealed greater radical scavenging activity (10% more) than viable cells. These opposing patterns may be caused by the inherent properties of ABTS and DPPH, which are hydrophilic and hydrophobic, respectively [31]. In the β-carotene bleaching inhibition assay, *L. brevis* KCCM 12203P (56–58%) showed higher values than *L. rhamnosus* GG (44–46%); however, there were no significant differences between sample conditions.

The protective ability of probiotics against oxidative stress is illustrated by metal ion chelation, enzyme inhibition, ROS scavenging, and reduction or inhibition of ascorbate autoxidation [32]. Antioxidative compounds of LAB have been considered as antioxidant enzymes, bioactive peptides, bacterial exopolysaccharides, and manganese ions. A few studies recently reported that some peptides of *L. rhamnosus* eliminated oxygen radicals and that cell surface protein or polysaccharides of *L. plantarum* C88 reduced free radicals [33]. Through molecular analysis, the *trxB1* and *uvrA* genes, which encode thioredoxin reductase and subunit A of the excinuclease ABC complex, respectively, have been suggested to play a key role in reducing oxidative and acid stress [34]. Moreover, several enzymatic reactions of intestinal microflora could produce bioactive dietary antioxidants by bioconversion processes using dietary substances [34].

Cytotoxicity and NO Productive Capacity of Viable and Heat-Killed LAB

MTT and NO assays were used to measure the cytotoxicity and NO productivity of *L. rhamnosus* GG and *L. brevis* KCCM 12203P in RAW 264.7 cells (Table 5). Results of viable cells at 10^9 CFU/ml and heat-killed cells 10^6 CFU/ml of two strains are not presented in Table 5 because of high cytotoxicity and low NO production, respectively. In MTT assay, it appeared that viable (10^6 CFU/ml) and heat-killed cells (10^8-10^7 CFU/ml) showed high viability of RAW 264.7 cells (> 80%). In addition, *L. rhamnosus* GG exhibited more cytotoxicity than *L. brevis* KCCM 12203P at all concentrations of viable cells.

The NO assay is a rapid and convenient tool for detection of immune response. NO is produced by not only macrophages but also various immune cells through the gene expression of inducible nitric oxide synthase (iNOS), and these cells are activated by several cytokines and microbial substances. The major functions of NO are anti-microbial, anti-tumor, tissue-damaging, anti-inflammatory, and immunosuppressive effects [35].

NO production of *L. rhamnosus* GG and *L. brevis* KCCM 12203P is shown in Table 5. It appeared that viable cells of both strains at high cell concentrations did not produce NO due to high cytotoxicity and that the LPS treatment group produced 19.95 μM of NO. In addition, both viable strains

Table 4. Comparison of results from three types of antioxidant assays between viable and heat-killed *L. rhamnosus* GG and *L. brevis* KCCM 12203P.

Antioxidant assays		Antioxidant activity (%)	
		L. rhamnosus GG	*L. brevis* KCCM 12203P
DPPH radical scavenging activity	Viable	23.55 ± 1.90^b	25.66 ± 2.30^a
	Heat-killed	20.82 ± 1.61^c	22.48 ± 2.13^{b,c}
ABTS radical scavenging activity	Viable	23.49 ± 2.92^b	12.97 ± 0.71^c
	Heat-killed	37.10 ± 2.18^a	22.07 ± 2.02^b
β-Carotene bleaching inhibition activity	Viable	44.50 ± 1.46^b	56.30 ± 1.74^a
	Heat-killed	46.15 ± 2.13^b	57.17 ± 1.40^a

$^{a-c}$Different superscripts in the same antioxidant assay signify significance differences ($p < 0.05$). All values are mean ± standard deviation of triplicate analysis.

Table 5. Cytotoxicity and nitric oxide production of viable and heat-killed *L. rhamnosus* GG and *L. brevis* KCCM 12203P in different cell concentrations.

Sample condition[1)]		Cell viability (%)	
		L. rhamnosus GG	*L. brevis* KCCM 12203P
Viable	10^8	33.58 ± 6.04^{h}	28.45 ± 3.30^{h}
	10^7	41.67 ± 1.32^{g}	96.85 ± 4.28^{d}
	10^6	101.82 ± 3.76^{c,d}	109.86 ± 0.70^{a,b}
Heat-killed	10^9	76.27 ± 2.55^{e}	62.54 ± 5.65^{f}
	10^8	104.68 ± 5.46^{b,c}	99.81 ± 1.59^{c,d}
	10^7	103.85 ± 5.89^{c}	114.80 ± 2.99^{a}
		Nitric oxide production (μM)	
LPS treatment		LPS (+), 1 ng/ml	LPS (–)
		19.95 ± 2.11^{b,c}	3.21 ± 0.51^{f}
Sample condition		*L. rhamnosus* GG	*L. brevis* KCCM 12203P
Viable	10^8	–1.01 ± 0.14^{g}	–0.07 ± 0.38^{g}
	10^7	0.96 ± 1.68^{g}	19.38 ± 2.29^{c}
	10^6	15.14 ± 2.31^{d}	7.27 ± 1.47^{e}
Heat-killed	10^9	18.46 ± 0.51^{c}	13.38 ± 1.75^{d}
	10^8	21.83 ± 1.25^{b}	26.54 ± 2.56^{a}
	10^7	0.46 ± 0.64^{g}	3.72 ± 1.27^{f}

[1]Viable and heat-killed lactic acid bacteria samples with different cell concentrations (CFU/ml).

[a-h]Different superscripts in the same assay result signify significance differences ($p < 0.05$). All values are mean ± standard deviation of triplicate analysis.

at 10^6 CFU/ml induced production of NO (15.14 μM and 7.27 μM, respectively). On the other hand, heat-killed cells produced NO at higher concentrations than the viable cells at the same cell concentration, except for a group treated with 10^6 CFU/ml cells. Lee *et al*. [28] reported that heat-killed *L. plantarum* SY11 and SY12 showed higher NO-inducing ability than live cells. In a group treated with 10^9 CFU/ml of heat-killed cells, *L. rhamnosus* GG (18.46 μM) produced more NO than *L. brevis* KCCM 12203P (13.38 μM), but in a group treated with 10^8 CFU/ml of heat-killed cells, *L. brevis* KCCM 12203P (26.54 μM) induced more NO production than *L. rhamnosus* GG (21.83 μM). Based on these results, it was determined that a concentration of 10^9 CFU/ml of heat-killed cells was optimum for the following immune-stimulating activity test because it exhibited a low cell viability, which was less than 80%, with high NO production.

Immune-Stimulating Activity of Heat-Killed *Lactobacillus* Species on RAW 264.7 Cells without LPS

To measure the immune-stimulating activity of heat-killed probiotics on RAW 264.7 cells, RNA was extracted and then synthesized to cDNA. Heat-killed bacterial samples were treated at 10^8 CFU/ml to determine the gene expression levels of immune-related cytokines such as tumor necrosis factor (TNF-α), iNOS, and interleukin (IL)-1β and IL-6. TNF-α initiates innate and adaptive immunity and induces cellular proliferation and IL-1, IL-6, and IL-8 production. IL-1β activates pro-inflammatory, acute-phase, and Th1 cellular responses. IL-6 is a versatile cytokine that controls the pro- and anti-inflammatory responses and differentiation of immune cells like T and B cells. Furthermore, IL-6 inhibits production of TNF-α and IL-1 by macrophages and stimulates development of Th2 cells [36, 37]

Fig. 2 indicates the RT-PCR results for gene expression levels of cytokines after treatment with heat-killed LAB strains. Based on the results of MTT and NO assay, the optimum cell concentration for test was established to a 10^8 CFU/ml. To evaluate the dose-dependent activity, each sample was diluted serially 0.5 × and 0.25 × 10^8 CFU/ml. In addition, it appeared that a higher concentration than 10^8 CFU/ml affected the viability of RAW 264.7 cell. The LPS(+) group showed the highest gene expression level in all cytokines. When compared to same concentration, *L. brevis* KCCM 12203P exhibited consistently the higher cytokine-stimulatory activities than *L. rhamnosus* GG. In addition, *L. brevis* KCCM 12203P revealed higher gene expression of iNOS and cytokines except for TNF-α than *L. rhamnosus* GG. Furthermore, both lactic acid bacteria showed dose-dependent activity. Heat-killed *L. rhamnosus* GG and *L. brevis* KCCM 12203P produced both IL-1β and IL-6, which are Th1 and Th2 cytokines, respectively. This indicates that these strains enhanced immune function by modulating balance of Th1/Th2 immune responses in activated macrophages [38].

Some researchers reported that a mixture of various heat-killed *L. acidophilus* strains strongly released TNF-α and NO [39] and that heat-killed *Enterococcus faecalis* alleviated atopic symptoms in mice by reducing the IgE level, expression level of CDNB/DFE-induced inflammatory cytokines, and infiltration of mast cells [40]. Kim *et al*. [41] showed that the viable and heat-killed LAB isolated from *mukeunji* showed different patterns of TNF-α and IL-6 production between species.

Several studies suggested that lipoteichoic acid (LTA), which is major component of the cell wall of gram-positive bacteria, is the primary factor for immune-stimulating responses. It is known that LTA has analogous biochemical and physiological characteristics and the minute structural

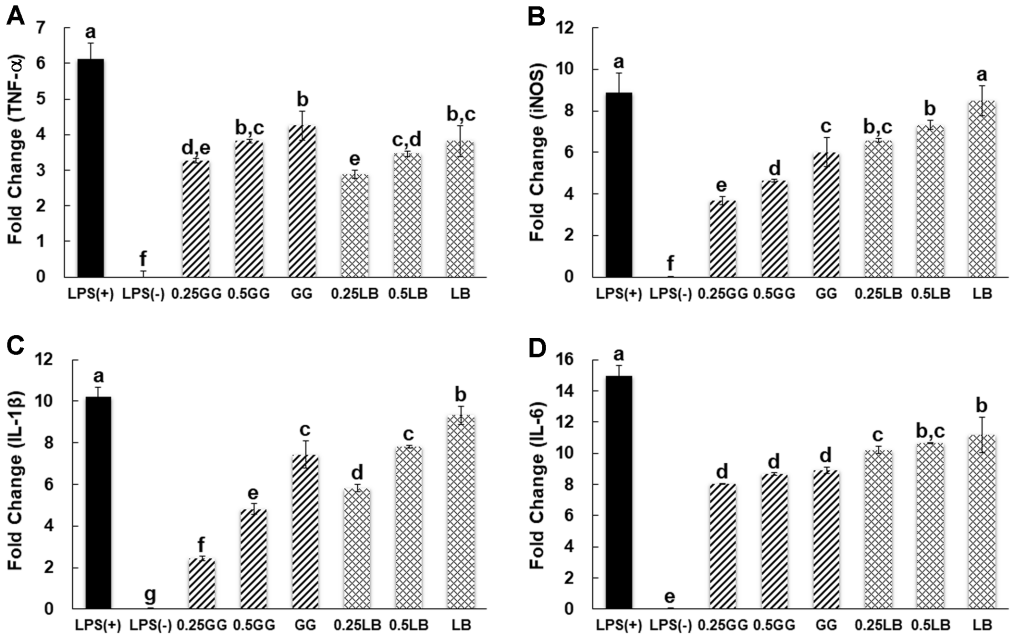

Fig. 2. Gene expression levels of immune-stimulating mediator under treatment with heat-killed *L. rhamnosus* GG and *L. brevis* KCCM 12203P on RAW 264.7 cells.

The relative gene expression levels of TNF-α (**A**), iNOS (**B**), IL-1β (**C**), and IL-6 (**D**) were represented. ■, LPS treatment with 1 ng/ml (LPS(+)); □, without LPS treatment (LPS(–)); ▨, *L. rhamnosus* GG (GG); ▩, *L. brevis* KCCM 12203P (LB). Each number (0.25 or 0.5) means the sample dilution ratio. The values are expressed as mean ± standard deviation of triplicate experiments and standardized against β-actin housekeeping gene. The different letters on error bars represent statistically significant difference between values ($p < 0.05$).

difference of LTA and LPS is dependent on species [42]. Therefore, this structure variance can lead to different immune responses and it signifies that immune-promoting properties of LAB are strain-specific properties.

In conclusion, *L. brevis* KCCM 12203P isolated from kimchi revealed higher tolerance against artificial gastric acid and bile salt conditions and showed higher adhesion activity on HT-29 human colon cancer cells. Furthermore, *L. brevis* KCCM 12203P did not produce carcinogenic β-glucuronidase. In antioxidant assays, *L. brevis* KCCM 12203P showed radical scavenging activity and lipid oxidation inhibition activity. Viable cells have higher antioxidant radical scavenge activity than heat-killed cells, as shown by the DPPH assay, while heat-killed cells showed higher antioxidant activity in the ABTS assay. However, in the β-carotene bleaching inhibition assay, there was no significant difference between live and heat-killed cells. The NO and MTT assays showed that 10^8 CFU/ml heat-killed LAB is a proper concentration for RT-PCR analysis. *L. brevis* KCCM 12203P showed a higher gene expression level of immune-modulating mediators such as iNOS, IL-1β, and IL-6 than *L. rhamnosus* GG, except for TNF-α. Both viable and heat-killed *L. brevis* KCCM 12203P cells revealed high antioxidant activities, and especially, heat-killed cells showed immune-stimulating activity. This study suggests that *L. brevis* KCCM 12203P could be applied in functional food and the pharmaceutical industry as a potential probiotic and immune-stimulating ingredient.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Boudoulas KD, Triposkiadis F, Stefanadis C, Boudoulas H. 2017. The endlessness evolution of medicine, continuous

increase in life expectancy and constant role of the physician. *Hellenic J. Cardiol*. **58:** 322-330.
2. Ziemer CJ, Gibson GR. 1998. An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. *Int. Dairy J*. **8:** 473-479.
3. Sommer F, Bäckhed F. 2013. The gut microbiota – masters of host development and physiology. *Nat. Rev. Microbiol*. **11:** 227-238.
4. Gamallat Y, Ren X, Walana W, Meyiah A, Xinxiu R, Zhu Y, *et al*. 2019. Probiotic *Lactobacillus rhamnosus* modulates the gut microbiome composition attenuates preneoplastic colorectal Aberrant crypt foci. *J. Funct. Foods* **53:** 146-156.
5. Wu Q, Shah NP. 2018. Restoration of GABA production machinery in *Lactobacillus brevis* by accessible carbohydrates, anaerobiosis and early acidification. *Food Microbiol*. **69:** 151-158.
6. Yang X, Zhou J, Fan L, Qin Z, Chen Q, Zhao L. 2018. Antioxidant properties of a vegetable-fruit beverage fermented with two *Lactobacillus plantarum* strains. *Food Sci. Biotechnol*. **27:** 1719-1726.
7. Daliri EB, Lee BH, Park BJ, Kim SH, Oh DH. 2018. Antihypertensive peptides from whey proteins fermented by lactic acid bacteria. *Food Sci. Biotechnol.* **27:** 1781-1789.
8. Koh WY, Utra U, Ahmad R, Rather IA, Park YH. 2018. Evaluation of probiotic potential and anti-hyperglycemic properties of a novel *Lactobacillus* strain isolated from water kefir grains. *Food Sci. Biotechnol.* **27:** 1369-1376.
9. Lv X, Miao L, Ma H, Bai F, Lin Y, Sun M, *et al.* 2018. Purification, characterization and action mechanism of plantaricin JY22, a novel bacteriocin against *Bacillus cereus* produced by *Lactobacillus plantarum* JY22 from golden carp intestine. *Food Sci. Biotechnol.* **27:** 695-703.
10. Cruz Rodrigues VC, Silva LGS Simabuco FM, Venema K, Antunes AEC. 2019. Survival, metabolic status and cellular morphology of probiotics in dairy products and dietary supplement after simulated digestion. *J. Funct. Foods* **55:** 126-134.
11. Ou CC, Lin SL, Tsai JJ, Lin MY. 2011. Heat-killed lactic acid bacteria enhance immunomodulatory potential by skewing the immune response toward Th1 polarization. *J. Food Sci.* **76:** 260-267.
12. Kanasugi H, Hasegawa T, Goto Y, Ohtsuka H, Makimura S, Yamamoto T. 1997. Single administration of enterococcal preparation (FK-23) augments non-specific immune responses in healthy dogs. *Int. J. Immunopharmacol*. **19:** 655-659.
13. Sakai Y, Tsukahara T, Bukawa W, Matsubara N, Ushida K. 2006. Cell preparation of *Enterococcus faecalis* strain EC-12 prevents vancomycin-resistant enterococci colonization in the cecum of newly hatched chicks. *Poult. Sci.* **85:** 273-277.
14. Sashihara T, Sueki N, Ikegami S. 2006. An analysis of the effectiveness of heat-killed lactic acid bacteria in alleviating allergic diseases. *J. Dairy Sci.* **89:** 2846-2855.
15. Kamiya T, Wang L, Forsythe P, Goettsche G, Mao Y, Wang Y, *et al.* 2006. Inhibitory effects of *Lactobacillus reuteri* on visceral pain induced by colorectal distension in Sprague-Dawley rats. *Gut* **55:** 191-196.
16. Lee NK, Han KJ, Son SH, Eom SJ, Lee SK, Paik HD. 2015. Multifunctional effect of probiotic *Lactococcus lactis* KC24 isolated from kimchi. *LWT-Food Sci. Technol.* **64:** 1036-1041.
17. Yang SJ, Lee JE, Lim SM, Kim YJ, LEE NK, Paik HD. 2019. Antioxidant and immune-enhancing effects of probiotic *Lactobacillus plantarum* 200655 isolated from kimchi. *Food Sci. Biotechnol.* **28:** 491-499.
18. Jang HJ, Song MW, Lee NK, Paik HD. 2018. Antioxidant effects of live and heat-killed probiotic *Lactobacillus plantarum* Ln1 isolated from kimchi. *J. Food Sci. Technol.* **55:** 3174-3180.
19. Kachouri F, Ksontini H, Kraiem M, Setti K, Mechmeche M, Hamdi M. 2015. Involvement of antioxidant activity of *Lactobacillus plantarum* on functional properties of olive phenolic compounds. *J. Food Sci. Technol.* **52:** 7924-7933.
20. Jeon EB, Son SH, Jeewanthi RKC, Lee NK, Paik HD. 2016. Characterization of *Lactobacillus plantarum* Lb41, an isolate from kimchi and its application as a probiotic in cottage cheese. *Food Sci. Biotechnol.* **25:** 1129-1133.
21. Chang CK, Wang SC, Chiu CK, Chen SY, Chen ZT, Duh PD. 2015. Effect of lactic acid bacteria isolated from fermented mustard on immunopotentiating activity. *Asian Pac. J. Prop. Biomed.* **5:** 281-286.
22. Son SH, Jeon HL, Yang SJ, Sim MH, Kim YJ, Lee NK, *et al*. 2018. Probiotic lactic acid bacteria isolated from traditional Korean fermented foods based on β-glucosidase activity. *Food Sci. Biotechnol.* **27:** 123-129.
23. Park YU, Kim MD, Jung DH, Seo DH, Jung JH, Park JG, *et al.* 2015. Probiotic properties of lactic acid bacteria isolated from Korean rice wine Makgeolli. *Food Sci. Biotechnol.* **24:** 1761-1766.
24. Kleeman EG, Klaenhammer TR. 1982. Adherence of *Lactobacillus* species to human fetal intestinal cells. *J. Dairy Sci.* **65:** 2063-2069.
25. Devi SM, Kurrey NK, Halami PM. 2018. In vitro anti-inflammatory activity among probiotic *Lactobacillus* species isolated from fermented foods. *J. Funct. Foods* **47:** 19-27.
26. Duary RK, Rajput YS, Batish VK, Grover S. 2011. Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J. Med. Res.* **134:** 664-671.
27. Monteagudo-Mera A, Rodríguez-Aparico L, Rúa J, Martínez-Blanco H, Navasa N, García-Armesto MR, *et al*. 2012. In vitro evaluation of physiological probiotic properties of different lactic acid bacteria strains of dairy and human origin. *J. Funct. Foods* **4:** 531-541.
28. Lee NK, Kim SY, Han KJ, Eom SJ, Paik HD. 2014. Probiotic potential of *Lactobacillus* strains with anti-allergic effects from kimchi for yogurt starters. *LWT-Food Sci. Technol.* **58:** 130-134.

29. Mroczyńska M, Libudzisz Z. 2010. Beta-glucuronidase and beta-glucosidase activity of *Lactobacillus* and *Enterococcus* isolated from human feces. *Pol. J. Microbiol.* **59:** 265-269.
30. Lin MY, Yen CL. 1999. Antioxidative ability of lactic acid bacteria. *J. Agric. Food. Chem.* **47:** 1460-1466.
31. Schaich KM, Tian X, Xie J. 2015. Reprint of "Hurdles and pitfalls in measuring antioxidant efficacy: a critical evaluation of ABTS, DPPH, and ORAC assays". *J. Funct. Foods* **18:** 782-796.
32. Amaretti A, di Nunzio M, Pompei A, Raimondi S, Rossi M, Bordoni A. 2013. Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities. *Appl. Microbiol. Biotechnol.* **97:** 809-817.
33. Rajoka MSR, Mehwish HM, Siddiq M, Haoin Z, Zhu J, Yan L, *et al*. 2017. Identification, characterization, and probiotic potential of *Lactobacillus rhamnosus* isolated from human milk. *LWT-Food Sci. Technol.* **84:** 271-280.
34. Davis CD, Milner JA. 2009. Gastrointestinal microflora, food components and colon cancer prevention. *J. Nutr. Btochem.* **20:** 743-752.
35. Bogdan C. 2001. Nitric oxide and the immune response. *Nat. Immunol.* **2:** 907-916.
36. Park HE, Lee WK. 2018. Immune enhancing effects of *Weissella cibaria* JW15 on BALB/c mice immunosuppressed by cyclophosphamide. *J. Funct. Foods* **49:** 518-525.
37. Mikkelsen K, Stojanovska L, Prakash M, Apostolopoulos V. 2017. The effects of vitamin B on the immune/cytokine network and their involvement in depression. *Maturitas* **96:** 58-71.
38. Tang C, Sun J, Liu J, Jin C, Wu X, Zhang X, *et al.* 2019. Immune-enhancing effects of polysaccharides from purple sweet potato. *Int. J. Biol. Macromol.* **123:** 923-930.
39. Choi EJ, Iwasa M, Han KI, Kim WJ, Tang Y, Han WC, *et al*. 2016. Effect of *Enterococcus faecalis* EF-2001 on experimentally induced atopic eczema in mice. *Food Sci. Biotechnol.* **25:** 1087-1093.
40. Lin WH, Yu B, Lin CK, Hwang WZ, Tsen HY. 2007. Immune effect of heat-killed multistrain of *Lactobacillus acidophilus* against *Salmonella* Typhimurium invasion to mice. *J. Appl. Microbiol.* **102:** 22-31.
41. Kim JH, Li J, Han SK, Qin P, Kim J, Park Y, *et al*. 2016. Characterization of macrophage-activating lactic acid bacteria isolated from *Mukeunji*. *Food Sci. Biotechnol.* **25:** 595-599.
42. Jeong JH, Jang S, Jung BJ, Jang KS, Kim BG, Chung DK, *et al*. 2015. Differential immune-stimulatory effects of LTAs from different lactic acid bacteria via MAPK signaling pathway in RAW 264.7 cells. *Immunobiology* **220:** 460-466.