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Immune-Enhancing Effect of Heat-Treated *Levilactobacillus brevis* KU15159 in RAW 264.7 Cells

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Abstract

Probiotics are alive microbes that present beneficial to the human's health. They influence immune responses through stimulating antibody production, activating T cells, and altering cytokine expression. The probiotic characteristics of *Levilactobacillus brevis* KU15159 were evaluated on the tolerance and adherence to gastrointestinal conditions. *L. brevis* KU15159 was safe in a view of producing various useful enzymes and antibiotic sensitivity. Heat-treated *L. brevis* KU15159 increased production of nitric oxide (NO) and phagocytic activity in RAW 264.7 cells. In addition, heat-treated *L. brevis* KU15159 upregulated the expression of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, at protein as well as mRNA levels. In addition, the mitogen-activated protein kinase (MAPK) pathway, which regulates the immune system, was activated by heat-treated *L. brevis* KU15159. Therefore, *L. brevis* KU15159 exhibited an immune-enhancing effect by the MAPK pathway in macrophage.

Keywords *Levilactobacillus brevis* · Probiotics · Immune-enhancing · RAW 264.7 cell · MAPK signaling pathway

Introduction

The ongoing COVID-19 pandemic has increased interest in improving immunity. The immune system protects the host from the external antigens and reduces susceptibility to diseases [1]. Induction of immune cells (dendritic cells, macrophages, and lymphocytes) that comprise the immune system acts an important role in enhancing immunity [2]. The gut microbiota communicates closely with the immune system to maintain immune homeostasis in the gastrointestinal tract of the host [3].

Supplementation with probiotics, alive microbes that confer several health benefits to the hosts, could re-establish homeostasis and restore the microbiota [4]. Probiotics influence immune responses by stimulating antibody production, activating T cells, and altering cytokine expression. They activate macrophages to secrete proinflammatory cytokines as well as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6; they stimulate the expression of inducible nitric oxide synthase (iNOS) [5].

Cytokines are regulated through the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-B (NF-κB) signaling pathways. MAPK regulates cell proliferation and cellular responses to cytokines [6]. The MAPK components include p38 MAPKs, extracellular signal-regulated kinases 1/2 (ERK 1/2), and c-Jun N-terminal kinase (JNK) [7]. NF-κB regulates genes related to both intrinsic and adaptive immune responses. Stimulated NF-κB is transported into the cell nucleus, and it regulates the mRNA expression of *iNOS* and *TNF-α* [8].

Lactobacillus strains are one of the most commonly used probiotics because they are commonly found in healthy intestines [9]. *Lactobacillus* strains were reclassified into 25 genera including *Lactiplantibacillus*, *Levilactobacillus*, and *Lacticaseibacillus* according to the new taxonomic conventions [10]. *Lactobacillus* strains exhibit anticancer [11], antioxidant [12], anti-inflammatory [13], and immune-enhancing [14] properties. To avoid sepsis that could be caused by live cells [11], it is necessary to consider the use of heat-killed *Lactobacillus* or its metabolites [15].

Levilactobacillus brevis KU15159 was obtained from homemade diced radish kimchi. The purpose of this

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investigation was to estimate the probiotic properties of *L. brevis* KU15159 and the immune-improving effect of heat-treated *L. brevis* KU15159 in macrophage.

Materials and Methods

Culture Media and Analytical Reagents

Lactobacilli MRS broth for bacterial cultures was acquired from BD Biosciences (Franklin Lakes, NJ, USA). For cell culture, phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin/streptomycin (P/S), DMEM, and RPMI 1640 were acquired from HyClone (Logan, UT, USA). The API ZYM® kit was obtained from BioMérieux (Marcy-l'Étoile, France). RevertAid First Strand cDNA Synthesis Kit and RNeasy® Mini Kit were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and QIAGEN (Hilden, Germany), respectively. SYBR Green PCR Master Mix for the semi-quantitative real-time RT-PCR was acquired from PikoReal 96 (Scientific Pierce, Waltham, MA, USA). The mouse iNOS ELISA kit was purchased from Abcam (Cambridge, USA). Mouse TNF-α, Mouse IL-1beta, and IL-6 ELISA kit were used from Invitrogen (Carlsbad, CA, USA). The DC protein assay kit and enhanced chemiluminescence detection kit were used from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and the antibodies were used from Santa Cruz Biotechnology (Dallas, TX, USA). Other materials were used from Sigma-Aldrich (St. Louis, MO, USA).

Probiotics and Sample Production

Lactiplantibacillus plantarum KU15149, *Levilactobacillus brevis* KU15159, and *L. brevis* KU15176 were used in this study [11]. *Lacticaseibacillus rhamnosus* GG utilized for a reference strain. For sample preparation, *Lactobacillus* strains were cultivated in lactobacilli MRS broth at 37 °C for 20 h, and the bacteria cells were obtained by centrifugation at 12,000 × *g* at 4 °C for 5 min. The bacterial cells were rinsed trice with PBS and treated at 80 °C for 30 min. And then, the samples were obtained by resuspension using cell growth media.

Tolerance of *Lactobacillus* Strains in Artificial Gastric Conditions

The resistance of *Lactobacillus* strains in artificial gastric conditions was evaluated, as depicted previously [16]. Tolerance in artificial gastric juice was confirmed by incubating the *Lactobacillus* strains in lactobacilli MRS broth balanced pH 2.5 with 3 g/L pepsin, at 37 °C for 3 h. Tolerance in bile acids was evaluated through incubation in lactobacilli MRS broth including 3 g/L oxgall, at 37 °C for 24 h. Tolerance (%) was computed based on the survived cells on lactobacilli MRS agar plates in comparison with the initial cells, as follows:

$$\text{Tolerance } (\%) = \frac{\log\ A}{\log\ B} \times 100$$

where *A* and *B* are the survived cells following treatment with artificial gastric conditions and the initial cells before treatment, respectively.

Intestinal Adhesion of *Lactobacillus* Strains

The adhesion capacity of *L. brevis* KU15159 to HT-29 cells was evaluated as the bacterial counts of bacteria attached to the cells. HT-29 cells (1×10^5 cells/mL) were inoculated in 24-well plates and cultured at 37 °C for 24 h. *Lactobacillus* strains were performed centrifugation at 12,000 × *g* at 4 °C for 10 min and rinsed trice with PBS; they were diluted with antibiotic-free RPMI medium. HT-29 cells were handled with *Lactobacillus* strains and cultured at 37 °C for 2 h; the cutlured cells were rinsed trice with PBS, treated with 1 mL of 1% (v/v) Triton X-100, and incubated at 37 °C for 10 min. The separated *Lactobacillus* strains were diluted in PBS and spread on lactobacilli MRS agar plates. After 24 h of incubation at 37 °C, the bacteria were counted.

Enzyme Production of *Lactobacillus* Strains

To estimate the enzymes produced by the *Lactobacillus* strains, the API ZYM® kit was utilized. The *Lactobacillus* strains were incubated in lactobacilli MRS broth at 37 °C for 20 h. The bacterial cultures were performed centrifugation at 12,000 × *g* at 4 °C for 5 min and washed twice with PBS. The cell pellet was suspended using PBS to 10^7 CFU/mL. Suspensions were put to each slot cultured at 37 °C for 4 h. Two microliters of separately Zym A and Zym B reagent was added. Enzyme production was determined on a scale of 0 to ≥ 40 nM as the color change.

Antibiotics Sensitivity of *Lactobacillus* Strains

The antibiotics sensitivity of the *Lactobacillus* strains was determined, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The disc diffusion method was used to measure the sensitivity to ampicillin (10 mg), chloramphenicol (30 mg), tetracycline (30 mg), doxycycline (30 mg), streptomycin (10 mg), kanamycin (30 mg), and gentamicin (10 mg). Each *Lactobacillus* strain at 10^5 CFU/mL was placed on lactobacilli MRS agar plates. Paper discs were placed on the plates and added 50 µL of

antibiotics. The plates were cultured at 37 °C for 24 h, and the inhibition zones were evaluated.

RAW 264.7 Cells and Culture Condition

RAW 264.7 cells were acquired from the Korean Cell Line Bank (KCLB, Seoul, Korea) and incubated in DMEM with 10% (v/v) FBS and 1% (v/v) P/S at 37 °C with 5% CO_2 incubator.

NO Production and Viability in RAW 264.7 Cells

Nitric oxide (NO) assays were performed to evaluate the immune-enhancing potential, as described earlier [17]. RAW 264.7 cells (2×10^5 cells/well) were seeded in a microwell plate and incubated for 4 h; 100 µL of diluted samples or 1 ng/mL LPS (positive control) was added. Later 24 h of incubation, 100 µL of the cell supernatant was blended with 100 µL of Griess reagent. The optical density was determined at 540 nm, and the NO production was determined using standard curve of sodium nitrite.

Cell viability was investigated applying an MTT assay. RAW 264.7 cells were inoculated in microplate at 100 µL of 2×10^5 cells/well and cultured for 4 h; 100 µL of diluted samples or 1 ng/mL LPS (positive control) was added and cultured for 24 h. The cells were rinsed two times with PBS, handled with 100 µL of 0.5 mg/mL MTT solution. After 30 min incubation, the MTT mixture was eliminated, and 200 µL of dimethyl sulfoxide (DMSO) was mixed. Optical density was measured at 570 nm, and viability was determined as follows:

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

where A_{sample} and A_{control} are the optical density of the treated sample and the untreated sample, respectively.

Phagocytic Activity of RAW 264.7 Cells

The neutral red uptake method was utilized to measure the effect of heat-treated *L. brevis* KU15159 on the phagocytic activity of macrophages [18]. RAW 264.7 cells were cultured at 2×10^5 cells/well in a 24-well plate and cultured for 4 h. Cultured RAW 264.7 cells were added 7 log CFU/mL of heat-treated *Lactobacillus* strains and incubated for 24 h. The supernatant was removed; 0.075% neutral red solution was put in the cells and stained for 1 h. Cells were washed thrice with PBS and lysed with lysis reagent (acetate and ethanol; 1:1). The optical density was measured at 540 nm.

RNA Extraction and Semi-Quantitative Real-Time RT-PCR

Total RNA was obtained from the RAW 264.7 cells, and cDNA was synthesized. The proinflammatory factor (*iNOS*) and proinflammatory cytokines (*TNF-α*, *IL-1β*, and *IL-6*) were confirmed by using SYBR Green PCR Master mix in a semi-quantitative real-time RT-PCR. *β-Actin* was used as the control. The used primers are represented in Table 1 [19]. The thermal cycling conditions were followed: 95 °C for 2 min, 40 cycles of 95 °C for 5 s, and 60 °C for 15 s. The amplification results were evaluated using the delta–delta Cq (ΔΔCq) method, and the purity of the PCR products was estimated based on the melting curve.

ELISA

RAW 264.7 cells were incubated with 1×10^6 cells/well seeding in a 6-well plate for 24 h. The cells were treated with 7 log CFU/mL of heat-treated *Lactobacillus* strains and incubated for 24 h. The levels of iNOS and cytokines were measured using the respective ELISA kit, as stated by the manufacturer's directions.

Western Blotting

The cells were washed along with chilly PBS and extracted with Pro-prep lysis buffer with protease inhibitor. The supernatant was obtained by centrifugation at 4 °C at 15,000 × *g* for 30 min. Thirty micrograms of proteins was split on a 10% SDS-PAGE gel and subsequently transported to a polyvinyl difluoride membrane. Transferred membranes were blocked into 5% skim milk for 1 h and reacted with primary antibodies (p-p38, p38, p-ERK 1/2, ERK 1/2, p-JNK, and JNK antibodies) at 4 °C overnight. The membranes were cross-reacted by secondary antibodies at 25 °C for 2 h. The protein bands

Table 1 Sequence of primers used for semi-quantitative RT-PCR

Primer		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

iNOS inducible nitric oxide synthase, *TNF-α* tumor necrosis factor-α, *IL* interleukin

were identified and were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

All data are displayed as the mean ± standard deviation of 3 independent experiments. One-way analysis of variance (ANOVA) was used to confirm significant differences. The mean values were used in Duncan's multiple range test for post hoc verification ($p < 0.05$).

Results

Tolerance in Artificial Gastric Conditions and Adhesion to HT-29 Cells of *Lactobacillus* Strains

Tolerance in artificial gastric conditions is a basic trait of probiotics; it was evaluated to determine whether *L. brevis* KU15159 could reach the intestines live, following ingestion. *L. brevis* KU15159 revealed a survival rate of 98.90% in artificial gastric juice and more than 100% in artificial bile condition (Table 2). This was similar to that observed for *L. rhamnosus* GG. Therefore, survival of *L. brevis* KU15159 is affected by gastric conditions but is not significantly affected by the bile salt concentration found in the human intestine.

To assess the adhesion rate of *L. brevis* KU15159, its survival rate under gastrointestinal tract conditions was evaluated. *L. brevis* KU15159 exhibited an adhesion rate of 7.66% to HT-29 cells, which was higher than the 6.37% exhibited by *L. rhamnosus* GG (Table 2). The adhesion rate of *Lactobacillus* strains typically ranges from 2 to 10% [20]. Therefore, *L. brevis* KU15159 can colonize the gastrointestinal tract.

Enzyme Production of *Lactobacillus* Strains

The API ZYM kit was used to identify enzymes produced by *L. brevis* KU15159. *L. brevis* KU15159 was positive for β-galactosidase, β-glucosidase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and valine arylamidase (Table 2). *L. brevis* KU15159 produced a large amount of β-glucosidase compared to *L. rhamnosus* GG, the reference strain. In contrast, *L. brevis* KU15159 did not show β-glucuronidase production, which can aid the production of cancer-causing agent [21]. Therefore, *L. brevis* KU15159 produced useful enzymes without being harmful.

Table 3 Antibiotic sensitivities of LAB strains

Antibiotics	*L. rhamnosus* GG	*L. brevis* KU15159
Ampicillin (10 μg)	S	S
Chloramphenicol (30 μg)	S	S
Tetracycline (30 μg)	S	S
Doxycycline (30 μg)	S	S
Streptomycin (10 μg)	R	R
Kanamycin (30 μg)	R	R
Gentamycin (10 μg)	R	I

Resistance was evaluated according to the CLSI breakpoints for Enterobacterales [22]

S susceptible, *I* intermediate, *R* resistant

Antibiotic Sensitivity of *Lactobacillus* Strains

Antibiotic sensitivity of a probiotic should be evaluated because antibiotic-resistant strains could become persistent and difficult to eradicate when necessary; in addition, antibiotic resistance could be transferred to pathogenic bacteria [23]. Table 3 shows the antibiotic sensitivities of *L. brevis* KU15159. *L. brevis* KU15159 showed similar results as *L. rhamnosus* GG, except for gentamycin (10 mg). Therefore, *L. brevis* KU15159 was consistent with the CLSI guidelines.

NO Production and Viability in RAW 264.7 Cells

The immune-enhancing effect of heat-treated *Lactobacillus* strains was assessed using an NO assay. If *Lactobacillus* strains influence the viability of RAW 264.7, they can alter the amount of NO production; therefore, the viability was evaluated using MTT analysis. LPS (1 ng/mL) was employed as the positive control to stimulate RAW 264.7 cells.

Table 2 Probiotic properties of *Lactobacillus* strains

Probiotic properties	*L. rhamnosus* GG	*L. brevis* KU15159
Tolerance in pH 2.5 with 3 g/L pepsin, for 3 h	99.28 ± 1.97[a]	98.90 ± 0.22[a]
Tolerance in 3 g/L oxgall, for 24 h	102.89 ± 2.01[a]	109.39 ± 0.93[a]
Adhesion to HT-29 cells (%)	6.37 ± 0.35[a]	7.66 ± 0.37[a]
Enzyme production	5 nM	5 nM
β-Galactosidase	5 nM	20 nM
β-Glucosidase	20 nM	10 nM
Leucine arylamidase	10 nM	5 nM
Naphthol-AS-BI-phosphohydrolase	20 nM	5 nM
Valine arylamidase		

[a]The data are the mean ± SD from three independent experiments

The NO production after LPS treatment was higher than the control group. Similarly, treatment with heat-treated *Lactobacillus* increased NO production (Fig. 1). Despite the higher amount of NO production at the concentration of 8 log CFU/mL, the viability of *L. brevis* KU15159 was less than 100%; therefore, 7 log CFU/mL was adopted in the subsequent experiments. At 7 log CFU/mL, *L. plantarum* KU15149 and *L. brevis* KU15159 increased NO production to a greater extent than *L. rhamnosus* GG. In contrast, *L. brevis* KU15176 exhibited lower values.

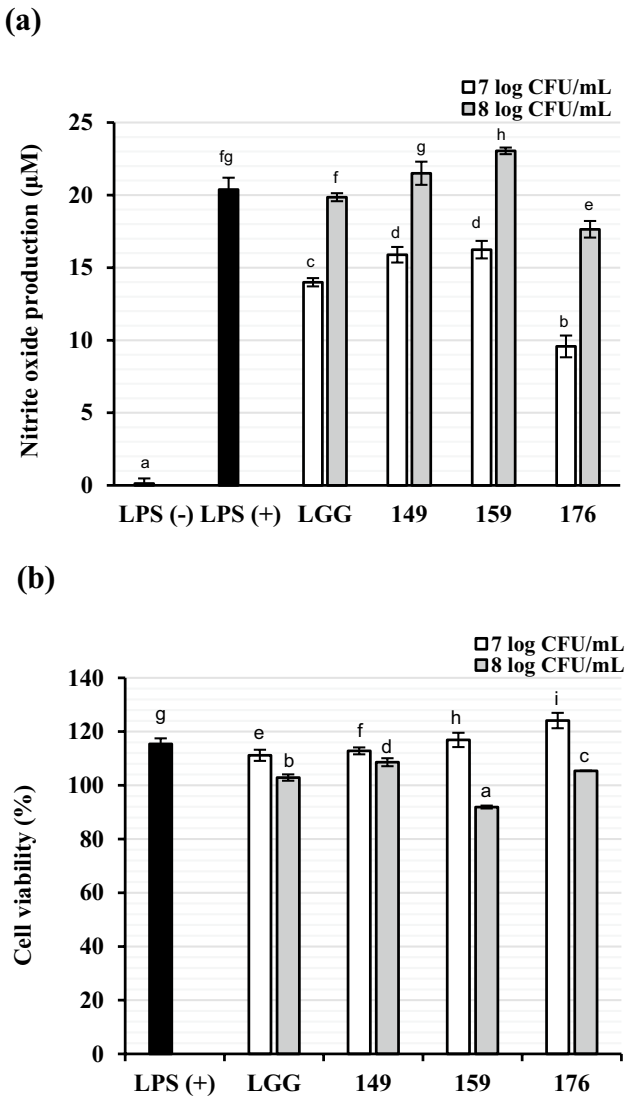

Fig. 1 Cytotoxicity of heat-treated *L. brevis* KU15159 (7 log CFU/mL) in RAW 264.7 cells. The cells were incubated with LPS (1 ng/mL) or heat-treated bacterial sample for 24 h. **a** Nitric oxide (NO) production evaluated using NO assay. **b** Cell viability assessed using MTT assay. LGG, *L. rhamnosus* GG; 149, *L. plantarum* KU15149; 159, *L. brevis* KU15159; 176, *L. brevis* KU15176. Different letters indicate significant differences for each characteristic ($p < 0.05$)

Changes in the Phagocytic Activity of Macrophages

Neutral red uptake assay was accomplished to assess the influence of *L. brevis* KU15159 on the phagocytic activity of RAW 267.4 cells (Fig. 2). LPS increased the phagocyte activity compared to that in the control group, which is supported by the increase in NO production in the LPS-treated RAW 267.4 cells. All *Lactobacillus* strains increased phagocytic activity, compared to that in the LPS treatment. However, *L. plantarum* KU15149 showed a slightly lower value, while the other strains showed a similar increase in phagocytic activity.

Expression of *iNOS*, *TNF-α*, *IL-1β*, and *IL-6*

The RT-PCR results indicated that heat-treated *Lactobacillus* strains regulated the expression of some immune-correlated genes (Fig. 3). The treatment with LPS (positive control) increased the expression of all genes compared to the control. The *iNOS* expression in the *L. rhamnosus* GG and *L. brevis* KU15176 groups was similar to that in the LPS group. *L. plantarum* KU15149 and *L. brevis* KU15159 increased the expression 48.19 and 45.13 times, compared to that in the control, respectively. This corroborates the results of the NO assay, because *iNOS* produces NO. In *TNF-α*, *IL-1β*, and *IL-6*, the *L. plantarum* KU15149 and *L. brevis* KU15159-treated groups showed higher expression levels, compared to the *L. rhamnosus* GG-treated group. In contrast, *L. brevis* KU15176 induced only a small increase in the overall expression.

The amount of protein present in the supernatant was measured using ELISA to confirm whether an increase in the expression of mRNA, as identified using RT-PCR, leads to

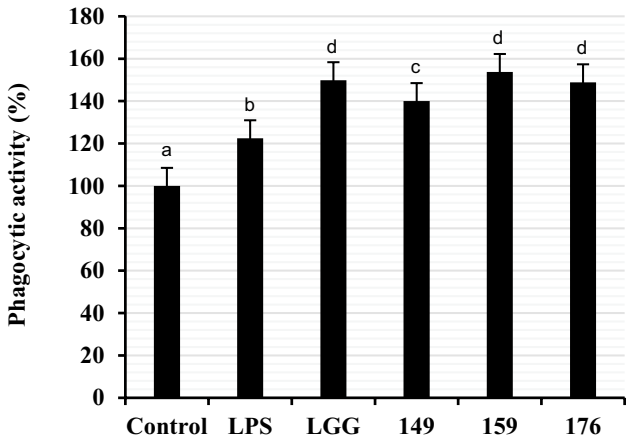

Fig. 2 Phagocytic activity of heat-treated *L. brevis* KU15159 in RAW 264.7 cells using neutral red uptake method. The cells were incubated with the LPS (1 ng/mL) or heat-treated bacterial sample (7 log CFU/mL) for 24 h. LGG, *L. rhamnosus* GG; 149, *L. plantarum* KU15149; 159, *L. brevis* KU15159; 176, *L. brevis* KU15176. Different letters indicate significant differences for each characteristic ($p < 0.05$)

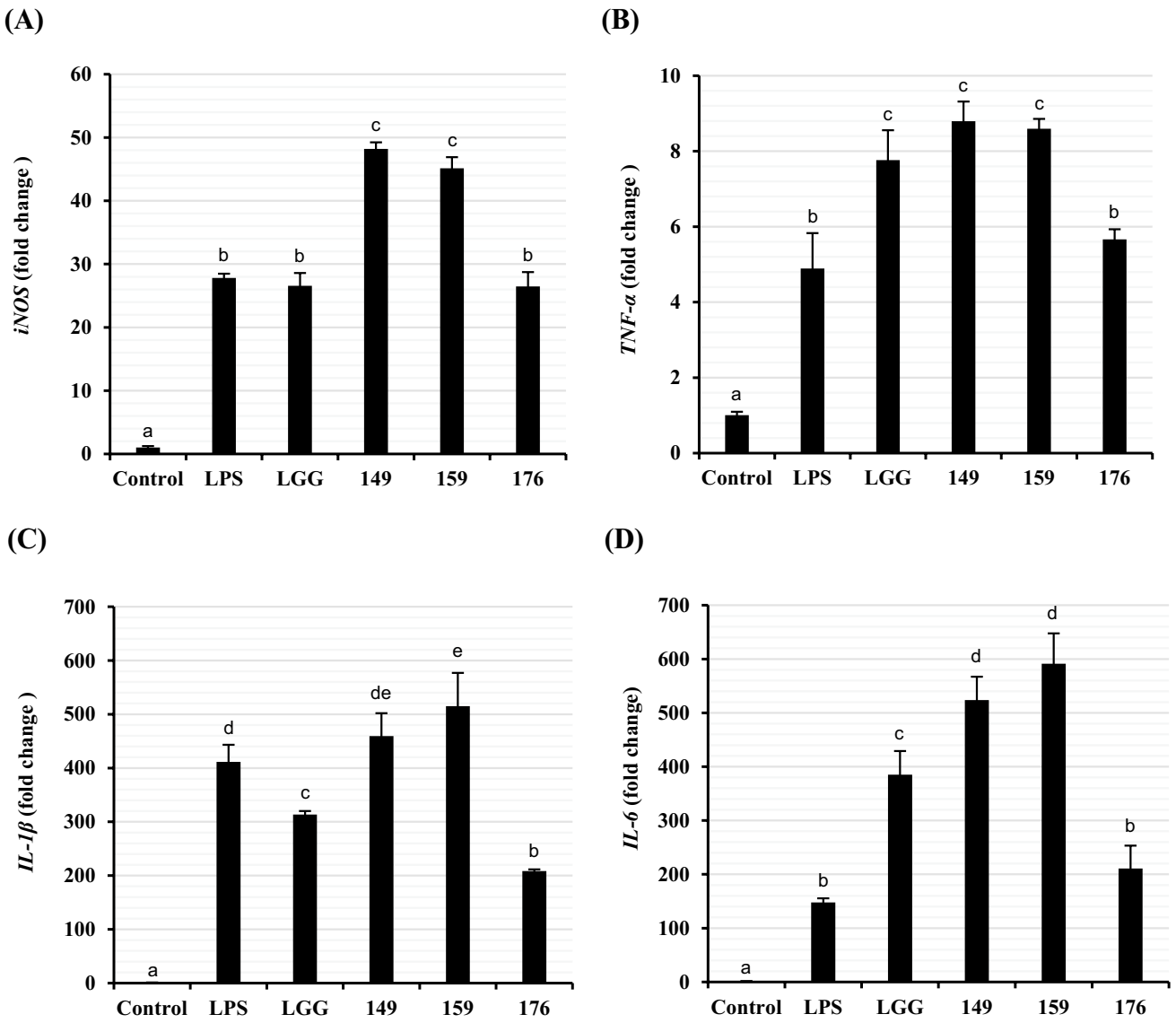

Fig. 3 The expression of immune-associated genes induced by heat-treated *L. brevis* KU15159 in RAW 264.7 cells. The cells were incubated with LPS (1 ng/mL) or heat-treated bacterial sample (7 log CFU/mL) for 24 h. **A** *iNOS*, **B** *TNF-α*, **C** *IL-1β*, and **D** *IL-6* expression levels were measured using RT-PCR. LGG, *L. rhamnosus* GG; 149, *L. plantarum* KU15149; 159, *L. brevis* KU15159; 176, *L. brevis* KU15176. Different letters indicate significant differences for each characteristic ($p < 0.05$)

a corresponding increase in the protein levels. All four factors increased the protein levels compared to that in treatment with LPS (positive control). The amounts of iNOS induced by *L. rhamnosus* GG, *L. plantarum* KU15149, and *L. brevis* KU15159, but not by *L. brevis* KU15176, were similar (Fig. 4). However, TNF-α and IL-1β production was higher in the *L. brevis* KU15159 treatment, compared to that in the other treatments.

Protein Expression of MAPK Pathway Components

The activation of factors associated with the MAPK signaling pathway (p38, JNK, and ERK1/2) was evaluated applying western blotting, to confirm the mechanism by which *L. brevis* KU15159 increased NO production and upregulated cytokines. LPS treatment increased the phosphorylation of p38, JNK, and ERK 1/2, compared to that in the control (Fig. 5). Similarly, *L. brevis* KU15159 activated all the proteins; this was more effective than LPS and LGG. *L. brevis* KU15159 increased the activation of p-p38/p38, p-JNK/JNK, and p-ERK 1/2/ERK 1/2 by 169.57-, 52.63-, and 88.22-fold, respectively. Therefore, the immune-enhancing effect of heat-treated *L. brevis* KU15159 occurs via the MAPK pathway.

Discussion

Probiotics must be safe for human consumption, have the ability to endure the gastrointestinal environment, adhere to intestinal epithelial cells, and perform various biological

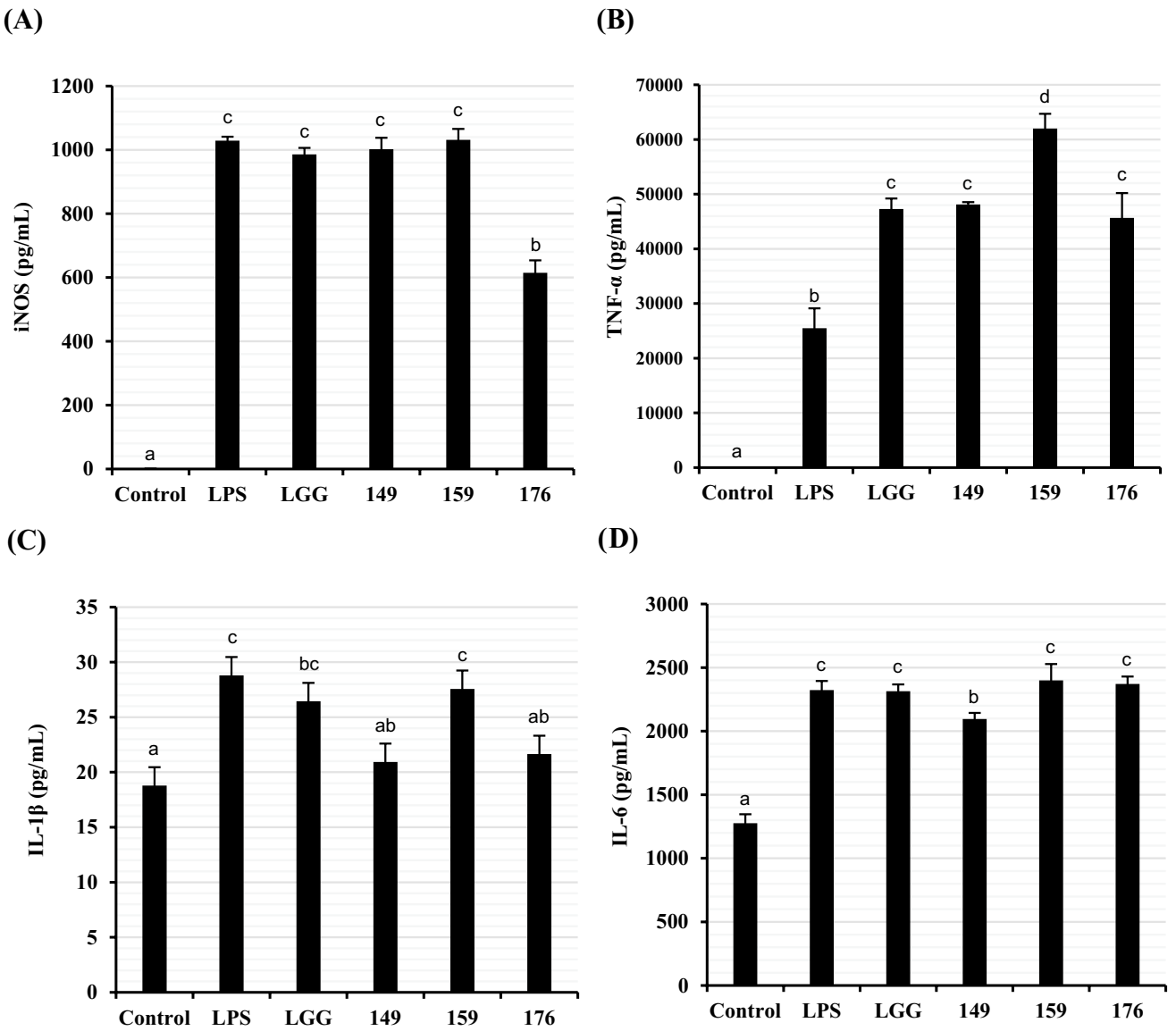

Fig. 4 The expression of immune-associated protein induced by heat-treated *L. brevis* KU15159 in RAW 264.7 cells. The cells were incubated with the LPS (1 ng/mL) or heat-treated bacterial sample (7 log CFU/mL) for 24 h. **A** iNOS, **B** TNF-α, **C** IL-1β, and **D** IL-6 expression levels evaluated using ELISA. LGG, *L. rhamnosus* GG; 149, *L. plantarum* KU15149; 159, *L. brevis* KU15159; 176, *L. brevis* KU15176. Different letters indicate significant differences for each characteristic ($p < 0.05$)

functions in the hosts [24]. Through a series of probiotic experiments, it was identified that *L. brevis* KU15159 can survive in the gastrointestinal environment and attach to intestinal cells. Additionally, *L. brevis* KU15159 can produce useful enzymes without the risk of transferring antibiotic resistance to harmful bacteria or producing harmful enzymes. Leucine arylamidase and valine arylamidase produced by *Lactobacillus* influence the decomposition of proteins and peptides, contributing to the flavor of the final product [25]. β-Galactosidase helps alleviate lactose intolerance in the gut and is important for probiotics [26, 27]. In addition, β-glucosidase contributes to taste by hydrolyzing bitter compounds and converting flavorless glucosides into aromatic compounds [28]. *L. brevis* KU15159 showed a higher production of β-glucosidase than *L. rhamnosus* GG.

The immune-improving effect of heat-treated *L. brevis* KU15159 was confirmed using NO and phagocytosis assays. Treatment of RAW 264.7 cells with 7 log CFU/mL of heat-treated *L. brevis* KU15159 increased NO production more effectively than the *L. rhamnosus* GG treatment. When macrophages are activated, they secrete NO to counteract free radicals; NO production is the important molecules in immune response. In addition, it is fatal to intracellular parasites and bacteria [29]. The induction of phagocytic activity by heat-treated *L. brevis* KU15159 was similar to that by *L.*

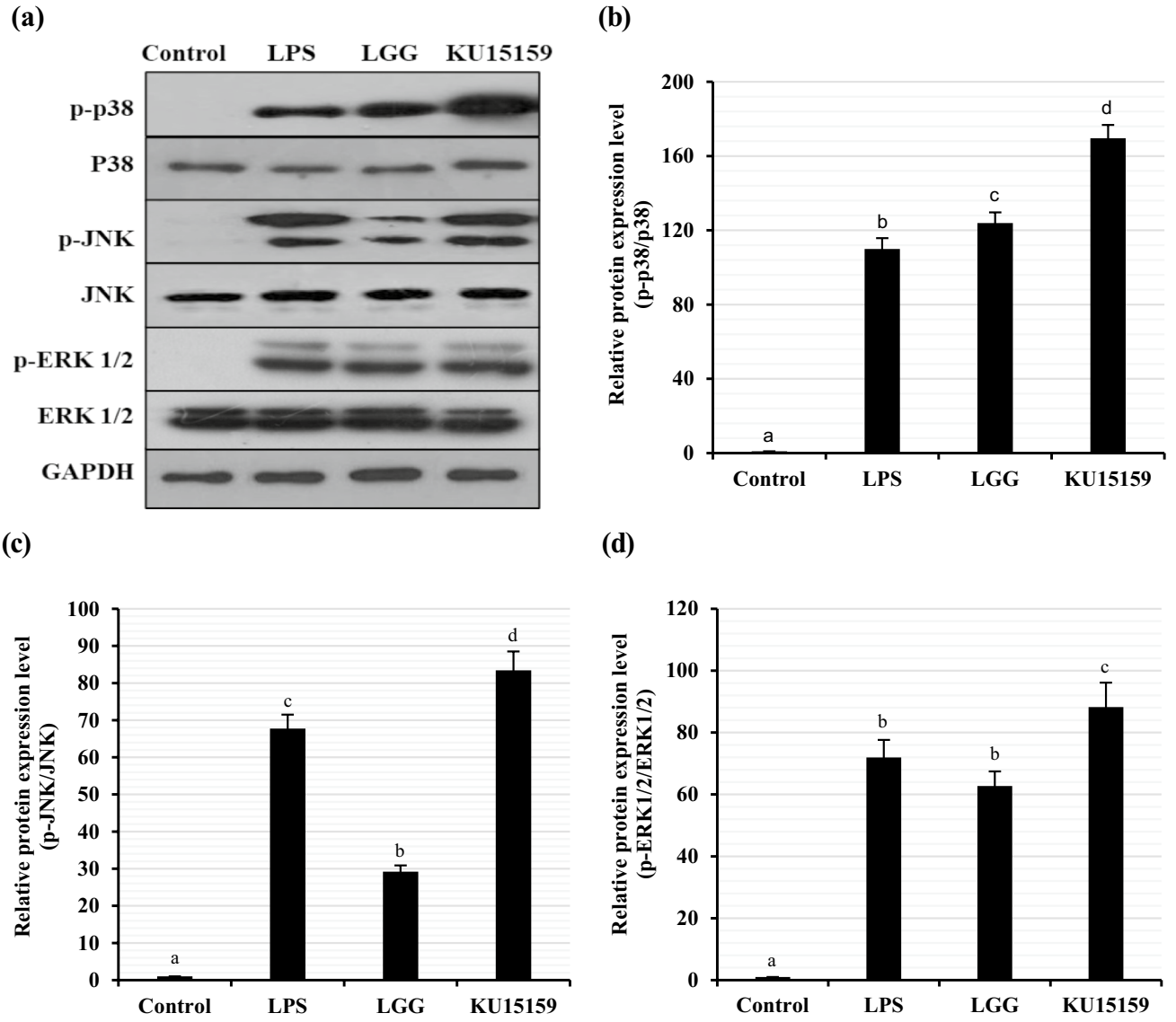

Fig. 5 The expression level of phosphorylated MAPKs induced by heat-treated *L. brevis* KU15159 in RAW 264.7 cells. The cells were incubated with LPS (1 ng/mL) or heat-treated bacterial sample (7 log CFU/mL) for 5 min. **a** Expression of proteins in the MAPK signaling pathway was investigated through western blotting. **b**–**d** Relative quantification of protein bands using ImageJ. LGG, *L. rhamnosus* GG; 159, *L. brevis* KU15159. Statistical differences are indicated using different letters ($p < 0.05$)

rhamnosus GG. The phagocytic activity of macrophages is important to defend their host against pathogens, which is crucial for immunity [30].

The increased expression of *iNOS* in RAW 264.7 cells treated with heat-treated *L. brevis* KU15159, compared to that in the LPS or *L. rhamnosus* GG treatment, further implicates the immune-enhancing effect of *L. brevis* KU15159. iNOS adjusts the function and differentiation of immune cells through nitration of principal molecules related to signaling or transcriptional pathways [31, 32]. In addition, heat-treated *L. brevis* KU15159 upregulated the expression of *TNF-α*, *IL-6*, and *IL-1β*. The upregulation of *TNF-α* by *L. brevis* KU15159 was higher than that in the other treatments. Macrophages secrete cytokines, as well as TNF-α, IL-6, and IL-1β, and have considerable effects on immune responses [33]. They can bind to particular receptors of other cells and activate them immunologically [24]. TNF-α protects the host against infectious pathogens [3], initiates the expression of adhesion molecules in endothelial cells and neutrophils, and activates leukocytes [34]. The amounts of iNOS, TNF-α, IL-6, and IL-1β in the heat-treated *L. brevis* KU15159 treatment were similar to or higher than that in the *L. rhamnosus* GG treatment.

Western blot analysis showed that heat-treated *L. brevis* KU15159 activated the MAPK pathway (Fig. 5). The MAPK signaling pathway is related to regulating mRNA expression

associated with the expression and release of inflammatory cytokines for modulating immune responses [35]. MAPKs are regulators of immune responses; their activity is regulated by reversible phosphorylation of threonine and tyrosine residues [36]. There are trinity-specific MAPK pathways, the p38, ERK, and JNK pathways [36]. Heat-treated *L. brevis* KU15159 phosphorylated p38, JNK, and ERK 1/2 more than LGG did. Therefore, elevated cytokine production in macrophage handled with heat-treated *L. brevis* KU15159 was associated with the activity of MAPK pathway.

L. plantarum KU15149 exhibited immune-enhancing potential. In the RT-PCR and ELISA data, *L. plantarum* KU15149 treatment had similar or slightly lower values than *L. brevis* KU15159. Unlike *L. brevis* KU15159, which affected cell viability at 8 log CFU/mL in the MTT assay, *L. plantarum* KU15149 did not affect viability. Therefore, it is necessary to evaluate the use of higher concentrations and longer treatment times. In addition, it is important to assess inflammatory reactions that could occur with increased bacterial concentrations. This applies to the immune-enhancing potential of *L. brevis* KU15159 as well, and in vivo tests are necessary to evaluate the effectiveness and the possibility of an inflammatory reaction.

In summary, *L. brevis* KU15159 was characterized for its probiotic properties, and heat-treated *L. brevis* KU15159 was investigated to detect its immune-enhancing effects in macrophage. Heat-treated *L. brevis* KU15159 augmented the phagocytic activity and the NO production in macrophage by regulating *iNOS*. The *TNF-α*, *IL-1β*, and *IL-6* expression was upregulated. At the protein level, *L. brevis* KU15159 showed an effect similar to that of LGG. Therefore, *L. brevis* KU15159 has immune-enhancing potential in vitro, but in vivo experiments are needed to clarify its effect.

Author Contribution C.-H. Hwang, K.-T. Kim, N.-K. Lee, and H.-D. Paik conceptualized this study. C.-H. Hwang conducted all the experiments, and C.-H. Hwang and N.-K. Lee drafted first version of the manuscript. K.-T. Kim, N.-K. Lee, and H.-D. Paik reviewed the draft manuscript, and all the authors revised the final version of the manuscript.

Declarations

Competing Interests The authors declare no competing interests.

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