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Protective Effects of a Novel Probiotic Strain of *Lactobacillus plantarum* JSA22 from Traditional Fermented Soybean Food Against Infection by *Salmonella enterica* Serovar Typhimurium

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Lactobacillus species have been shown to enhance intestinal epithelial barrier function, modulate host immune responses, and suppress the growth of pathogenic bacteria, yeasts, molds, and viruses. Thus, lactobacilli have been used as probiotics for treating various diseases, including intestinal disorders, and as biological preservatives in the food and agricultural industries. However, the molecular mechanisms used by lactobacilli to suppress pathogenic bacterial infections have been poorly characterized. We previously isolated *Lactobacillus plantarum* JSA22 from buckwheat *sokseongjang*, a traditional Korean fermented soybean food, which possessed high enzymatic, fibrinolytic, and broad-spectrum antimicrobial activity against foodborne pathogens. In this study, we investigated the effects of *L. plantarum* JSA22 on the growth of *S.* Typhimurium and *S.* Typhimurium-induced cytotoxicity by stimulating the host immune response in intestinal epithelial cells. The results showed that co-incubation of *S.* Typhimurium and *L. plantarum* JSA22 with intestinal epithelial cells suppressed *S.* Typhimurium infection, *S.* Typhimurium-induced NF-κB activation, and IL-8 production, and lowered the phosphorylation of both Akt and p38. These data indicated that *L. plantarum* JSA22 has probiotic properties, and can inhibit *S.* Typhimurium infection of intestinal epithelial cells. Our findings can be used to develop therapeutic and prophylactic agents against pathogenic bacteria.

Keywords: *Lactobacillus plantarum* , *Salmonella* Typhimurium, probiotic, intestinal epithelial cells, prophylactic agents

Introduction

Salmonella enterica is a foodborne pathogen that can cause a broad range of infections, including gastroenteritis, enteric fever, fecal infections, and septicemia [38]. *Salmonella* invasion into the intestinal epithelium is a multistep process that consists of entry into the epithelial cells by first inducing internalization within phagocytes and subsequent dissemination within the epithelial cell cytoplasm [8]. *S.* Typhimurium intimately adheres to, invades, survives, and replicates within host epithelial cells to cause actin cytoskeletal rearrangements and the production of several proinflammatory mediators by activating host cell signal transduction pathways and molecular pathways [2].

In the presence of *Salmonella*, the host cells recognize pathogen-associated molecular patterns such as flagellin, lipopolysaccharide, and lipoproteins, which are recognized by TLR5, TLR4, and TLR2, respectively. The bacterial components initiate a series of cellular signaling cascades in the host cells, leading to the activation of PI3K/Akt and several types of MAPK pathways mediated by p38, ERK, and JNK protein kinases [14]. Subsequently, transcription factors such as NF-κB and AP-1 are activated and translocated to the nucleus and trigger the production of proinflammatory cytokines and chemokines that stimulate the adaptive immune responses [37].

Furthermore, the global prevalence of antibiotic-resistant *S. enterica* strains has increased in recent years [10]. The

increased prevalence of antibiotic resistance is especially worrisome, because *S. enterica* is a common cause of human gastroenteritis worldwide [34]. Therefore, development of effective treatments or prophylactic agents against *Salmonella* infections is of considerable research interest.

Probiotics, especially *Lactobacillus* species, can inhibit the intestinal colonization and infection of pathogenic bacteria such as *Salmonella* spp. and may provide therapeutic benefits by controlling or eliminating the proliferation of the undesired pathogen strains [5]. However, the underlying molecular mechanisms that mediate the protective effects of probiotics have not been thoroughly characterized. Although the specific mechanisms mediating the protective effects of probiotics remain unknown, a previous study indicated that the probiotic bacteria could reduce intestinal microbial infections by competing with pathogens for attachment sites on the intestinal wall, producing lactic acid bacteria and antibacterial compounds, and stimulating and modulating the host immune system [26].

Food-based sources of probiotics have assumed greater significance in recent years, because various food products can predominantly harbor native and probiotic lactobacilli that exert beneficial effects on human health and thus can be used for both nutritional and therapeutic purposes [5, 15]. For example, naturally fermented soybean food products are commonly used as a good potentially beneficial probiotic [12].

We previously demonstrated that *L. plantarum* JSA22, isolated from a traditionally fermented soybean food product, exhibits high enzymatic, fibrinolytic, and antagonistic activity against foodborne pathogens such as *S. enterica* [1]. The mechanisms underlying the antibacterial activity of this *Lactobacillus* strain, especially the production of bacteriocin and non-bacteriocin antibacterial substances, have not been extensively studied. The main objective of the study was to determine the effects of the isolated probiotic strain, *L. plantarum* JSA22, in preventing and treating *S.* Typhimurium infections. Furthermore, the molecular mechanism of antimicrobial activity against *S.* Typhimurium was assessed to evaluate whether it can be used as a probiotic strain. Our data indicated that *L. plantarum* JSA22 can be potentially used as a probiotic strain and can inhibit *S.* Typhimurium infection of intestinal epithelial cells.

Materials and Methods

Bacterial Strains and Culture Conditions

L. plantarum JSA22 [1] and *L. rhamnosus* GG (used as a reference strain) were grown in de Man–Rogosa–Sharpe (MRS) broth (Difco, Becton Dickinson, Sparks, MD, USA) and incubated anaerobically at 37°C for 36 to 72 h. Pathogenic bacterial strains (*Bacillus cereus* ATCC 27348, *Staphylococcus aureus* DSM 346, *Escherichia coli* DSM 30083 and *S. enterica* IFO 3313) were cultured in Luria-Bertani (LB) broth (Difco, Becton Dickinson) or on LB agar medium at 37°C for 24 h.

Acid, Bile Salt, and Heat Tolerance Assays

To study the effects of acid, bile salt, and heat on the growth rate of lactobacilli, *Lactobacillus* cultures (approximately 2×10^8 CFU/ml) were harvested by centrifugation at 13,000 ×*g* for 5 min and washed twice with MRS medium. For the acid stress assay, the supernatant was removed, and the pellet was resuspended in MRS broth at pH 2; the pH was adjusted using 1 N HCl. The acid-treated samples were collected after 1, 2, and 3 h incubation at 37°C. For the bile salt stress assay, all cultures were inoculated in MRS medium containing 0.3% of bile salt, and incubated at 37°C for 1, 3, and 6 h. For the heat stress assay, *Lactobacillus* cells grown in MRS medium were heated for 30, 60, and 120 min at 65°C. For all stress assays, viable cell counts were performed using serial dilutions in MRS broth. For enumeration, the serial dilutions were plated on MRS agar plates, followed by incubation at 37°C for 48 h. The growth of *Lactobacillus* strains on MRS agar plates was used to identify acid-tolerant isolates.

Reverse Transcription PCR Analysis of Bile Salt Hydrolase Gene Expression

Total RNA was isolated from the bacterial cells by using an RNeasy plus mini kit (Qiagen, Hilden, Germany) by following the manufacturer's protocol. First-strand cDNA was synthesized using 1 μg of isolated RNA template and amfiRivert Platinum cDNA synthesis Master Mix (GenDEPOT; Barker, Texas, USA). A subset of genes was amplified with amfiEco Taq DNA polymerase and the following gene-specific primers were used: 16S rRNA gene, 248 bp: F-5′-ATTCATAGTCTAGTTGGAGGT-3′ and R-5′-CCTGAACTGAGAGAATTTGA-3′; bile salt hydrolase (*bsh*) gene, 231 bp: F-5′-ATCACCGCTACATTGGTTGG-3′ and R-5′-AGTCCG CCCATTCCTCTACT-3′; *bsh* gene, 975 bp: F-5′-ATGTGTACTGCC ATAACTTAT-3′ and R-5′-TTAGTTAACTGCATAGTATTG-3′ [17]. The amplified products were separated using 1% agarose gel and visualized by staining with Safe-Pinky DNA gel staining solution. Cycling conditions were as follows: 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec.

Cell Surface Hydrophobicity Assay

The optical density of the cultures was adjusted to 0.4 to obtain an approximate concentration of 10^7 CFU/ml. Approximately, 1 ml of bacterial suspension was added to 50 μl of hydrocarbon solution (n-hexadecane or xylene) and vortexed vigorously for 1 min. Then, the aqueous and oleophobic phases were separated by incubating the solutions for 1 h at RT. After phase separation, the optical density of the aqueous phase was measured and compared with

the initial value. Hydrophobicity was calculated using the following equation: Percent hydrophobicity = [(A540 initial – A540 aqueous phase)/A540 initial] × 100

Antagonistic Activity Against Pathogenic Bacteria

Antimicrobial effects of *Lactobacillus* strains against several pathogenic bacteria were determined using the agar-well diffusion assay [44]. Bacterial cultures grown overnight in LB broth were inoculated (3% (v/v)) in soft (0.7%) nutrient agar (NA; Difco, Becton Dickinson), which was melted and then cooled down to a temperature between 45°C and 50°C. After vigorous homogenization, the inoculated agar media were poured into Petri dishes, and wells of 3 mm diameter were bored into the inoculated agar plates. The petri dishes were incubated for 24 h at 37°C and the diameters of the inhibition halos were recorded in centimeters.

Mammalian Cell Culture

The human epithelial colorectal adenocarcinoma (Caco-2) cell line (ATCC HTB-37) was maintained in MEM (Gibco BRL Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% FBS (HyClone Laboratories, Logan, Utah, USA) inactivated at 56°C for 30 min, and 1% (v/v) penicillin-streptomycin (10,000 IU/ml; HyClone Laboratories). CCD-18Co normal human intestinal fibroblasts (ATCC CRL-1 459) were cultured in MEM enriched with 10% FBS, 1% sodium-pyruvate, 1% non-essential amino acids, and 1% antibiotics. The cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h.

Adhesion Assay

Cell adhesion assay was carried out according to the methods described previously [5, 24]. Briefly, Caco-2 and CCD-18Co cells were seeded into 24-well plates at 2 × 10^5 cells/well for 24 h and then infected with bacteria at a multiplicity of infection (MOI) of 10:1. The plates were centrifuged at 500 ×*g* for 10 min, followed by incubation for 45 min at 37°C with 5% CO_2 atmosphere. Unbound bacteria were removed by aspiration, and the wells were washed three times with fresh MEM, and the cells were lysed with 0.1% Triton X-100 (v/v). Cell lysates were serially diluted in PBS buffer and plated onto LB agar to enumerate bacteria.

Invasion Assay

Caco-2 and CCD-18Co cell monolayers grown on 24-well plates at a density of 2 × 10^5 cells/well were infected with bacterial strains at an MOI of 10, and centrifuged at 500 ×*g* for 10 min. After incubation for 45 min at 37°C with 5% CO_2, the cells were washed three times with fresh MEM and incubated in MEM containing gentamycin (100 μg/ml) for 90 min to kill the remaining extracellular bacteria. Following incubation, the plates were washed three times with PBS, the cells were treated with a 1% Triton X-100 (v/v) solution for 15 min at RT, and the lysates were serially diluted and then plated onto LB agar plates. The number of cells infected by bacteria (invasion rates) was calculated as a percentage of the control (*S.* Typhimurium), which was set at 100%.

Cell Cytotoxicity Assay

For performing LDH release assay, the cellular supernatants were evaluated during bacterial infection. Mammalian cells were seeded in 24-well plates at a density of 2 × 10^5 cells/well for 24 h before treatment. After 2 h of incubation with bacteria, supernatants of the infected cells containing released LDH were collected, centrifuged at 13,000 ×*g* for 10 min at 4°C, and evaluated for LDH activity. The percentage of Caco-2 and CCD-18Co cell death was determined by measuring LDH release in confluent cultures using the LDH Cytotoxicity Assay Kit II (Abcam Inc., Cambridge, MA, USA), following instructions provided by the manufacturer. The percentage of cytotoxic activity was calculated as 100 × (experimental LDH release – spontaneous LDH release) / (maximum LDH release – spontaneous LDH release), wherein spontaneous LDH release was the level detected in a non-confluent cell culture.

Immunofluorescence Study

Monolayers of CCD-18Co cells (1 × 10^4 cells/well) grown on glass coverslips in 24-well plates were infected with bacterial strains for 45 min. After infection, the CCD-18Co cells on the coverslips were washed three times with PBS, fixed with 3.7% paraformaldehyde in PBS for 20 min, and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min. The permeabilized cells were incubated with 3% (w/v) BSA in PBS for 1 h at RT to suppress nonspecific binding. The cells were incubated with *Salmonella* SopB antibody overnight at 4°C, followed by incubation with Alexa Fluor 488-conjugated rabbit anti-goat secondary antibody in 3% BSA for 1 h at RT in the dark. The samples were the washed three times with PBS. Mounting medium containing DAPI was dispensed onto the microscope slide and the coverslip with the cells was placed onto the slide. The cells were visualized using a fluorescence microscope (Leica DMI6000; Leica, Germany).

Isolation of Total, Cytosolic, and Nuclear Protein Extracts

To detect *Salmonella* SopB expression in the total cellular proteins, CCD-18Co cells were seeded into two 100-mm-diameter culture dishes (2 × 10^6 cells/dish) and then infected with bacteria at an MOI of 1:10 for 1 h. The cell monolayers were washed three times with PBS and detached by using a cell scraper. The cells were then lysed in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, and 1 mM EDTA) containing a protease inhibitor cocktail, and centrifuged at 13,000 ×*g* for 5 min at 4°C to collect whole cell lysate.

Cytoplasmic and nuclear protein extracts were prepared as described by Pieper *et al.* [33] with some modifications. CCD-18Co cells were seeded into 100-mm-diameter culture dishes at 5 × 10^6 cells/dish for 24 h and then infected with bacteria at an MOI of 50 for 1 h. The cells were washed three times with PBS and harvested by scraping in hypotonic buffer A (10 mM HEPES at pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT) to obtain cytosolic and nuclear fractions. After centrifugation of the samples at 3,300 ×*g* for 5 min at 4°C, the supernatants were collected and

used as cytoplasmic extracts. The pellets were then resuspended in hypertonic buffer B (20 mM HEPES (pH 7.9), 0.2 mM EDTA, 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.4 M NaCl, 0.5 mM DTT) and incubated on ice for 10 min [39]. After centrifugation at 13,000 ×*g* for 10 min, the supernatants were collected and used as nuclear protein extracts. The total protein concentrations in the isolated fractions were determined and normalized by using the Bradford protein assay data (Bio-Rad, Berkeley, CA, USA).

Western Blotting and Antibodies

The nitrocellulose membranes were blocked with 3% BSA in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h and then incubated for 1 h with primary antibody. The following primary antibodies were used: polyclonal anti-SopB antibody (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), polyclonal anti-p65 antibody (1:500; Santa Cruz Biotechnology Inc.), polyclonal anti-phospho-Akt antibody (1:500; Abcam Inc., Cambridge, MA, USA), polyclonal anti-Akt antibody (1:500; Abcam Inc.), monoclonal anti-phospho-p38 MAPK (Thr180/Tyr182) (1:1000; Cell Signaling Technology, Beverly, MA, USA), polyclonal anti-p38 MAPK antibody (1:1000; Cell Signaling Technology), polyclonal anti-lamin-B antibody (1:1,000; Santa Cruz Biotechnology Inc.), polyclonal anti-GAPDH antibody (1:3,000; Abcam Inc.), and polyclonal anti-beta actin antibody (1:5000; Abcam Inc.). After washing the membranes three times with TBST, the secondary antibody of goat anti-mouse IgG horseradish peroxidase (HRP) conjugate, goat anti-rabbit IgG HRP conjugate, or rabbit anti-goat IgG HRP conjugate (1:3,000; Bio-Rad) was incubated with the membranes for 1 h. The blots were developed using a BM chemiluminescence blotting substrate (POD; Roche, Mannheim, Germany).

Measurement of IL-8 Production

IL-8 levels in culture supernatants were determined by using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam Inc.) according to the manufacturer's protocol. After development of the colorimetric reaction, the absorbance was measured at 450 nm by using a Synergy Mx microplate reader (BioTek Instruments, Winooski, VT, USA). A standard curve was generated by plotting the mean absorbance for each standard concentration (X-axis) against the target protein concentration (Y-axis). By comparing the absorbance of the samples with the standard curve, the concentration of IL-8 in the samples was determined.

Statistical Analysis

Statistically significant differences were determined by calculating the *P* values of Student's *t*-test. *P* values of < 0.05 were considered statistically significant.

Results and Discussion

Antimicrobial Activity and Probiotic Characteristics of *L. plantarum* JSA22

Lactobacilli may confer health benefits to the host, and there is accumulating evidence that they are effective in controlling symptoms or preventing intestinal disease in both animals and humans owing to their ability to restore the balance of the normal microbiota in the intestine, inhibit pathogenic bacteria adhesion to the intestinal wall, and prevent the inflammatory processes [35].

In our previous study, we demonstrated that *L. plantarum* strain JSA22 isolated from buckwheat *sokseongjang*, a Korean traditional fermented soybean food, had probiotic properties and significant antibacterial activity against different pathogenic bacteria [1]. We correctly confirmed the evaluation of the probiotic potential value and antipathogenic activity of *L. plantarum* strain JSA22. In this study, we used *L. rhamnosus* strain GG (LGG) as the positive control, because this probiotic strain has been isolated from the human gastrointestinal tract and has been extensively studied [7]. This probiotic strain has been shown to be resistant to acid and bile, have strong adhesive properties to human and rabbit intestinal mucosal cells, mediate suppression of bacterial enzyme activity, and produce antimicrobial substances [22].

Therefore, we tested whether *L. plantarum* strain JSA22 could improve the survival rate of this strain grown under heat, bile salts, and acidic pH stress conditions. The viability of acid-adapted *L. plantarum* strain JSA22 was highly similar to that of *L. rhamnosus* GG (Fig. 1A). Consistent with the results shown in Fig. 1A, the survival studies data indicated that *L. plantarum* JSA22 and the probiotic bacterial strain *L. rhamnosus* GG showed high survival rates under bile and heat stress (Figs. 1B and 1C). Probiotic strains that secrete bile salt hydrolase (BSH), a bacterial enzyme involved in host lipid metabolism, are of interest because of their potential use for treating hypercholesterolemia. BSH is an enzyme produced by commensal bacteria in the intestinal microflora and a involved in host lipid metabolism as important regulators of gene expression in the liver and intestines [18, 21]. Thus, the enhanced BSH activity by the various probiotic strains may offer potential as a biological alternative to pharmaceutical interventions to treat hypercholesterolemia [21]. In this study, RNA expression of the *bsh* gene in *L. plantarum* JSA22 was assessed using RT-PCR, and 16S rRNA was used as a positive control (Fig. 1B). The PCR products of the expected size were detected in *L. plantarum* JSA22. Recent study demonstrated that acid-resistant strains can be produced by subjecting acid-sensitive strains to prolonged exposure to pH 2.0 [4]. Furthermore, a previous study showed that *L. plantarum* strain WCFS1 was able to grow and survive in acidic conditions, and cells harvested

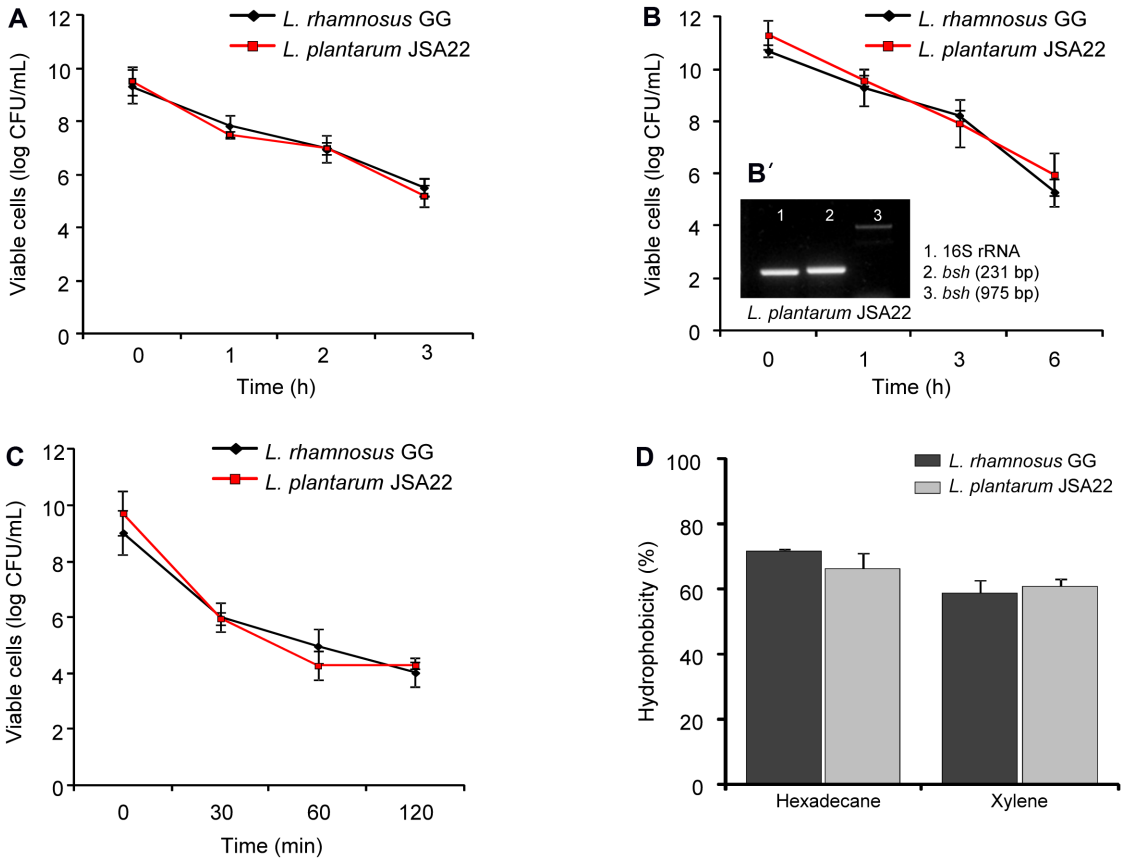

Fig. 1. Assessment of probiotic characteristics of *L. plantarum* strain JSA22.

Viability (log CFU/ml) of *L. rhamnosus* GG and *L. plantarum* JSA22 strains in (**A**) acid (pH 2.0), (**B**) bile (0.3% bile salts), and (**C**) heat (65°C) stress. (**B′**) The mRNA expression levels of bile salt hydrolase (*bsh*) gene in *L. plantarum* JSA22 were assessed using RT-PCR analysis. (**D**) Cell surface hydrophobicity of *L. rhamnosus* GG and *L. plantarum* JSA22 strains in different hydrophobic solvents. Experiments were performed at least three times, and representative data are shown. Error bars show the standard deviation.

from stationary phase of growth consistently displayed a higher tolerance to gastric juice treatment and a high survival rate in the GI-tract assay [11]. These results indicate that *L. plantarum* JSA22 is capable of surviving stress factors such as heating, bile salts, and low pH.

A comparison of cell-surface hydrophobicity of probiotic strains and pathogens can indicate their colonizing ability [6, 19]. A previous study indicated that probiotic strains *Bifidobacterium longum* B6 and *L. rhamnosus* GG had high hydrophobicity and strong binding ability to Caco-2 cells, and that the strains effectively inhibited the adhesion of *Listeria monocytogenes*, *S.* Typhimurium, and *S. aureus* to Caco-2 cells [40]. Thus, bacterial cell surface hydrophobicity strongly influences competitive inhibition, indicating that the *in vitro* adhesion assay data may be indicative of *in vivo* colonization. In this study, *n*-hexadecane and xylene were used to evaluate the hydrophobic bacterial cell surface properties of *L. plantarum* JSA22 and *L. rhamnosus* GG (Fig. 1D). The percent hydrophobicity of the tested strains ranged between 72% and 58%. Previous studies indicate that strains with percent hydrophobicity of 40% or higher have hydrophobic molecules such as surface array proteins, wall intercalated proteins, cytoplasmic membrane protein, and lipids on the bacterial cell surface [43]. Thus, the hydrophobicity of *L. plantarum* strain JSA22 may have influenced its high affinity towards xylene and *n*-hexadecane. Taken together, these data indicate that *L. plantarum* JSA22 has the ability to colonize the intestinal epithelium, which is of potential probiotic value.

Probiotic strains can also be used to improve food safety by treating food-producing animals with probiotics to reduce foodborne pathogens in those animals and their products [38]. Previously reported studies show that *L. rhamnosus* GG inhibits *E. coli*, *S.* Typhi, *B. cereus*, and *S. aureus*, and that *L. rhamnosus* GG produces bacteriocins and antimicrobial peptides that inhibit the growth of

pathogenic bacteria [29]. We next examined the *in vitro* antibacterial activity of both *L. plantarum* JSA22 and *L. rhamnosus* GG against pathogenic bacteria, including *B. cereus*, *S. aureus*, *E. coli*, and *S. enterica*. Both *L. plantarum* JSA22 and *L. rhamnosus* GG had similar antagonistic activity towards all four pathogenic bacteria and produced clear inhibition zones of more than 1.8 mm in agar-spot plates (Fig. 2). As compared with *L. rhamnosus* strain GG, *L. plantarum* JSA22 showed higher inhibitory activity against *E. coli* and *S. enterica*. Thus, *L. plantarum* JSA22 may be used as a candidate broad-spectrum antimicrobial agent for the treatment of bacterial diseases. Overall, our results indicated that *L. plantarum* JSA22 could be used as a potential probiotic strain and that the antimicrobial activity of the strain could be exploited to treat or prevent foodborne infections.

Influence of *L. plantarum* JSA22 on Adhesion, Invasion, and Cytotoxicity of *S.* Typhimurium

The probiotic strains may mediate antimicrobial actions by competing for limited nutrients and by inhibiting adhesion, invasion, and proliferation of pathogens in the intestinal epithelium [3]. Probiotics are also known to adhere to intestinal epithelial cells, thereby competitively excluding the adhesion of pathogenic intestinal bacteria [5]. Thus, adhesive probiotics can inhibit the intestinal colonization and attachment of pathogens, a phenomenon termed as competitive exclusion [2]. As shown in Fig. 3A, the adhesion of *S.* Typhimurium to CCD-18Co and Caco-2 cell lines was significantly reduced by treatment with *L. plantarum* JSA22 or *L. rhamnosus* GG. However, the adhesion of *S.* Typhimurium to host cells was markedly higher in *S.* Typhimurium infections performed in the absence of *Lactobacillus* strains (Fig. 3A). Thus, *L. plantarum* strain JSA22 significantly inhibits *S.* Typhimurium attachment to intestinal epithelial cell lines.

We performed invasion assays to determine the effect of *L. plantarum* JSA22 on *S.* Typhimurium entry and proliferation in intestinal cells. To assess this, *L. plantarum* JSA22 and *S.* Typhimurium strains were co-incubated with either a

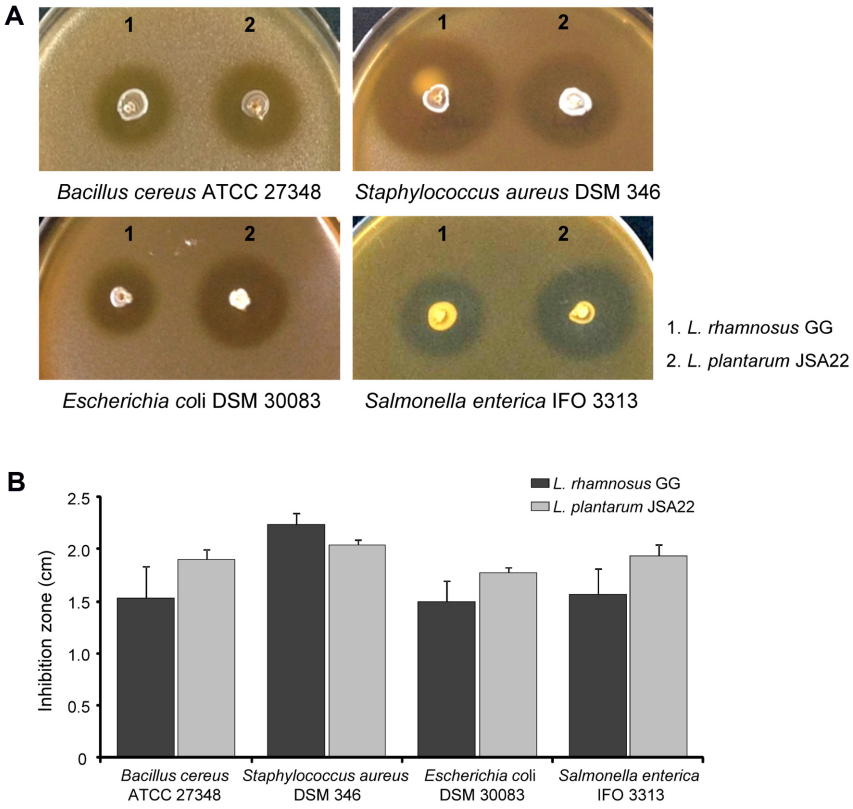

Fig. 2. Antagonistic activity of *L. plantarum* JSA22 against pathogenic bacteria.

(**A**) Agar spot test showing the antibacterial activity of *L. rhamnosus* GG and *L. plantarum* JSA22 strains against pathogenic bacteria. Inhibition zones were observed after 24–48 h of incubation under appropriate conditions. The clear area without bacterial growth is the zone of inhibition. (**B**) The inhibition zone diameters of the different pathogenic microbes were measured and recorded. Error bars represent the mean standard deviations for three independent experiments.

CCD-18Co cell line monolayer or Caco-2 cell line monolayer and gentamicin protection assays were performed. The *S.* Typhimurium invasion rate for CCD-18Co (54%) and Caco 2 cells (64%) was reduced in the presence of the probiotic bacterial strain *L. rhamnosus* GG (Fig. 3B). The *S.* Typhimurium invasion rate of CCD-18Co (48%) and Caco-2 cells (70%) was significantly reduced in the presence of *L. plantarum* strain JSA22 as compared with that of the positive control (inoculation with *S.* Typhimurium alone). Thus, we speculated that *L. plantarum* strain JSA22 suppressed the invasion of intestinal cells by *S.* Typhimurium by interfering with the adhesion of *S.* Typhimurium on the host cells. Previous studies have also reported reduced adhesion and invasion of pathogenic bacteria in the presence of probiotic strains due to competitive exclusion of the pathogens [23, 38].

Apoptosis and the maintenance of cell membrane integrity are crucial for maintaining intestinal epithelial barrier function and for blocking bacterial invasion [28]. In this study, we investigated the effects of *L. plantarum* JSA22 on preventing or promoting apoptosis of intestinal cell lines infected with *S.* Typhimurium. The viability of epithelial cells was indirectly assessed by measuring LDH release as an indicator of epithelial cell damage and apoptosis. We also speculated that the apoptosis level may correlate with the adhesion of pathogenic bacteria. As shown in Fig. 4, the low LDH release by epithelial cells incubated with *L. plantarum* JSA22 or *L. rhamnosus* GG was significantly lower than that of the LDH-positive control (lysed) and *S.* Typhimurium strain (no lactobacilli). The LDH levels of CCD-18Co monolayers infected with *L. plantarum* JSA22 at MOI 100 was 4-fold lower than that of CCD-18Co monolayer infected with *S.* Typhimurium alone. Similarly, the percent cytotoxicity of Caco-2 monolayer infected with *L. plantarum* JSA22 at MOI 100 was 4.6 fold lower than that of Caco-2 monolayer infected with *S.* Typhimurium alone. Cytotoxicity percentages of CCD-18Co and Caco-2 cell lines infected with *L. rhamnosus* GG infection at a MOI 100 were 10% and 24%, respectively. Thus, *L. plantarum* JSA22 induces protection against *S.* Typhimurium-induced cell cytotoxicity in intestinal epithelial cells. These data show that although *L. plantarum* JSA22 suppresses *S.* Typhimurium-induced cell membrane damage by potentially inhibiting its adherence to host epithelial cells, the co-incubation of the two strains with the intestinal cell lines increased epithelial cell membrane leakage. In accordance with a recent report that showed that *L. rhamnosus* GG prevented cytokine-induced apoptosis of human intestinal epithelial cells and mouse colonocytes [41], we found that both *L. rhamnosus* GG and *L. plantarum* JSA22 prevented *S.* Typhimurium-induced cytotoxicity. Accordingly, our results demonstrate that *L. plantarum* JSA22 can prevent *S.* Typhimurium-induced cell membrane damage and aggravate bacteria-evoked delayed apoptotic cell death involving membrane damage.

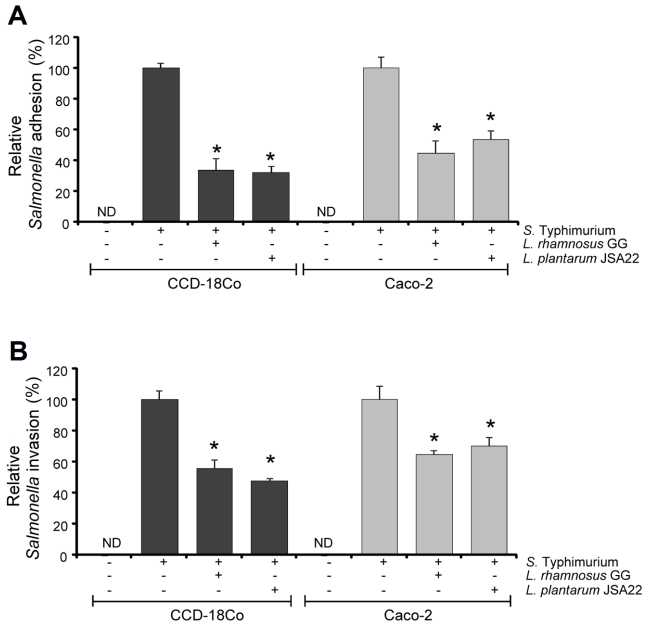

Fig. 3. Effect of *L. plantarum* JSA22 on the adhesion and invasion of *S.* Typhimurium.

Adhesion (**A**) and invasion (**B**) rates for *S.* Typhimurium-infected monolayers of CCD-18Co and Caco-2 cells were calculated as percentages of adhered or invaded *S.* Typhimurium (control), which was set as 100%. All of the experiments were performed at least three times, and representative data are shown. Data are shown as means (± SD). *, $p < 0.05$. ND, not detected.

Effect of *L. plantarum* JSA22 on *S.* Typhimurium Growth and Survival

Salmonella encodes various effector proteins that mediate the invasion of intestinal epithelial cells. For example, the type III protein secretion system (T3SS) is encoded by a chromosomal region called *Salmonella* Pathogenicity Island I (SPI-1), which enables production of various effector proteins, including SopB, that are translocated into the intestinal epithelial cells. The translocated effector proteins then modulate the intestinal epithelial cell actin cytoskeleton to promote internalization of bacteria into the intestinal epithelial cells. Notably, the SopB protein levels persist in the intestinal epithelial cells for up to 12 h post infection. Furthermore, SopB meditates host cell invasion in the intracellular phase of *Salmonella* infection [32].

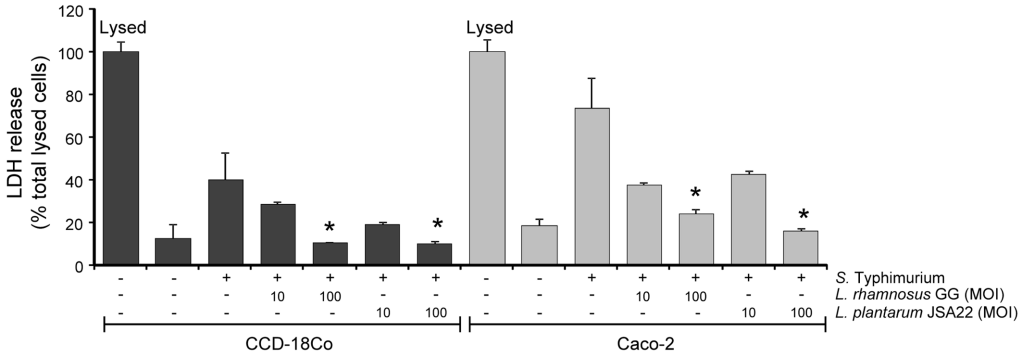

Fig. 4. Protective effect of *L. plantarum* JSA22 against *S.* Typhimurium-induced cell cytotoxicity.
LDH released from damaged epithelial cells was measured to estimate the cytotoxicity. CCD-18Co and Caco-2 monolayers treated with *S.* Typhimurium (60 min, 37°C, 5% CO_2) were used to assess the *S.* Typhimurium-mediated cytotoxicity. Pretreatment of CCD-18Co and Caco-2 cells with *L. plantarum* JSA22 and *L. rhamnosus* GG protected against *S.* Typhimurium-induced cell damage. The data are presented as means (± SD) for three independent experiments. Analysis was performed using Student's *t*-test. *, $p < 0.05$.

To visually examine the invasion of *S.* Typhimurium into the host cells co-infected with *Lactobacillus* strains, we stained the infected CCD-18Co cells with anti-SopB antibody to detect *Salmonella*. As shown in Fig. 5A, *Salmonella* attachment to the host cell surface was significantly reduced in the presence of *L. plantarum* JSA22 or *L. rhamnosus* GG, whereas *Salmonella* infection of intestinal epithelial cells was markedly higher in cell cultures infected with *S.* Typhimurium alone. In CCD-18Co cells with NI, the adhesion of *Salmonella* to the intestinal epithelial surface was not detectable. The confocal images further corroborated our data and indicated reduced *S.* Typhimurium infection of intestinal epithelial cells in the presence of *Lactobacillus* strains. Consistent with the confocal microscopy data, the SopB expression assay data showed that SopB levels were lower in the protein extracts of epithelial cells infected with *S.* Typhimurium in the presence of either *L. plantarum* JSA22 or *L. rhamnosus* GG. The SopB levels of epithelial cells infected with the *S.* Typhimurium alone were considerably higher, indicating that *L. plantarum* strain JSA22 may mediate the inhibition of *Salmonella* survival and replication within host cells.

Previous studies have reported that various probiotic components down-regulate *sopB* gene expression in *S.* Typhimurium, thereby preventing its invasion into intestinal epithelial cells [9]. The lactic-acid-producing bacteria treatments, particularly at low bacterial doses, suppress the expression of most SPI-1-encoded virulence genes (*hilA, hilC, hilD, sopB, sopD, sopE2, sipA, sipC, avrA*, and *sptP*) in *Salmonella*-infected chicken cecum [42].

Our data were also in agreement with these findings and showed down-regulation of *sopB* gene expression in *S.* Typhimurium that was co-incubated with *L. plantarum* JSA22. Based on these results, we speculated that *L. plantarum* JSA22 secretes proteins that mediate the down-regulation of virulence-related genes of *S.* Typhimurium. The antimicrobial effect of probiotics against intestinal pathogens may therefore be primarily mediated by the suppression of virulence gene expression of pathogenic bacteria.

Inhibition of Akt Phosphorylation, p38 MAPK Phosphorylation, and NF-κB Activation by *L. plantarum* JSA22

Recent studies showed that *L. brevis* G-101 inhibits the phosphorylation of both IRAK1 and AKT *via* the MyD88-PI3K signaling pathway, which eventually activates NF-κB and MAPKs [13]. Together, these data indicate that some probiotic *Lactobacillus* strains may be useful for preventing inflammatory response-mediated damage during bacterial infections. Kuijl *et al.* [20] also showed that *Salmonella* effector protein SopB induces the activation of Akt, which subsequently activates Rab14 and AS160 phosphorylation to block phagosomal maturation, and thereby contributes to anti-apoptotic signaling.

Therefore, to investigate the role of intracellular signaling events in mediating the antiproliferative activity of *L. plantarum* JSA22, Akt and p38 MAPK phosphorylation and NF-κB activation were evaluated in CCD-18Co cells stimulated with *S.* Typhimurium. As shown in Figs. 6A and 6B, the levels of Akt and p38 phosphorylation were significantly decreased in the total cellular proteins of host cells infected with *L. plantarum* JSA22 or *L. rhamnosus* GG, whereas Akt and p38 activation was markedly increased in *S.* Typhimurium-infected cells without *Lactobacillus*. Quantitative analysis of the relative signal intensity based on the ratios of phospho-Akt/total Akt indicated a 10-fold lower level of

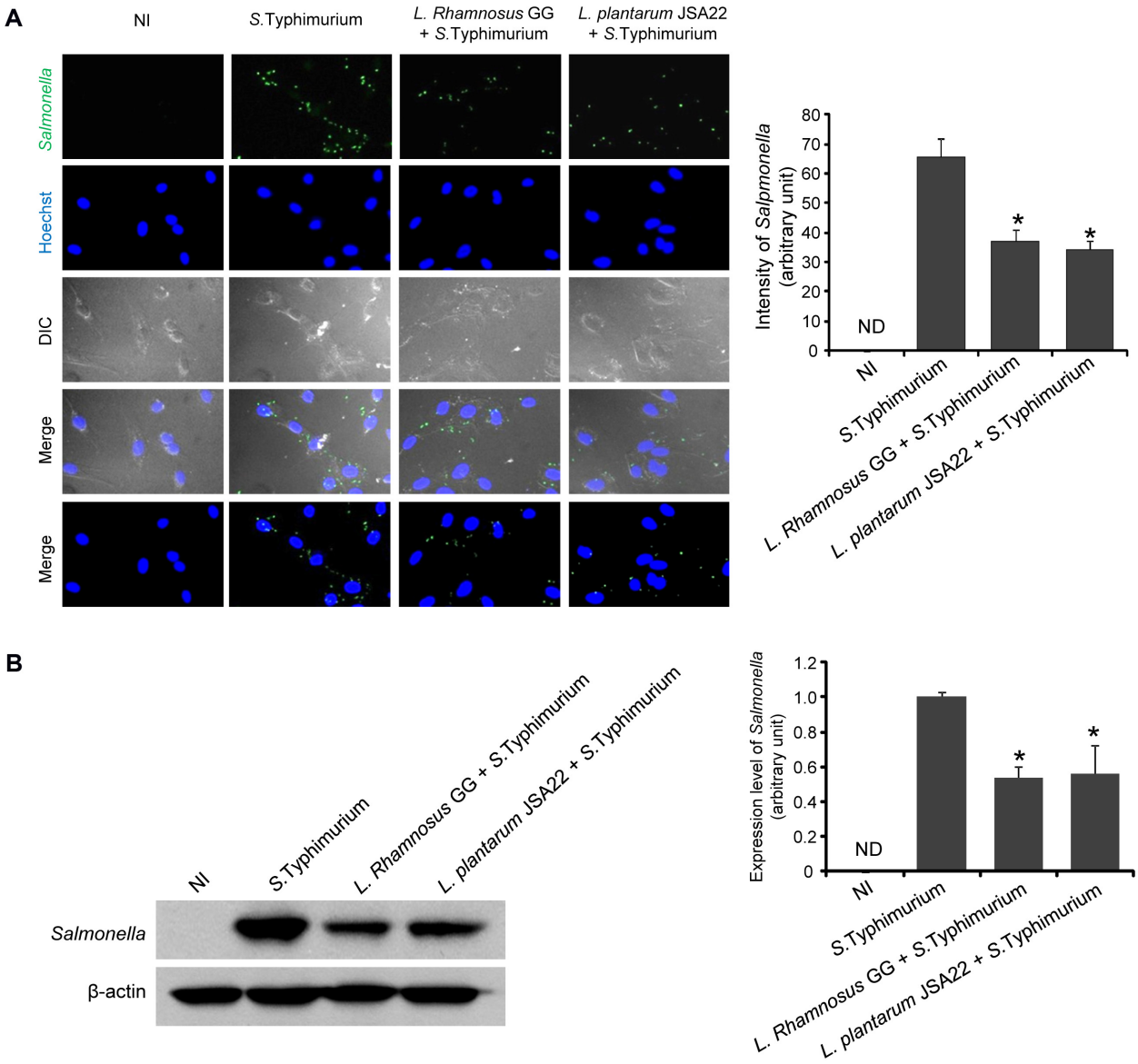

Fig. 5. *L. plantarum* JSA22 inhibits *S.* Typhimurium infection.

(**A**) For visualization of *Salmonella* invasion, CCD-18Co cells infected with bacterial strains were stained with FITC-conjugated anti-SopB antibodies. The epithelial cell nuclei were visualized by performing DAPI staining, and *Salmonella* was stained with FITC-conjugated anti-SopB antibodies. Images from three different confocal planes (at least 30 cells) per sample were analyzed to estimate SopB expression (Right panel). (**B**) The expression levels of SopB in the total cellular proteins were determined using immunoblot analysis with an anti-SopB antibody. The data were normalized relative to β-actin as a protein loading control (right panel). The expression levels of SopB in the total cellular proteins were quantified by performing densitometry analysis. Each band in the blots was normalized by using β-actin densitometry data, and the levels of SopB are expressed as the mean ± SD from three separate experiments. NI: non infected, ND: not detected. *, $p < 0.05$.

Akt phosphorylation in host cells infected with both *S.* Typhimurium and *L. plantarum* strain JSA22 as compared with host cells infected with *S.* Typhimurium in the absence of *Lactobacillus* (Fig. 6A). Analysis of the signal intensity for the phospho-p38/total p38 ratio showed the *S.* Typhimurium-infected epithelial cells had 2-fold higher p38 phosphorylation than that of epithelial cells infected with *S.* Typhimurium co-incubated with either *L. plantarum* JSA22 or *L. rhamnosus* GG strains (Fig. 6B). These results indicate that *L. plantarum* JSA22 promotes survival of intestinal epithelial cells through inhibition of the pro-apoptotic Akt factor and by suppressing p38 activation.

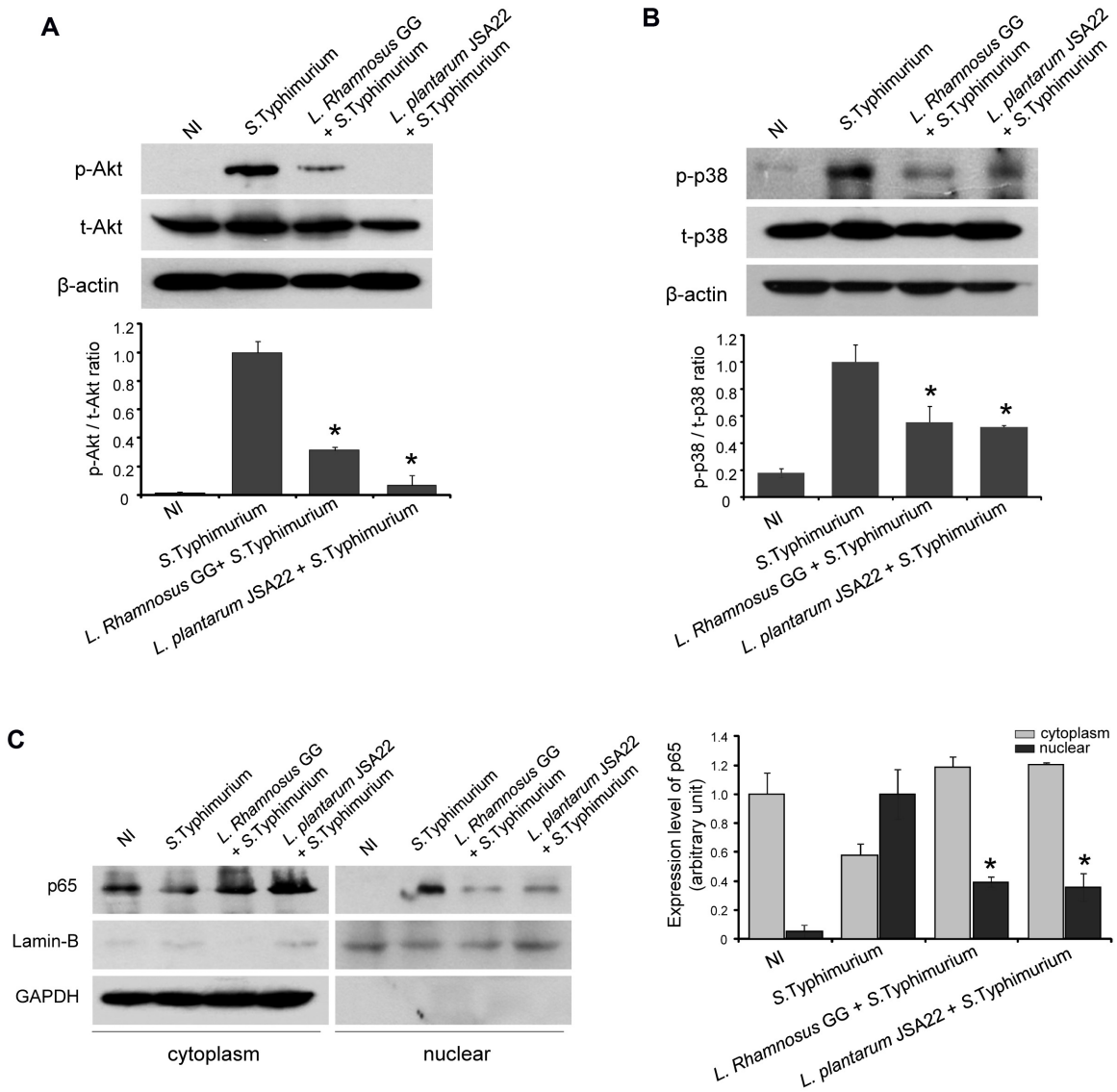

Fig. 6. Inhibition of *S*. Typhimurium-induced inflammatory mediators and NF-κB activation by *L. plantarum* JSA22.
(**A**, **B**) The expression levels of p-AKT and p-p38 in total cellular proteins from all strains were analyzed by performing western blotting using primary antibodies specific for p-AKT and p-p38, followed by incubation with HRP-conjugated secondary antibody. An anti-β-actin antibody was used to estimate actin expression as a loading control. The intensity of each band was quantified by performing densitometry analysis and the ratios of phosphorylated and total proteins were calculated. (**C**) The expression of NF-κB p65 was determined by performing immunoblot analysis with an anti-NF-κB p65 antibody for staining the cytoplasmic and nuclear fractions obtained from infected CCD-18Co cells. The anti-GAPDH and anti-lamin B antibodies were used as cytoplasmic and nuclear protein markers, respectively. The expression levels of p65 in the nuclear and cytoplasmic fractions were quantified by performing densitometry analysis (right panel). The data shown are representative of at least three independent experiments. The data are shown as means (± SD). NI: non infected. *, $p < 0.05$.

A previous study indicated that probiotic *L. rhamnosus* GR-1 modulated immune responses of bladder cells against enterotoxigenic *E. coli* by promoting NF-κB activation, which mediates the release of inflammatory mediators. Furthermore, the urogenital probiotic *L. rhamnosus* GR-1 increased the TLR4 expression in the bladder cells [16]. To test whether *L. plantarum* JSA22 activated the host innate immune response in a NF-κB-dependent manner, we assessed the nuclear translocation of NF-κB in CCD-18Co cells. As shown in Fig. 6C, host cell infection with the *S*. Typhimurium strain alone resulted in increased NF-κB p65 subunit nuclear translocation. Activation of NF-κB was

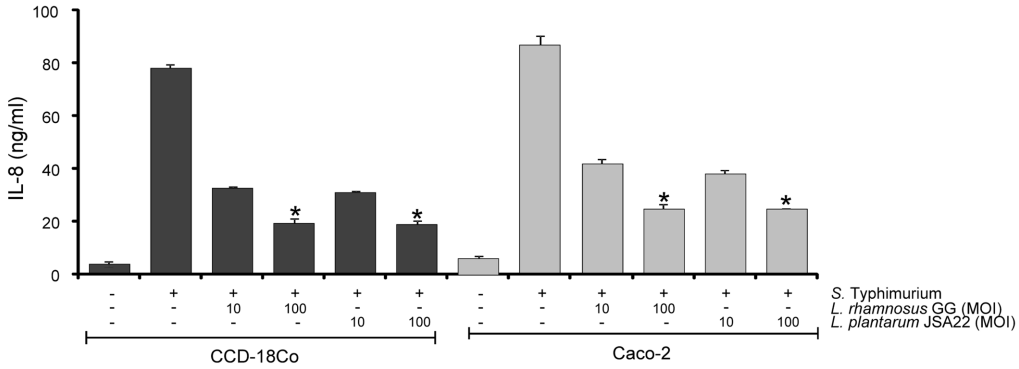

Fig. 7. Modulation of *S*. Typhimurium-induced IL-8 production by *L. plantarum* JSA22.
IL-8 expression of CCD-18Co and Caco-2 cells that were either untreated or infected with *S*. Typhimurium and lactobacilli (*L. plantarum* JSA22 or *L. rhamnosus* GG) for 3 h was determined by performing ELISA using culture supernatants. The data are shown as means (± SD) for three individual experiments. *, $p < 0.05$.

markedly lower in cells co-infected with *L. plantarum* JSA22 and *S*. Typhimurium, and the *L. rhamnosus* GG strain induced significantly decreased levels of NF-κB-dependent gene activation. These results indicate that *L. plantarum* JSA22 inhibits *Salmonella* infection by significantly reducing *Salmonella*-induced activation of the NF-κB pathway in intestinal epithelial cells. Together, these results indicate that *L. plantarum* strain JSA22 may prevent bacterial growth by inhibiting the Akt, p38 MAPK, and NF-κB (p65-dependent manner) pathways in *Salmonella*-stimulated host cells.

Reduction of IL-8 production by *L. plantarum* JSA22

The intestinal epithelial cells are considered the major reservoir of *Salmonella*, as they are known to produce and release a variety of proinflammatory cytokines that contribute to the protective immune responses against infections with virulent *Salmonella* strains [36]. In addition, *Salmonella* infections stimulate the production of pro-inflammatory cytokines, such as IL-8, IL-6, IL-18, TNF-α, MCP-1, and IL-6, in the host cells [25]. A recent study showed that several probiotic *Lactobacillus* strains prevent or attenuate the secretion of IL-8, and that the strains may mediate anti-inflammatory effects that directly modulate the host immune function and influence inflammatory diseases [31].

Therefore, we assessed whether the infection of the *L. plantarum* JSA22 strain could induce the production of IL-8 in *S*. Typhimurium-infected CCD-18Co and Caco-2 cell lines. As shown in Fig. 7, ELISA analysis showed that the expression of IL-8 in CCD-18Co and Caco-2 cells infected with *L. plantarum* strain JSA22 was significantly lower than that in cells infected with *S*. Typhimurium alone. As compared with the the *S*. Typhimurium-infected control cells, CCD-18Co and Caco-2 cells infected with *L. plantarum* JSA22 at an MOI 100 secreted IL-8 at levels that were 3-fold and 4-fold lower, respectively. CCD-18Co and Caco-2 cell monolayers treated with NI did not secrete IL-8 at levels detectable by the assay. These results demonstrate that *L. plantarum* JSA22 may reduce the immune response of host cells by decreasing IL-8 production during the course of *Salmonella* infection.

L. rhamnosus GG was recently reported to have a similar anti-inflammatory effect by suppressing tumor necrosis factor alpha production in *H. pylori*-treated macrophage, and surprisingly, *B. breve* Bb99 induced IL-8 production in epithelial cells with or without *H. pylori* exposure [28, 30]. Our results are consistent with those reported in a previous study, which showed that specific *B. breve* strains may induce proinflammatory responses [27].

In conclusion, *L. plantarum* JSA22 isolated from buckwheat *sokseongjang*, a Korean traditional fermented soybean food, had desirable characteristics for use as a potential probiotic strain. *L. plantarum* JSA22 inhibited the bacterial growth, adherence, and cell invasion of *S*. Typhimurium. In addition, *L. plantarum* could suppress the inflammatory response of intestinal epithelial cells by inhibiting IL-8 expression and suppressing *Salmonella*-induced NF-κB activation, which in turn reduced the phosphorylation of Akt and p38. Taken together, our data indicate that *L. plantarum* JSA22 could be used as a probiotic agent for treating inflammatory bowel diseases and may have other uses, including enhancing food safety and antimicrobial and industrial applications. Further studies are required to identify and characterize novel antagonistic substances such as bacteriocins, bacteriocin-like substances, and antibacterial lipopeptides that are secreted by *L. plantarum* JSA22. Thus, this study the data

indicate that *L. plantarum* JSA22 can be potentially used as a probiotic strain and this may enhance host immune responses against bacterial infection.

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