이학석사 학위논문

Induction of human beta defensin-2 by *Bacillus subtilis* lipoproteins in human intestinal epithelial cells

인간 장 상피세포에서

고초균 지질단백질에 의한

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협동과정 유전공학전공

소윤주

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이 논문을 이학석사 학위논문으로 제출함

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소윤주의 석사학위논문을 인준함

2023년 1월

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ABSTRACT

Induction of human beta defensin-2 by *Bacillus subtilis* lipoproteins in human intestinal epithelial cells

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Objectives

Human intestinal epithelial cells (IECs) play an important role in maintaining homeostasis of the gut. They produce antimicrobial peptides (AMPs) to eliminate the intestinal pathogens or generate mucin to generate the mucus layers that protect intestinal epithelium. *Bacillus subtilis*, frequently found in the healthy human gut, is utilized in several food and pharmaceutical industries since they are approved by the food and drug administration as generally recognized as safe. Although their protective effects on gastrointestinal epithelium, such as protection against pathogens and improving intestinal diseases, are widely reported, the key molecule of *B. subtilis* responsible for the beneficial effects remains elusive. Therefore, the aims of this study are (i) to elucidate the effects of *B. subtilis* on the induction of

AMP in human IECs, and (ii) to identify the key molecules responsible for the induction of AMP production and their underlying mechanisms.

Methods

Human IECs, Caco-2 cells, were stimulated with live and heat-killed *B. subtilis*, and the mRNA expressions of AMPs, including human beta-defensin (HBD)-1, HBD-2, or HBD-3, were determined using real-time RT-PCR. Microbe-associated molecular patterns (MAMPs) of *B. subtilis*, including lipoteichoic acid (LTA), peptidoglycan (PGN), and lipoprotein (LPP), were isolated and used for stimulating Caco-2 cells to compare the inducibility of HBD-2 expression. The cells were treated with transcriptional and translational inhibitors to examine the relationship between mRNA expression and protein levels of HBD-2. LPPs were treated with heat, DNase I, lipase, or proteinase K, and subjected to Coomassie blue or silver staining. The HBD-2 level of differentiated Caco-2 cells was measured by enzyme-linked immunosorbent assay. Caco-2 cells were pre-treated with inhibitors blocking toll-like receptor (TLR) 2, mitogen-activated protein (MAP) kinase, and NF-κB for 1 h and stimulated with LPP to determine the molecular mechanism of HBD-2 induction. Bacterial pathogens, including *Bacillus cereus* and *Staphylococcus aureus*, were incubated with HBD-2 induced by *B. subtilis* LPP treatment, and the bacterial growth was examined by spectrometry. In addition, goblet cell-like LS174T cells were stimulated with *B. subtilis* MAMPs, and the mRNA and protein levels of MUC2 were determined by real-time RT-PCR and Western blot, respectively. Moreover, bacterial cell wall components were stimulated to human primary colon cells, SNU-61 and SNU-407 cells, and the mRNA expression of HBD-2 was measured.

Results

Both live and heat-killed *B. subtilis* increased the HBD-2 mRNA expression in a dose- and time-dependent manner. Among *B. subtilis* MAMPs, LPP potently increased the mRNA expression and protein secretion of HBD-2, while LTA and PGN did not show such effects. Heat-killed bacteria and LPP from other strains of

B. subtilis also increased the HBD-2 mRNA expression. Heat-killed *B. subtilis* increased the HBD-2 mRNA expression, but other heat-killed bacteria failed to induce HBD-2 expression. In addition, LPPs incubated with lipase or proteinase K decreased the LPP-induced HBD-2 mRNA expression, suggesting that lipid and protein moieties of LPP are crucial for inducing HBD-2 expression. The recognition of LPP and secretion of HBD-2 in polarized Caco-2 cells occurred on the apical side, and polarized cells expressed increased mRNA levels of various AMPs, including HBD-2, HBD-3, LL-37, and RegIIIα, upon LPP stimulation. LPP induced HBD-2 expression through TLR2 mediated JNK/p38 and NF-κB pathway. In addition, secreted HBD-2 efficiently inhibited the growth of *B. cereus* and *S. aureus*. Moreover, LPP from *B. subtilis* also enhanced the mRNA and protein levels of MUC2 in LS174T cells. This upregulation of HBD-2 and MUC2 was also observed in human primary intestinal cells.

Conclusion

Taken together, this study demonstrated that AMPs and MUC2 are potently increased by *B. subtilis* treatment in human IECs. Among *B. subtilis* MAMPs, LPP might be a key molecule responsible for HBD-2. LPP upregulated HBD-2 induction by TLR2-mediated JNK/p38 and NF-κB pathway, contributing to the growth inhibition of intestinal pathogens.

Keywords: *Bacillus subtilis*, lipoprotein, intestinal epithelial cell, human beta defensin-2, mucin

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Abbreviations

IEC	Intestinal epithelial cell
GI	Gastrointestinal
TLR	Toll-like receptor
MAMP	Microbe-associated molecular pattern
AMP	Antimicrobial peptide
HBD	Human beta defensin
MUC	Mucin
LTA	Lipoteichoic acid
PGN	Peptidoglycan
LPP	Lipoprotein

I. Introduction

Human gastrointestinal (GI) tract is the place where diverse microorganisms exist and form complex bacterial communities [1]. Various cells in human GI tracts, including epithelial, mesenchymal, endothelial, and immune cells, interact with gut microbiota and maintain the host-commensal microbial balance by regulating immune responses [2]. Among diverse cell types, intestinal epithelial cells (IECs), including intestinal epithelial stem cells, Paneth cells, and goblet cells, provide the biochemical and physical barrier which separates commensal bacteria from host cells (**Fig. 1**) [3]. IECs express various pattern-recognition receptors (PRRs), including toll-like receptors (TLRs) and NOD-like receptors, which recognize intestinal microbes, and their expression vary depending on the site of the intestine [4, 5]. The recognition of bacterial microbe-associated molecular patterns (MAMPs) by TLRs triggers the downstream signaling pathway, such as mitogen-activated protein (MAP) kinase pathway and NF-κB activation to regulate gut homeostasis [6]. Therefore, the importance of bacterial components in the protection of intestinal epithelium has been widely reported. For instance, lipoteichoic acid (LTA) of *Lactobacillus rhamnosus* GG ameliorated intestinal epithelial injury through TLR2-mediated pathway [7]. Moreover, MAMPs of probiotics such as flagellin or pili are known to be recognized by TLR5 and TLR4, respectively, thereby upregulating the production of antimicrobial peptides (AMPs), tight junction protein, and cytokines [8]. Furthermore, NOD signaling induced by peptidoglycan (PGN) administration can protect the intestine from intestinal diseases by modulating tissue repair and wound healing [9].

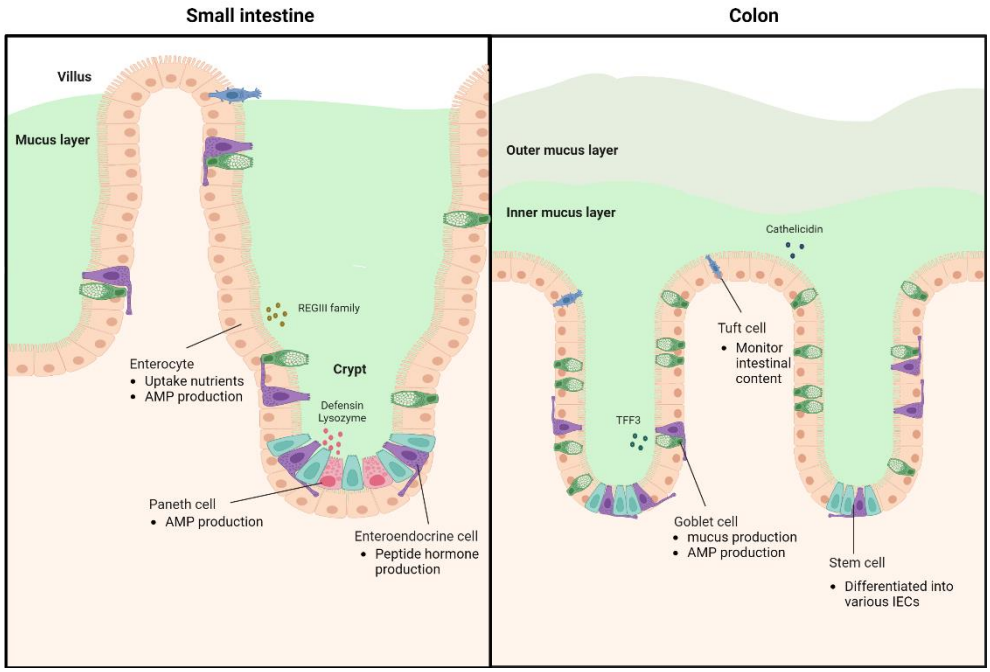

Figure 1. Intestinal epithelial cells (IECs) and their functions. Human intestine is composed of various intestinal cells with diverse function. Enterocytes are responsible for nutrient uptake and antimicrobial peptide (AMP) production such as RegIII family proteins. Enteroendocrine cells are the producers of peptide hormones. Goblet cells can produce AMPs and their major role is the production of mucin, which is important for the formation of mucus layers. Tuft cells can monitor the intestinal contents. Also, intestinal epithelial stem cells are in the crypt of small intestine and colon. They can be differentiated into multiple cell types maintaining intestinal epithelium. In the crypt of the small intestine, Paneth cells produce most of AMPs such as defensins and lysozyme, which contribute to the elimination of pathogens.

Paneth cells and enterocytes of the intestine secrete multiple AMPs such as α- and β-defensins, lysozyme, and RegIII family proteins for modulating the microbiome [10]. The secretion of AMPs remains at the basal level during homeostasis, but the amount of secreted AMPs can be significantly increased upon the bacterial infection [11]. Among various AMPs, human beta defensin (HBD)-2 and HBD-3 are induced by cytokines and microbial infection [12, 13]. In addition, they can induce further inflammatory responses via their chemotactic activity [11]. IECs of the colonic crypts secrete LL-37, the only identified cathelicidin in humans, which has broad antibacterial activity against Gram-positive and Gram-negative bacteria [11]. Both beta-defensins and cathelicidin eliminate the bacteria by permeabilizing the bacterial membrane and thereby protecting host cells from pathogens [14].

Mucus layers also play a crucial role in protecting the intestinal epithelium from the bacteria of the lumen. Goblet cells in small and large intestines are prominent producers of mucin (MUC), a glycoprotein that provides a habitat for commensal bacteria and a protective shield to protect host cells from direct contact with bacteria [15, 16]. In colon and small intestine, transmembrane mucins including MUC3, MUC12, MUC13, MUC15, and MUC17, form a glycocalyx, and secretory gel-forming mucins such as MUC2 form the mucus layer outside of the glycocalyx layer [17]. Among all mucins, MUC2 has been considered a critical factor for gut protection since MUC2 is the major component of the mucus layer [18]. Therefore, the relationship between MUC2 and intestinal diseases has been largely studied. For instance, previous studies revealed that MUC2 expression levels were

downregulated in patients with colitis [19] and colorectal carcinoma [20]. Moreover, MUC2 deficiency led to severe inflammation, colorectal cancer, and ulcerative colitis [21, 22]. Therefore, proper regulation of MUC2 production is vital for preventing intestinal diseases [17].

Bacillus subtilis is an aerobic Gram-positive bacterium that is found in fermented foods such as natto [23]. Since they are approved by Food and Drug Administration as generally recognized as safe bacteria [24], they are widely used in cosmetic and pharmaceutic industries [25, 26]. *B. subtilis* can strengthen intestinal barrier functions by increasing the expression of tight junction proteins [27-29] and reducing pro-inflammatory cytokines, which can impair the intestinal barrier [30, 31]. Oral administration of *B. subtilis* ameliorates *Salmonella*-induced intestinal disease or dextran sodium sulfate-induced colitis [32]. Most importantly, *B. subtilis* positively affected antibacterial, antiviral, and anticancer abilities [33]. For instance, *B. subtilis* inhibited the adherence of GI pathogens, including *Salmonella enteritidis*, *Listeria monocytogenes*, and *Escherichia coli*, to IECs [34]. Notably, several compounds produced by *B. subtilis* including cyclic lipopeptides are known to improve gut health with their broad spectrum of antimicrobial activities [35]. In addition, bacteriocin secreted by *B. subtilis* has been reported to show antimicrobial activities against *Klebsiella* sp. [36] and several Gram-positive bacteria [37].

Although *B. subtilis* is considered harmless, there are still theoretical risks to using

whole bacteria as therapeutic approaches since it can induce side effects such as systemic infections, harmful metabolic activities, and excessive inflammation [38]. Therefore, effector molecules that contribute to the protection of gut barrier functions should be elucidated. For this reason, the goal of this study is (i) to figure out the effect of *B. subtilis* on AMP production and (ii) to identify the major cell wall component responsible for the induction of AMPs.

II. Materials and Methods

2.1. Reagents and chemicals

DNase I was purchased from Roche Molecular Biocheimcals (Laval, QC, Canada). Thiazolyl blue tetrazolium bromide (MTT reagent), actinomycin D, cycloheximide, JNK V inhibitor, Proteinase K, and lipoprotein lipase from *Pseudomonas* sp. were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-human TLR2 antibody and its isotype control antibody were purchased from Invivogen (San Diego, CA, USA). The inhibitors of ERK (PD98059) and p38 (SB203580) were purchased from Calbiochem (San Diego, CA, USA). APC anti-human TLR2 antibody and its isotype control antibody were purchased from Biolegend (San Diego, CA, USA). NF-κB inhibitor and antibodies specific to MUC2 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Southern Biotech (Birmingham, AL, USA). Tryptic Soy Broth (TSB) was purchased from BD Biosciences (San Diego, CA, USA). All other materials were from Sigma-Aldrich unless stated otherwise.

2.2. Cell culture

The human epithelial cell line, Caco-2, was obtained from American Type Culture Collection (Manassas, VA, USA) and LS174T cells were kindly provided by Prof. Ki Won Lee (Seoul National University, Seoul, Republic of Korea). The cells were maintained in complete Dulbecco's modified Eagle medium (DMEM; Welgene,

Daegu, Republic of Korea) with 10% fetal bovine serum (FBS; GIBCO, Burlington, ON, Canada) and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA) at 37℃ in a 5% CO2-humidified incubator. For the polarization, Caco-2 cells were plated on 12-mm Transwell® with 0.4 μm pore polycarbonate membrane insert (Costar, Corning, NY, USA) and incubated for up to 21 days. The polarization was confirmed by measuring trans-epithelial electrical resistance (>400 Ω/cm2) with EVOM2 (World Precision Instruments, Sarasota, FL, USA). Primary human intestinal cells, including SNU-61 and SNU-407 cells, were obtained from Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in complete Roswell Park Memorial Institute (RPMI) 1640 (Welgene) containing 10% FBS and 1% penicillin-streptomycin at 37°C in a CO_2-humidified incubator.

2.3. Preparation of heat-killed bacteria

B. subtilis ATCC 6633 was purchased from the American Type Culture Collection. *B. subtilis* KCTC 3135, 3014, 3239 and *Lactobacillus plantarum* KCTC 10887-BP were purchased from Korean Collection for Type Cultures (Jeongeup, Republic of Korea). *Staphylococcus aureus* USA 300 was obtained from Nebraska Transposon Mutant Library (Omaha, NE, USA). All *B. subtilis* strains and *S. aureus* USA 300 were grown in TSB media at 37°C in a shaking condition, and *L. plantarum* was grown in Mann, Rogosa, and Sharpe (MRS) media at 37°C in a static condition until they reached mid-log phase. Bacterial pellets were harvested, followed by washing with phosphate-buffered saline (PBS) and incubated at 70°C for 2 h. To confirm that the bacteria were completely killed, all heat-killed bacteria were plated on proper

agar plates for 24 h. No bacterial colony was observed (data not shown).

2.4. Isolation of bacterial MAMPs

2.4.1. Isolation of LTA

Bacterial pellets were harvested by centrifugation and washed with PBS. LTA from *B. subtilis* ATCC6633 was isolated as previously described [39]. Briefly, bacterial pellets were resuspended in 0.1 M sodium citrate buffer (pH 4.7) and disrupted by ultrasonication (Vibracell VCX500; Sonics and materials, Newtown, CT, United States). The bacterial lysates were mixed with n-butanol and aqueous phase was lyophilized. The lyophilized pellet was dialyzed using a semi-permeable dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, United States) and equilibrated with 0.1 M sodium acetate buffer containing 15% 1-propanol (pH 4.7). After hydrophobic interaction chromatography, the LTA fractions were collected by phosphate assay and then dialyzed. The pool was subjected to anion-exchange chromatography, and LTA fraction was collected. After dialysis, LTA was lyophilized and quantified by measuring dry weight. No endotoxins, nucleic acids, or proteins were detected in the purified LTA.

2.4.2. Isolation of PGN

For purification of PGN, the bacterial pellet was suspended to 1 M NaCl in PBS and then disrupted using glass beads. The bacterial lysates were centrifuged to remove glass beads and cell debris. The supernatants were centrifuged at 20,400 × g and the

pellet containing PGN, LTA, and lipoprotein (LPP) was collected. The pellet was resuspended with 0.5% SDS in PBS and incubated at 60℃ for 30 min to remove protein, non-covalent bound LPP, and lipopolysaccharide (LPS). After washing with PBS, the insoluble PGN was suspended with 1 M NaCl and washed with PBS, followed by treatment with DNase I and RNase at 37℃ for 2 h. After adding trypsin containing 10 mM $CaCl_2$ in 1 M Tris-HCl (pH 7.0), PGN was incubated at 37℃ for 18 h. The PGN was washed with PBS and incubated with 5% trichloroacetic acid (Sigma) in PBS at 26℃ for 18 h. After centrifugation, PGN was washed with distilled water and treated with cold acetone to remove LTA or LPS. Insoluble PGN was washed with distilled water and quantified by measuring the dry weight. Soluble PGN was prepared by incubation with 50 U of mutanolysin and 0.2 M phosphate buffer (pH 6.0) at 37℃ for 16 h. No endotoxins were detected in the purified PGN.

2.4.3. Isolation of LPP

To isolate of LPPs, bacterial pellets were resuspended in Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, pH 7.6) buffer containing protease inhibitors and disrupted with ultrasonication. The bacterial lysates were suspended in 2% Triton X-114 in TBS and incubated at 4℃ for 2 hours. After centrifugation, bacterial debris was removed, and the supernatant was incubated at 37℃ for phase separation. The lysates were centrifuged at 37℃, and the aqueous phase was discarded. Then, the equal volume of TBS was mixed with the Triton X-114 phase and incubated at 37℃ for 15 min to separate the Triton X-114 phase. After repeating the previous step three times, the Triton X-114 phase was mixed with methanol and incubated at -20℃

overnight for precipitation. The precipitated LPPs were dissolved in 10 mM octyl-β-d-glucopyranoside and quantified using BCA protein assay kits (Pierce, Rockford IL, USA). No endotoxins were detected in the purified PGN.

2.5. Identification of the LPP component responsible for HBD-2 upregulation

Isolated LPP was treated with proteinase K (50 μg/ml) or DNase I (50 μg/ml) at 37℃ for 1 h or heat at 100℃ for 10 min. To inactivate lipids, LPP was incubated with lipases (50 μg/ml) at 37℃ for 12 h. Each LPP was separated by 10% SDS-polyacrylamide (PAGE) gel and stained with Coomassie and silver staining to determine the protein composition changes. In addition, Caco-2 cells were stimulated with each LPP to figure out the functional moieties of LPPs.

2.6. Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR)

Total RNA was isolated from cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized from total RNA using random hexamers (Roche, Basel, Switzerland) and reverse transcriptase (Promega, Madison, WI, USA). Real-time RT-PCR was performed using Applied Biosystems real-time RT-PCR system (Applied Biological Materials, Waltham, Massachusetts, USA). Relative mRNA expression levels were normalized with GAPDH and assessed by $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study are shown in **Table 1**.

Table 1. Primer sequences used in this study

Gene		Sequence (5' → 3')
HBD-1	Forward	GGGCACCCCTACAAAAGGAA
	Reverse	TGGCAAAATGGAAGATGCTAGTC
HBD-2	Forward	CTTCACTCAGGAGCAGCAAGC
	Reverse	ACACCAGTGCTGTCCTGTGACA
HBD-3	Forward	GCCATGAAGTTGCTGACTGC
	Reverse	TGAAGTTGGCGGCTGGTAAT
Human LL-37	Forward	GACACAGCAGTCACCAGAGGAT
	Reverse	TCACAACTGATGTCAAAGGAGCC
Human RegIIIα	Forward	TATGGCTCCCACTGCTATGCCT
	Reverse	TCTTCACCAGGGAGGACACGAA
Human RegIIIγ	Forward	CTCCCTGGTGAGGAGCATTAGT
	Reverse	AGTGCTACTCCACTCCCATCCA
MUC2	Forward	CAGGATGATGTCTGCCTCGC
	Reverse	TTAGGAACCACCTCCACGCAG
GAPDH	Forward	TGCTACTGACAACGTGGCTT
	Reverse	CCAGGAAAGCTGGGCAACTA

2.7. Enzyme-linked immunosorbent assay (ELISA)

Caco-2 cells were plated on a 96-well plate overnight and treated with indicated stimuli. Culture supernatants were collected, and the protein levels of HBD-2 in the culture supernatants were measured using ELISA kits (PeproTech) according to the manufacturer's instruction.

2.8. Western blot analysis

LS174T cells were plated on a 6-well plate and then stimulated with indicated stimuli for 24 h. The cells were washed with PBS and lysed with HEPES lysis buffer (containing 25 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% NP-40, pH 7.5). For MUC2 detection, culture supernatants of cells were collected and precipitated as previously described [40]. Protein concentration of lysates was measured with a BCA protein assay kit and equal concentration of proteins was subjected to SDS-PAGE gel and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking the membrane with TBST (TBS with 0.05% Tween-20) containing 5% skim milk for 1 h at room temperature, the membrane was incubated with antibodies MUC2 or β-actin at 4°C overnight. After washing three times with TBST, the membrane was incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were visualized by ECL reagents (Amersham Biosciences, Princeton, NJ, USA)

2.9. Flow cytometry

Cultured Caco-2 cells were detached using an enzyme-free cell dissociation solution (Sigma-Aldrich, St. Louis, MO, USA) to prevent the denaturation of surface proteins. After detachment, cells were washed twice with cold PBS and divided into two groups. For the intracellular staining (ICS) group, cells were fixed and permeabilized for 20 min on ice using a fixation/permeabilization solution (BD Biosciences). After washing with PBS, Fc region of both membrane-bound staining (MBS) and ICS group was blocked using anti-mouse CD16/32 antibody (Biolegend) for 15 min on ice. After washing with PBS, cells were stained with APC-conjugated anti-human TLR2 antibody and its isotype control (Biolegend) for 30 min on ice and then washed twice with PBS. The stained cells were fixed using 1% paraformaldehyde (PFA) and the expression of TLR2 in cell membrane and the intracellular region was analyzed using flow cytometry (FACSVerse, BD Biosciences, San Jose, CA, USA).

2.10. Measurement of bacterial growth

Caco-2 cells were cultured in DMEM without antibiotics and then treated with 1 μg/ml of LPP for 24 h. The culture media were collected and then centrifuged to remove the cell debris. The culture supernatant was stored at -80℃ for further experiments. *B. cereus* KCTC13153 and *S. aureus* USA300 were cultured in TSB media at 37℃ in aerobic condition. After 18 h, both bacteria were sub-cultured at 1% and then plated onto 96-well plates. Various concentrations of supernatant were prepared by 2-fold serial dilution. The supernatant was treated to both bacteria and

the growth of bacteria was determined by measuring optical density at 600 nm using a microplate reader.

2.11. Immunofluorescence staining of MUC2

LS174T cells were plated on a coverslip in a 24-well plate and incubated overnight. On the next day, the cells were treated with indicated stimuli for 24 h and fixed with 4% PFA for 15 min. For permeabilization, cells were treated using 0.3% Triton X-100 in TBS (TBST) for 5 min and blocked with 5% BSA in TBST. Permeabilized cells were incubated with anti-MUC2 antibody at 4°C overnight and stained with FITC-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h. For nuclei staining, the cells were treated with HOECHST 33258. Immunofluorescence staining of MUC2 was visualized with LSM 800 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) and analyzed with LSM Browser software.

2.12. Statistical analysis

All data are shown as mean value ± standard deviation from triplicates unless otherwise stated. Treatment groups were compared with an appropriate control group, and statistical analysis was determined using Student's *t*-test. Statistical significance was determined when $P < 0.05$.

III. Results

3.1. Both live and heat-killed *B. subtilis* potently increase HBD-2 mRNA expression in a dose- and time-dependent manner

To investigate whether live or heat-killed *B. subtilis* induce HBD induction in human intestinal epithelial cells, Caco-2 cells were treated with multiplicity of infection (MOI) 1:10, 1:100, and 1:1000 of each *B. subtilis* and the mRNA expression of HBD-1, HBD-2, and HBD-3 was measured by real-time RT-PCR. As shown in **Fig. 2A-C**, only HBD-2 mRNA expression was significantly upregulated with both live and heat-killed *B. subtilis*. HBD-2 mRNA expression was increased in a dose-dependent manner, while HBD-1 and HBD-3 expressions were constant. In addition, the expression level of HBD-2 was higher in the heat-killed *B. subtilis* treatment group than in the live *B. subtilis* treatment group. Moreover, to elucidate the time kinetics of each AMP expression, the cells were stimulated with live or heat-killed *B. subtilis* at MOI 1:100 for indicated time points. The mRNA expression of HBD-2 was highest at 6 h post treatment in both groups and decreased thereafter. However, the expression of HBD-1 and HBD-3 did not change during 0 to 12 h of stimulation **(Fig. 2D-F)**. These results suggest that both live and heat-killed *B. subtilis* potently induced HBD-2 mRNA expression in human intestinal epithelial cells.

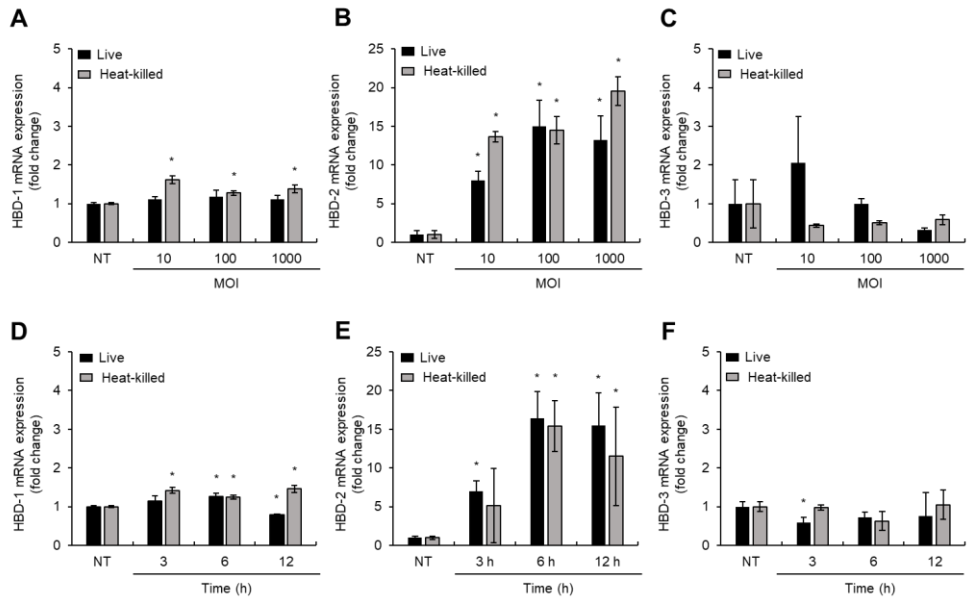

Figure 2. Both live and heat-killed *B. subtills* increase HBD-2 mRNA expression. (A-C) Caco-2 cells (3×10^5 cells/well) were stimulated with various concentrations of live- and heat-killed *B. subtilis* for 6 h. (D-F) Caco-2 cells (3×10^5 cells/well) were treated with live- or heat-killed *B. subtilis* at multiplicities of infection (MOI) 1:100 for indicated time points. After treatment, total RNA was isolated, and the mRNA expression of AMPs was analyzed using real-time RT-PCR. All results were expressed as mean $\pm$ standard deviation (S.D.) of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment.

3.2. *B. subtilis* lipoproteins potently induce HBD-2 production in Caco-2 cells

To figure out the central molecule responsible for the increase of HBD-2 expression, a culture supernatant of *B. subtilis* was collected and treated to Caco-2 cells. The culture supernatant potently increased the HBD-2 mRNA expression in a dose-dependent manner (**Fig. 3A**), suggesting that the effector molecules can be released from *B. subtilis*. Since bacterial MAMPs can be released into culture supernatants and are known to have immunoregulatory abilities [41-43], it can be postulated that they could contribute to the upregulation of HBD-2. Therefore, each MAMP including LTA, PGN, or LPP, were isolated from *B. subtilis* and equal concentration of each MAMP was treated to Caco-2 cells to investigate the HBD-2 induction ability. Interestingly, LPP significantly increased the mRNA expression of HBD-2 while the effect of LTA or PGN on HBD-2 expression was negligible (**Fig. 3B)**. In addition, LPP upregulated HBD-2 secretion without affecting the viability of Caco-2 cells (**Fig. 3C** and **D**). These results indicate that LPPs from *B. subtilis*, but not LTA or PGN, potently induce HBD-2 production in Caco-2 cells.

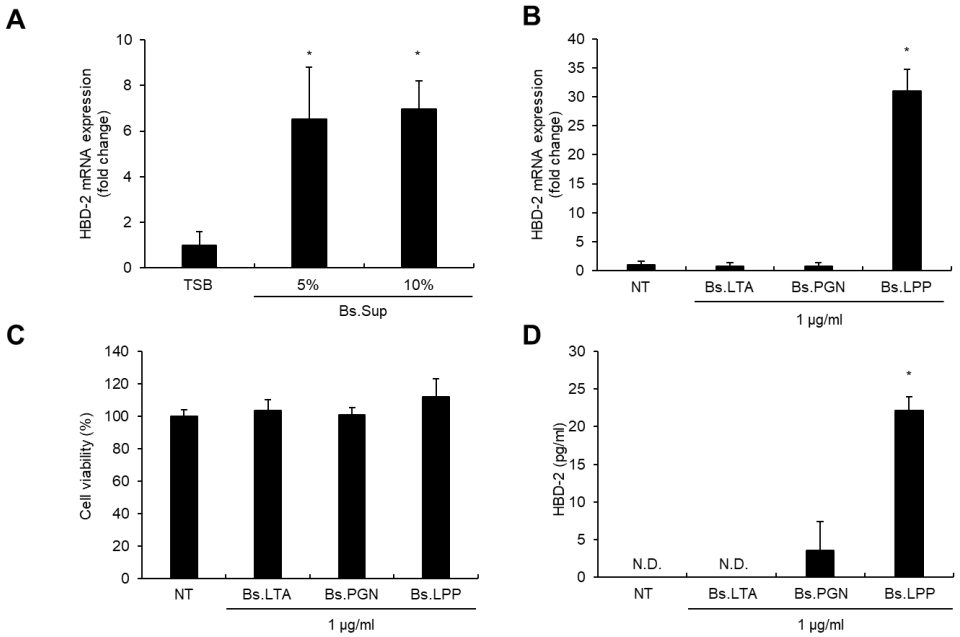

Figure 3. *B. subtilis* lipoprotein potently induces HBD-2 production in Caco-2 cells. Caco-2 cells (3×10^5 cells/well) were treated with (A) indicated concentrations of *B. subtilis* culture supernatants (Bs.sup) or 1 μg/ml of *B. subtilis* lipoteichoic acid (Bs.LTA), peptidoglycan (Bs.PGN), or lipoprotein (Bs.LPP) for 6 h. Total RNA was isolated and mRNA expression of HBD-2 was measured using real-time RT-PCR. (C, D) Caco-2 cells (1×10^6 cells/well) were plated on 96 well plate and stimulated with Bs.LTA, Bs.PGN, or Bs.LPP for 24 h. (C) Cell viability was measured using MTT reagent. (D) After treatment, culture supernatants were collected, and the HBD-2 concentration was measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment; TSB, tryptic soy broth; N.D., non-detected.

3.3. Lipoprotein from various *B. subtilis* strains induces the HBD-2 mRNA expression in Caco-2 cells

To figure out whether other *B. subtilis* strains have same effects on HBD-2 upregulation, three strains of *B. subtilis* were obtained to identify the effect of each strain on HBD-2 mRNA expression. Four strains, including KCTC6633, KCTC3014, KCTC3135, and KCTC3239, which were commonly used in *B. subtilis* studies [44], were inactivated by heat, and then used for Caco-2 cell stimulation. As a result, all heat-killed *B. subtilis* strains differentially induced the HBD-2 mRNA expression in Caco-2 cells (**Fig. 4A**). The heat-killed *B. subtilis* of KCTC6633 and KCTC3239 strains significantly increased the mRNA expression of HBD-2, while the effect of KCTC3135 strain was moderate. To compare the immunostimulatory effect, LPPs were isolated from each strain and then used for Caco-2 cell stimulation. As a result, all LPPs tested substantially induced HBD-2 mRNA expressions. Opposite to the whole bacteria stimulation, LPP from KCTC3135 strain induced the most potent increase in HBD-2 mRNA expression (**Fig. 4B**). However, LPP from KCTC3014 or KCTC3239 seemed to induce less HBD-2 mRNA expression. These data suggest that all *B. subtilis* strains potently upregulated the HBD-2 mRNA expression, but the induction rate varied depending on the strains.

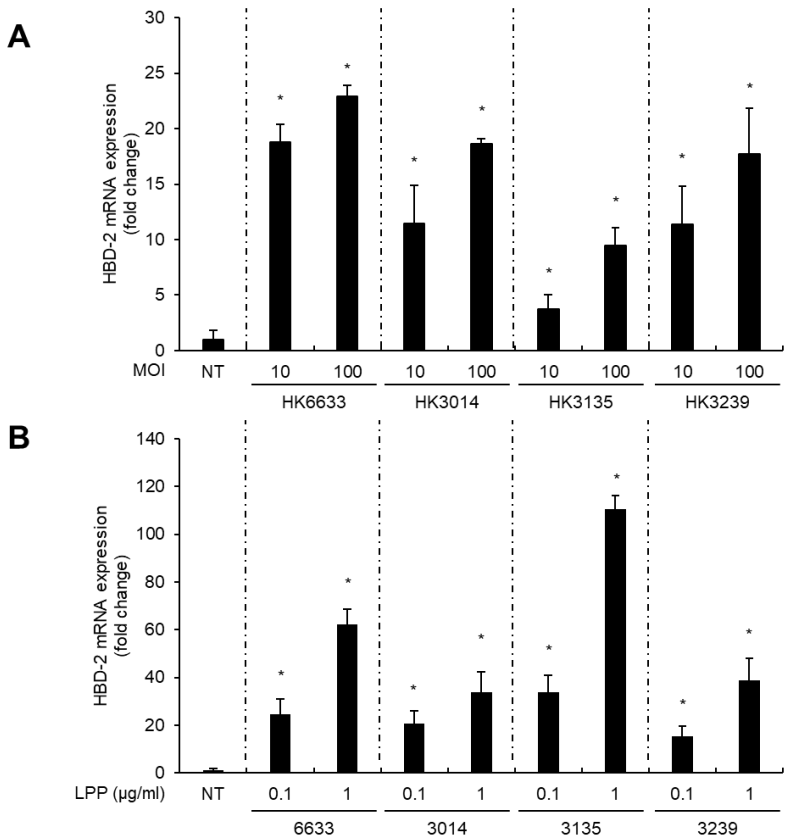

Figure 4. Other *B. subtilis* strains and their lipoproteins also induce the mRNA expression of HBD-2. (A) Caco-2 cells (3×10^5 cells/well) were treated with various KCTC strains of heat-killed *B. subtilis* at MOI 10 or 100 for 6 h. (B) Caco-2 cells were treated with 0.1 or 1 μg/ml of LPP from various *B. subtilis* strains for 6 h. Total RNA was isolated, and the mRNA expression of HBD-2 was analyzed using real-time RT-PCR. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment.

3.4. Lipoprotein from various bacteria differentially induces HBD-2 mRNA expression in Caco-2 cells

Since intestine is the place where various bacteria are introduced by food uptake [45], any probiotics or pathogens delivered to the GI tract can differentially regulate AMP production. Therefore, probiotics *B. subtilis* and *L. plantarum* and representative pathogens *B. cereus* and *S. aureus* were heat-killed and then used to stimulate Caco-2 cells. As a result, only heat-killed *B. subtilis* significantly upregulated the mRNA expression of HBD-2, while all other bacteria did not affect the expression level (**Fig. 5A**). To examine the effect of LPPs from each species on HBD-2 mRNA expression, LPPs from four species were isolated, and then utilized for Caco-2 cells stimulation. Interestingly, LPP from *B. subtilis* and *S. aureus* potently upregulated HBD-2 mRNA expression while *L. plantarum* LPP induced relatively low expression of HBD-2 (**Fig. 5B**). LPP from *B. cereus* showed a moderate effect on HBD-2 upregulation. Taken together, these data indicate that various bacterial species and their LPPs differentially regulate the HBD-2 mRNA expression. Among them, heat-killed *B. subtilis* and LPP from *B. subtilis* are the potent stimulator for HBD-2 induction.

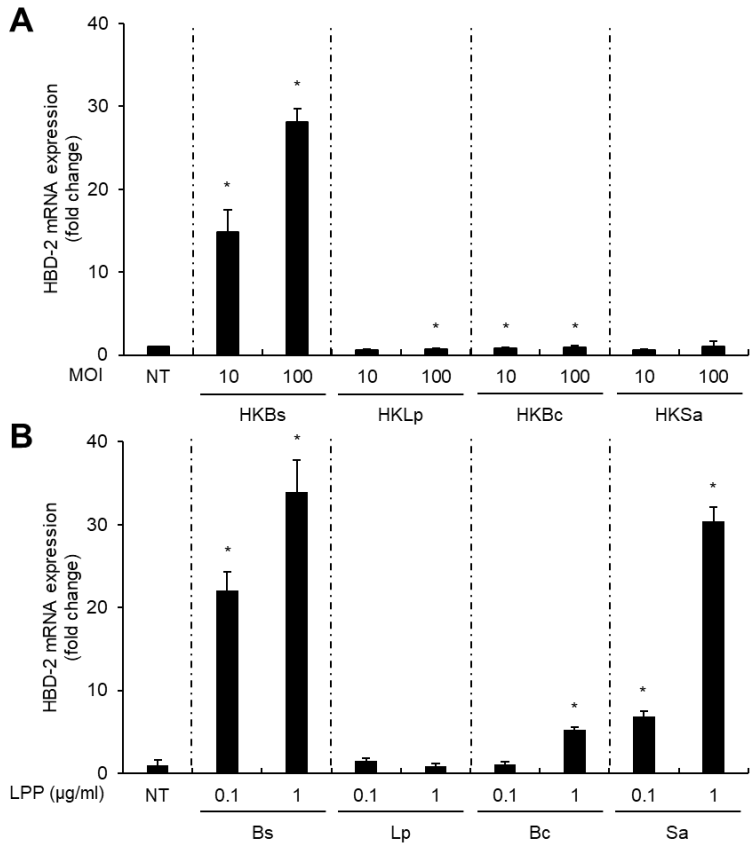

Figure 5. Various bacteria and their lipoprotein differentially induce HBD-2 mRNA expression in Caco-2 cells. (A) Caco-2 cells (3×10^5 cells/well) were stimulated with MOI 10 or 100 of various heat-killed bacteria for 6 h. (B) Caco-2 cells (3×10^5 cells/well) were treated with 0.1 or 1 μg/ml of LPP from various bacteria for 6 h. Total RNA was isolated, and the mRNA expression of HBD-2 was determined using real-time RT-PCR. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment; HK, heat-killed; Bs, *Bacillus subtilis*; Lp, *Lactobacillus plantarum*; Bc, *Bacillus cereus*; Sa, *Staphylococcus aureus*.

3.5. *B. subtilis* lipoprotein increases HBD-2 protein production in Caco-2 cells

To investigate the relationship between HBD-2 mRNA expression and protein production, Caco-2 cells were pre-treated with actinomycin D (ActD) and cycloheximide (CHX), RNA synthesis inhibitor and protein synthesis inhibitor, respectively [46], and stimulated with LPP. As a result, ActD significantly decreased the LPP-induced HBD-2 mRNA expression, while pre-treatment of CHX significantly increased the mRNA expression (**Fig. 6A**). This is in concordance with a previous report showing that mRNA can be stabilized by CHX treatment [47]. Both ActD and CHX pre-treatment down-regulated the HBD-2 production of Caco-2 cells without affecting the cell viability (**Fig. 6B** and **C**), suggesting that the mRNA expression of HBD-2 is positively correlated with the protein production of HBD-2. To investigate the kinetics of LPP-induced HBD-2 production, Caco-2 cells were stimulated with various concentrations of *B. subtilis* LPP, and the HBD-2 mRNA expression was measured using real-time RT-PCR. As shown in **Fig. 7A**, LPP dose-dependently increased HBD-2 mRNA expression in Caco-2 cells. Time kinetics of LPP-induced HBD-2 expression showed that the expression of HBD-2 peaked at 6 to 9 h and decreased from 12 h after the treatment (**Fig. 7B**). Concordant with mRNA expression, LPP dose-dependently increased HBD-2 protein secretion in Caco-2 cells (**Fig. 7C)**. These results suggest that LPP from *B. subtilis* upregulates HBD-2 expression in a dose- and time-dependent manner.

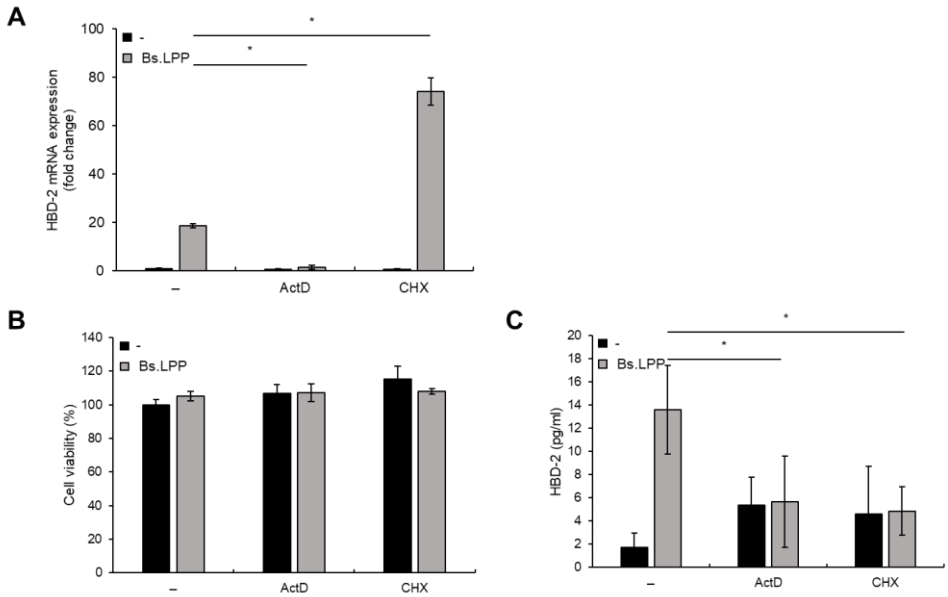

Figure 6. mRNA expression of HBD-2 correlates with the HBD-2 protein secretion in Caco-2 cells. (A) Caco-2 cells (3×10^5 cells/well) were pre-treated with 1 μg/ml of actinomycin D (ActD) or 10 μg/ml of cycloheximide (CHX) for 1 h. After pre-treatment, Caco-2 cells were stimulated with 1 μg/ml of LPP for 6 h. Total RNA was isolated, and the mRNA expression of HBD-2 was measured using real-time RT-PCR. (B, C) Caco-2 cells (1×10^6 cells/well) were pre-treated with 1 μg/ml of ActD or 10 μg/ml of CHX for 1 h and stimulated with 1 μg/ml of Bs.LPP for 24 h. (B) Cell viability was measured by MTT assay. (C) HBD-2 concentration in the cell culture supernatant was measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment.

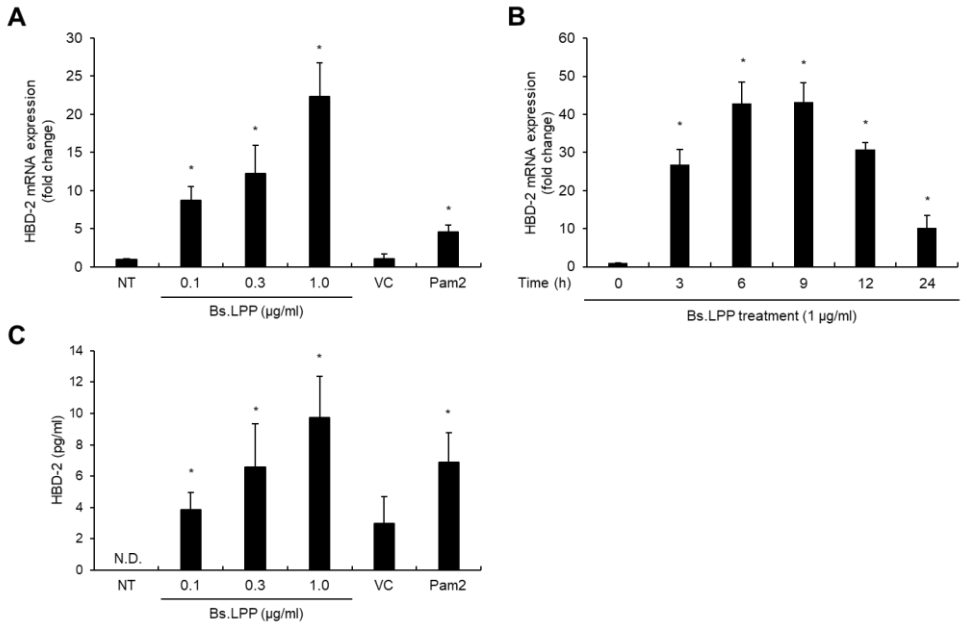

Figure 7. Lipoprotein increases HBD-2 mRNA expression and protein secretion in a dose- and time-dependent manner. (A, B) Caco-2 cells (3×10^5 cells/well) were treated with (A) various dose of Bs.LPP for 6 h or (B) 1 μg/ml of Bs.LPP for indicated time periods. Total RNA was isolated, and the mRNA expression of HBD-2 was measured using real-time RT-PCR. (C) Caco-2 cells (1×10^6 cells/well) were treated with various dose of Bs.LPP for 24 h. Culture supernatants were collected, and the HBD-2 concentration was analyzed by ELISA. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment; N.D., non-detected; VC, vehicle control; Pam2, Pam2CSK4.

3.6. Lipid and protein moieties of lipoprotein are important for HBD-2 induction

To determine whether the induction of HBD-2 is due to LPP or impurities in the extract, LPP from *B. subtilis* was treated with heat, DNase I, lipoprotein lipase, and proteinase K. Each LPP was visualized using Coomassie blue and silver staining. As shown in **Fig. 8A**, heat and lipase stimulation did not alter the protein composition of LPP. However, DNase I treatment deleted the protein of ~50 kDa and ~35 kDa while proteinase K treatment significantly altered the protein composition of LPP. Silver staining data showed that proteinase K treatment dramatically decreased the protein concentration in ~60 kDa and ~35 kDa. To examine whether each denaturation affects the HBD-2 mRNA expression, Caco-2 cells were treated with denatured LPPs, and the mRNA expression of HBD-2 was measured. As a result, LPP treated with lipase or proteinase K decreased the mRNA expression of HBD-2, while heat and DNase I treatment did not reduce the expression level (**Fig. 8B**). These data indicate that LPP is the primary factor responsible for the HBD-2 induction and both protein and lipid moieties of LPP are important for HBD-2 mRNA upregulation in Caco-2 cells.

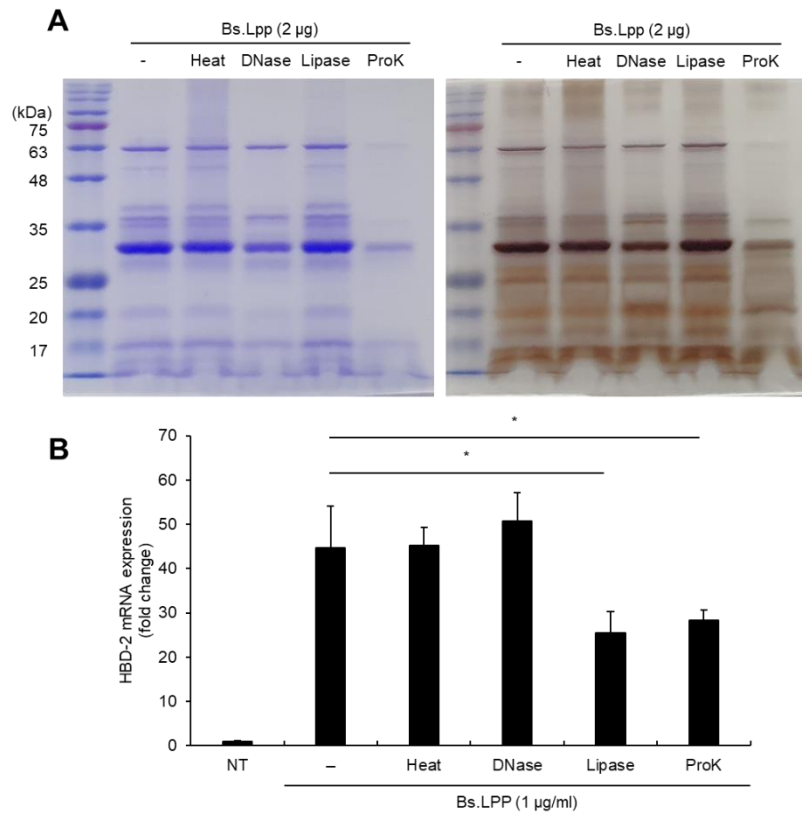

Figure 8. Lipid and protein moieties of lipoprotein are important for HBD-2 upregulation. (A) Bs.LPP was treated with heat, DNase I, lipoprotein lipase, and proteinase K (ProK). The detailed information is described at Material and Method section. Bs.LPPs treated with each method were visualized with Coomassie blue staining (left panel) and silver staining (right panel). (B) Caco-2 cells (3×10^5 cells/well) were stimulated with 1 μg/ml of each LPP for 6 h. The mRNA expression of HBD-2 was measured using real-time RT-PCR. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment.

3.7. *B. subtilis* lipoprotein induces HBD-2 production on apical side of differentiated Caco-2 cells

It has been reported that Caco-2 cells can differentiate into cells that morphologically and functionally express the characteristic of mature enterocytes [48]. Therefore, Caco-2 cells were cultured in transwell plates for 3 weeks, and the transepithelial electrical resistance (TEER) was measured to confirm the monolayer formation and cell polarization (**Fig. 9A**). As shown in **Fig. 9B**, TEER reached a plateau after 4 days of culture and remained constant until 21 days of differentiation, suggesting that Caco-2 cells were fully differentiated. In addition, to figure out the direction of LPP recognition and HBD-2 secretion, differentiated Caco-2 cells were treated with LPP apically or basolaterally. As a result, apical treatment of LPP increased the HBD-2 production in the apical compartment, while HBD-2 was not detected in basolateral side (**Fig. 9C**). Interestingly, basolateral treatment of LPP could not induce HBD-2 secretion on both apical and basolateral compartment (**Fig. 9D**). Collectively, these results indicate that both recognition of LPP and secretion of HBD-2 occurred on the apical side of differentiated Caco-2 cells.

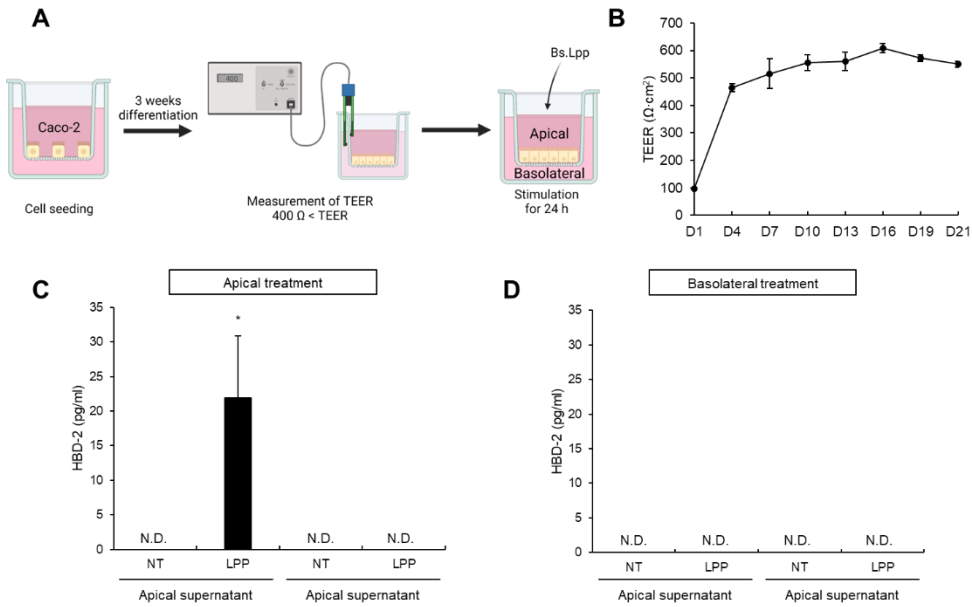

Figure 9. *B. subtilis* lipoproteins induce HBD-2 production on apical side in differentiated Caco-2 cells. (A) Schematic of the experiments. Caco-2 cells (1×10^5 cells/well) were seeded on transwell plate and cultured for 21 days for differentiation. (B) Transepithelial electrical resistance (TEER) values were measured for 21 days in three-day interval using EVOM2. Differentiated cells were treated with Bs.LPP (C) apically or (D) basolaterally for 24 h. Culture supernatants were collected, and the concentration of HBD-2 was measured using ELISA. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment; N.D., non-detected.

3.8. Differentiated Caco-2 cells potently express several AMPs gene expression by *B. subtilis* lipoprotein treatment

To demonstrate the effect of *B. subtilis* LPPs on the various AMPs expression in differentiated IECs, both non-differentiated and differentiated Caco-2 cells were treated with LPP, and the mRNA expression levels of various AMPs such as HBD-1, HBD-2, HBD-3, LL-37, and RegIII family, were analyzed. The expression of HBD-1 in differentiated cells was slightly increased, but the increasing rate was similar to that of polarizing cells (**Fig. 10A**). Interestingly, the mRNA expression of HBD-2 was significantly upregulated after differentiation, which was about 10-fold higher than in non-differentiated cells **(Fig. 10B**). In addition, LPP treatment increased the expression of HBD-3 and LL-37, which were not altered in non-differentiated Caco-2 cells. (**Fig. 10C** and **D**). The expression of RegIIIα was slightly upregulated, but the RegIIIγ expression was not changed by LPP stimulation (**Fig. 10E** and **F**). These data suggest that the differentiated IECs more potently respond to LPP stimulation than non-differentiated IECs do.

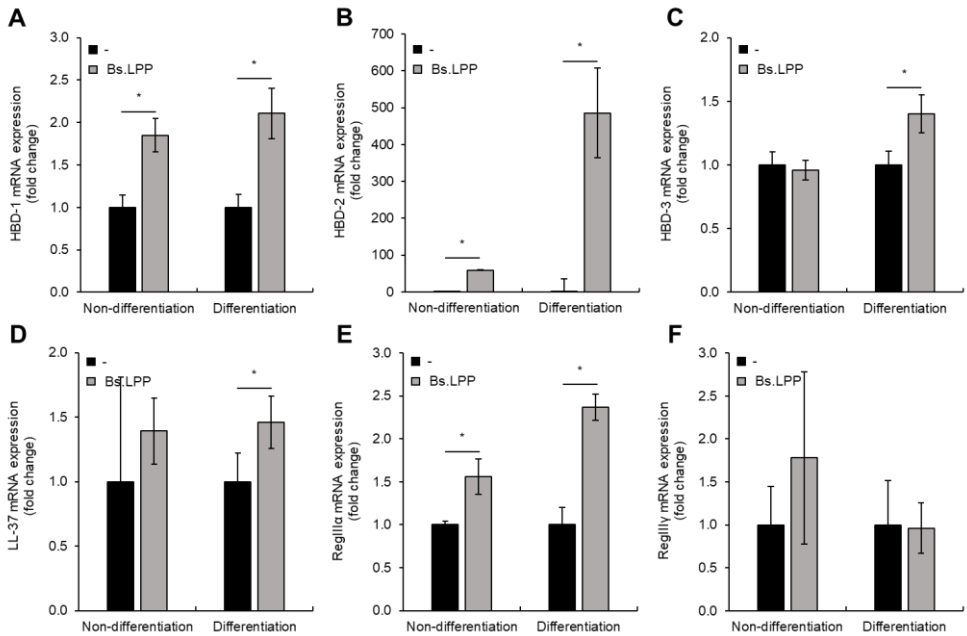

Figure 10. Differentiated Caco-2 cells express several AMPs gene expression by *B. subtilis* lipoprotein treatment. Caco-2 cells (3×10^5 cells/well) were plated on 6-well plate and separated into two groups: non-differentiation group and differentiation group. Cells of the non-differentiated group were stimulated with 1 μg/ml of Bs.LPP for 6 h. Cells of the differentiation group were culture for 21 days for polarization, and the media was changed every three days. After differentiation, cells were stimulated with 1 μg/ml of Bs.LPP for 6 h. Total RNA was isolated, and the mRNA expressions of (A) HBD-1, (B) HBD-2, (C) HBD-2, (D) LL-37, (E) RegIIIα, (F) RegIIIγ were measured using real-time RT-PCR. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control.

3.9. TLR2 signaling is involved in lipoprotein-induced HBD-2 expression

Previous studies identified that bacterial LPPs are mainly recognized by TLR2 [49, 50]. Therefore, Caco-2 cells were stained with anti-human TLR2 antibody with or without permeabilization to figure out the expression pattern of TLR2. As a result, intracellular TLR2 expression was 2-fold higher than the isotype control antibody staining group. However, the TLR2 expression was about 4-fold higher in the membrane staining group, suggesting that TLR2 is mainly expressed on the cell membrane **(Fig. 11A**). In addition, to investigate whether the induction of HBD-2 expression by LPP is mediated by TLR2 signaling pathway, the cells were pre-treated with TLR2 neutralizing antibody or its isotype control and then stimulated with LPP. As a result, pre-treatment of anti-TLR2 antibody down-regulated the mRNA expression of HBD-2 induced by LPP stimulation, while the isotype control antibody did not affect the mRNA expression (**Fig. 11B**). These data suggest that the TLR2 pathway is involved in the upregulation of HBD-2 expression induced by LPP.

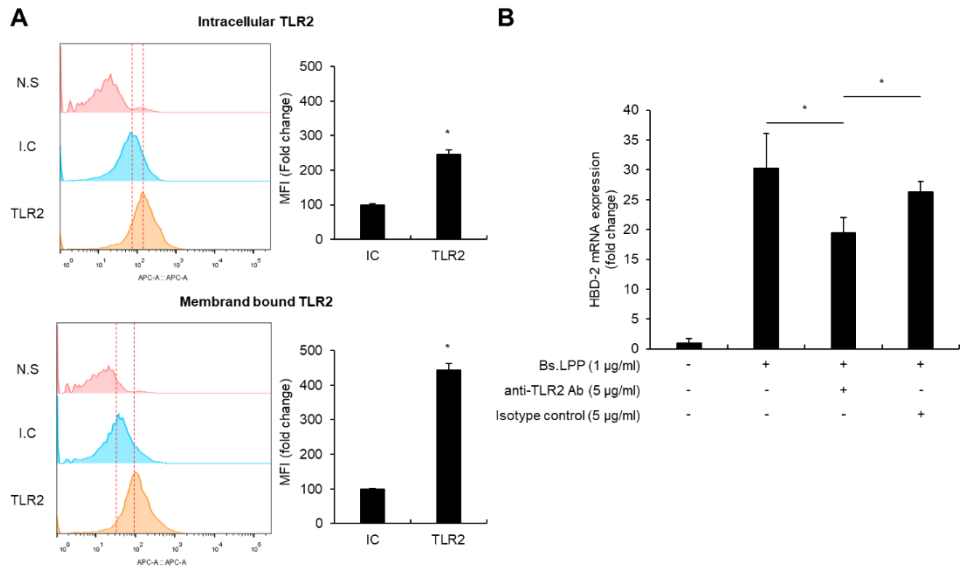

Figure 11. *B. subtilis* lipoprotein induces HBD-2 mRNA expression via TLR2. (A) Caco-2 cells (3×10^5 cells) were stained with APC-conjugated anti-human TLR2 (TLR2) or its isotype control (I.C) antibody. For intracellular staining, cells were permeabilized before antibody staining. Protein expression of TLR2 in cytosol and membrane was analyzed using flow cytometry. The APC-positive cells were shown as histogram (left panel) and the ratio of mean fluorescence intensity (MFI) was shown as a graph (right panel). N.S. means non-staining group. (B) Caco-2 cells (3×10^5 cells/well) were pre-treated with 5 μg/ml of anti-human TLR2 neutralizing antibody or its isotype control for 1 h, and then stimulated with 1 μg/ml of LPP for 6 h. RNA was isolated, and the mRNA expression of HBD-2 was measured by real-time RT-PCR. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control.

3.10. JNK/p38 and NF-κB pathways are involved in lipoprotein-induced HBD-2 expression in Caco-2 cells

It has been well documented that TLR2 activation leads to the downstream signaling pathway, such as MAP kinase and NF-κB translocation, to initiate proper immune responses [51]. Therefore, to investigate the intracellular mechanism of the HBD-2 induction, Caco-2 cells were pre-treated with several inhibitors and then stimulated with LPP. As shown in **Fig. 12A** and **B**, MAP kinase inhibitors, including JNK inhibitor V (JNK inhibitor) and SB203580 (p38 inhibitor), but not PD98059 (ERK inhibitor), significantly reduced the HBD-2 mRNA expression without affecting cell viability. Mainly, JNK inhibitor V potently inhibited the LPP-induced mRNA expression of HBD-2 more than other MAP kinase pathway inhibitors did, suggesting that the JNK pathway is the most important for the induction of HBD-2 expression. Also, BAY11-7082, an NF-κB inhibitor, dramatically decreased the HBD-2 mRNA expression (**Fig. 12C**). These results suggest that LPP upregulates HBD-2 expression through the MAP kinase and NF-κB pathway.

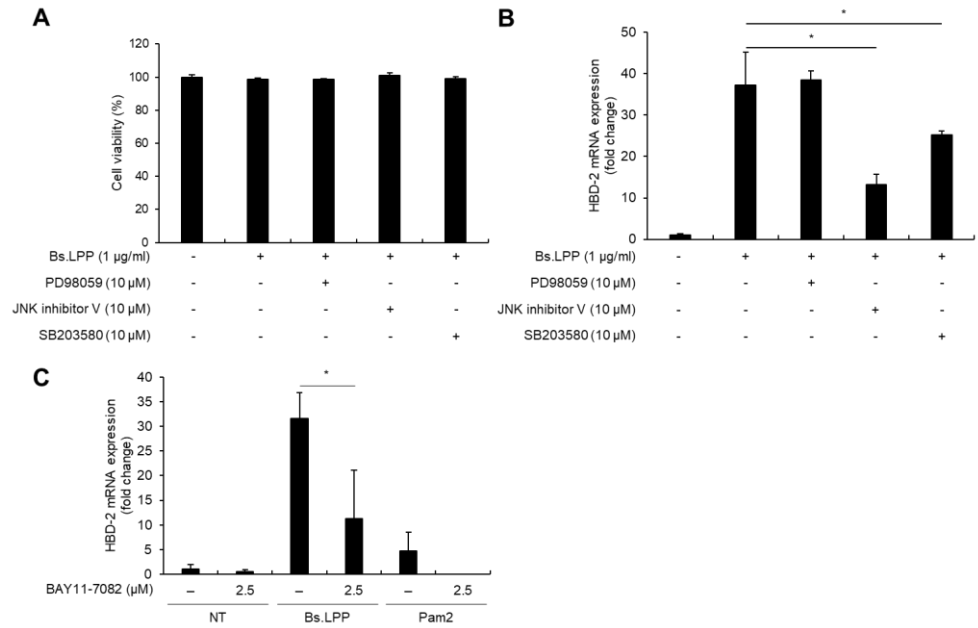

Figure 12. JNK/p38 pathway and NF-κB signaling pathway are involved in lipoprotein-mediated HBD-2 upregulation. (A) Caco-2 cells (1×10^6 cells/well) were pre-treated with three MAP kinase inhibitors for 1 h, and then stimulated with 1 μg/ml of Bs.LPP for 24 h. Cell viability was analyzed by MTT assay. (B, C) Caco-2 cells (3×10^5 cells/well) were pre-treated with (B) MAP kinase inhibitors or (C) NF-κB inhibitors for 1 h, and then treated with 1 μg/ml of Bs.LPP for 6 h. mRNA expression of HBD-2 was measured by real-time RT-PCR. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment; Pam2, Pam2CSK4.

3.11. Lipoprotein-induced HBD-2 inhibits the growth of intestinal pathogens

It has been demonstrated that HBD-2 has bactericidal effects against pathogenic bacteria, thereby protecting the host from microbial infection [52]. Therefore, to examine whether HBD-2 induced by LPP treatment contributes to the elimination of intestinal pathogens, Caco-2 cells were treated with *B. subtilis* LPP for 24 h in an antibiotics-free medium, and the culture supernatant was collected (**Fig. 13A)**. The supernatant was diluted with 2-fold serial dilution and then treated to *B. cereus*, which causes food poisoning [53], or *S. aureus*, which causes secondary GI disorders [54-56]. As shown in **Fig. 13**, the culture supernatant of LPP-treated Caco-2 cells dose-dependently inhibited the growth of *B. cereus* and *S. aureus*. HBD-2 showed lower inhibitory effects against *B. cereus*, and the inhibitory rate was nearly 10% (**Fig. 13B**). However, culture supernatant of LPP-treated Caco-2 cells efficiently reduced the growth of *S. aureus* with 20% inhibition rate (**Fig. 13C**). Collectively, these results indicate that HBD-2 secretion by LPP treatment in Caco-2 cells efficiently inhibits the growth of intestinal pathogens.

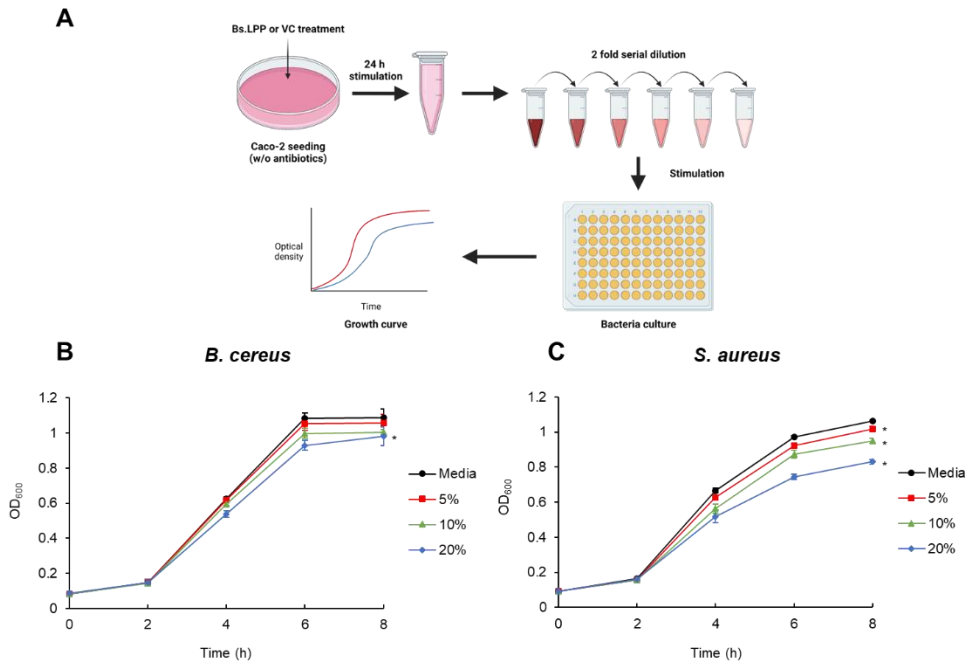

Figure 13. Lipoprotein-induced HBD-2 efficiently inhibits the growth of bacterial pathogens. (A) Schematic of the experiment. Caco-2 cells (1×10^6 cells/well) were stimulated with 1 μg/ml of Bs.LPP for 24 h in antibiotics-free medium. The culture media was collected, and then centrifuged to remove cell debris. The bacteria which were sub-cultured 1% were plated onto 96 well plates and treated with conditioned media in 2-fold serial dilution. The optical density (OD) of (B) *B. cereus* and (C) *S. aureus* USA300 were measured at each time points using microplate reader at 600 nm wavelengths. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control.

3.12. Lipoprotein from *B. subtilis* significantly increases MUC2 production in LS174T cells

It has been reported that the mucus layer plays essential role in maintaining healthy epithelium by acting as a reservoir and a distributor of AMPs [57, 58] or protecting host cells from intestinal pathogens. Therefore, to demonstrate whether *B. subtilis* MAMPs contribute to the MUC2 expression, goblet cell-like LS174T cells, which are suitable for mucin analysis with their high MUC2 production ability [59, 60], were stimulated with each *B. subtilis* MAMP and the MUC2 expression was analyzed. As shown in **Fig. 14A**, among MAMPs, LPP mostly upregulated the MUC2 mRNA expression. In addition, western blot data showed that the LPP significantly enhanced the MUC2 secretion in LS174T cells (**Fig. 14B**). Since MUC2 has been known to form a dense mucus layer outside of the IECs [61], MUC2 expression on the cell surface was visualized using confocal laser microscopy. **Fig. 14C** indicated that MUC2 protein was potently increased by LPP treatment in LS174T cells, while other MAMPs showed negligible effects. Collectively, these results demonstrate that LPP from *B. subtilis* potently increases the MUC2 production in LS174T cells.

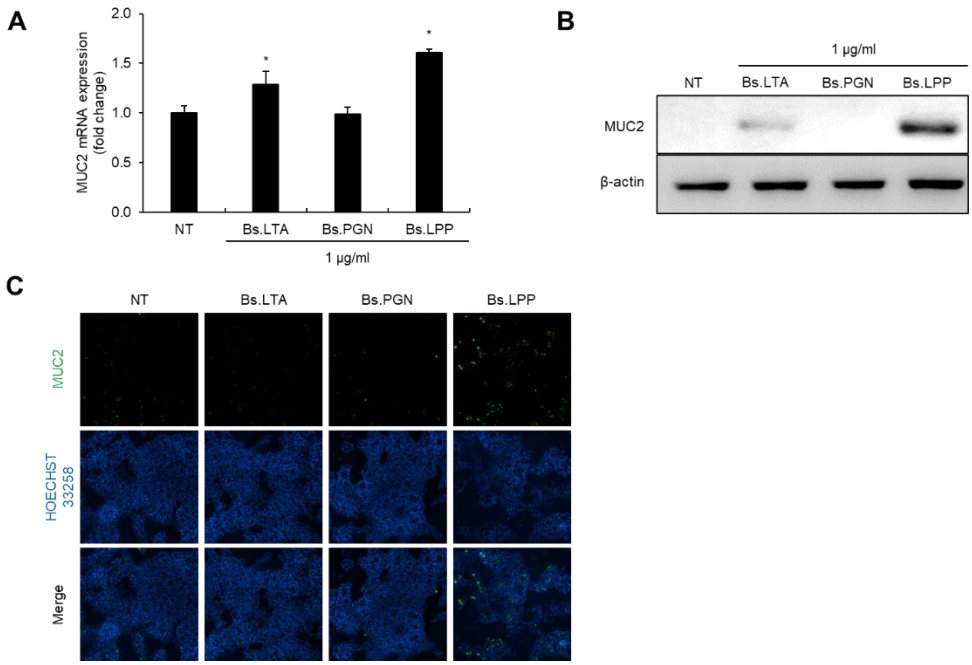

Figure 14. *B. subtilis* lipoprotein significantly increases MUC2 production in LS174T cells. LS174T cells (3×10^5 cells/well) were stimulated with 1 μg/ml of *B. subtilis* LTA, PGN, or LPP for 6 h. (A) The mRNA expression of MUC2 was measured by real-time RT-PCR. (B) LS174T cells (3×10^5 cells/well) were treated with indicated stimuli for 24 h. After treatment, culture supernatants were collected, and the proteins were subjected to western blotting using specific antibodies to the MUC2 and β-actin. (C) LS174T cells (1×10^5 cells/well) were plated onto a cover glass and stimulated with 1 μg/ml of *B. subtilis* LTA, PGN, or LPP for 24 h. Cells were fixed and then incubated with anti-MUC2 antibody. The cells were stained using FITC-conjugated secondary antibody (green) and the nuclei were stained with HOECHST33258 (blue). The fluorescence was analyzed by confocal laser scanning microscope. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment.

3.13. Lipoprotein from *B. subtilis* dose-dependently upregulates MUC2 production in LS174T cells

To investigate the kinetics of the MUC2 induction, LS174T cells were stimulated with various concentrations of LPP for 6 h. Real-time RT-PCR results indicated that LPP increased the MUC2 mRNA expression in a dose-dependent manner in LS174T cells (**Fig. 15A**). In addition, MUC2 secretion of LS174T cells was also dose-dependently upregulated by treatment of LPP while octyl-β-glucopyranoside, the vehicle control, did not induce the MUC2 secretion (**Fig. 15B**). To examine the MUC2 expression on the cell surface, LS174T cells were stimulated with various concentrations of LPP, and the fluorescence of the MUC2 was measured by confocal microscopy. As a result, LPP dose-dependently increased the MUC2 production. Interestingly, Z-stack analysis showed that the MUC2 was expressed not in the intracellular region but in the extracellular surfaces. As shown in **Fig. 15C**, the fluorescence was not detected at the 20 to 25 μm position where the nuclei were strongly stained with HOECHST33258, but the fluorescence of MUC2 was strongly detected at the 35 to 40 μm position where the fluorescence of HOECHST33258 was disappeared. These data indicate that LPP dose-dependently increases the mRNA expression and protein secretion of MUC2, leading to the formation of the mucus layer outside of the intestinal epithelial cells.

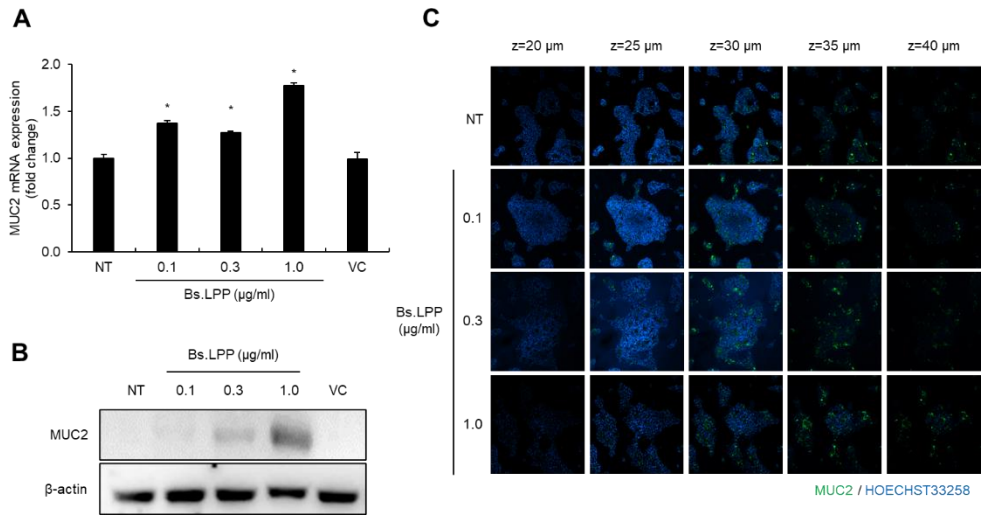

Figure 15. *B. subtilis* lipoprotein dose-dependently induces MUC2 production in LS174T cells. (A) LS174T cells (3×10^5 cells/well) were stimulated with indicated concentrations of Bs.LPP for 6 h. The mRNA expression of MUC2 was measured using real-time RT-PCR. (B) LS174T cells (3×10^5 cells/well) were stimulated with indicated concentrations of Bs.LPP for 24 h. The culture supernatant was collected, and the protein was precipitated. Proteins from supernatant and cell lysates were subjected to western blotting using specific antibodies against MUC2 and β-actin. (C) LS174T cells (1×10^5 cells/well) were seeded onto cover glass and stimulated with indicated concentrations of Bs.LPP for 24 h. Cells were fixed and then incubated with anti-MUC2 antibody. The cells were stained using FITC-conjugated secondary antibody (green) and the nuclei were stained with HOECHST33258 (blue). The fluorescence was analyzed by confocal laser scanning microscope in different Z-stack positions. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicate statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment; VC, vehicle control.

3.14. Lipoprotein from *B. subtilis* potently enhances HBD-2 and MUC2 production in human primary intestinal epithelial cells

To confirm that the increase of HBD-2 and MUC2 production by LPP treatment was general phenomena, primary IECs SNU-407 and SNU-61 cells were used for experiments. SNU-407 cells and SNU-61 cells were stimulated with *B. subtilis* LTA, PGN, or LPP, and the mRNA expressions of HBD-2 and MUC2 were measured by real-time RT-PCR. Interestingly, similar to previous data (**Fig. 3** and **Fig. 14**), HBD-2 mRNA expression was strongest by LPP treatment than other MAMPs in both primary IECs (**Fig. 16A** and **B**). In addition, LPP also potently upregulated the MUC2 mRNA expression in both SNU-407 and SNU-61 cells (**Fig. 16C** and **D**). These results demonstrate that the induction of HBD-2 and MUC2 mRNA expression is not limited to Caco-2 cells but also applied to primary IECs, suggesting this is a general phenomenon.

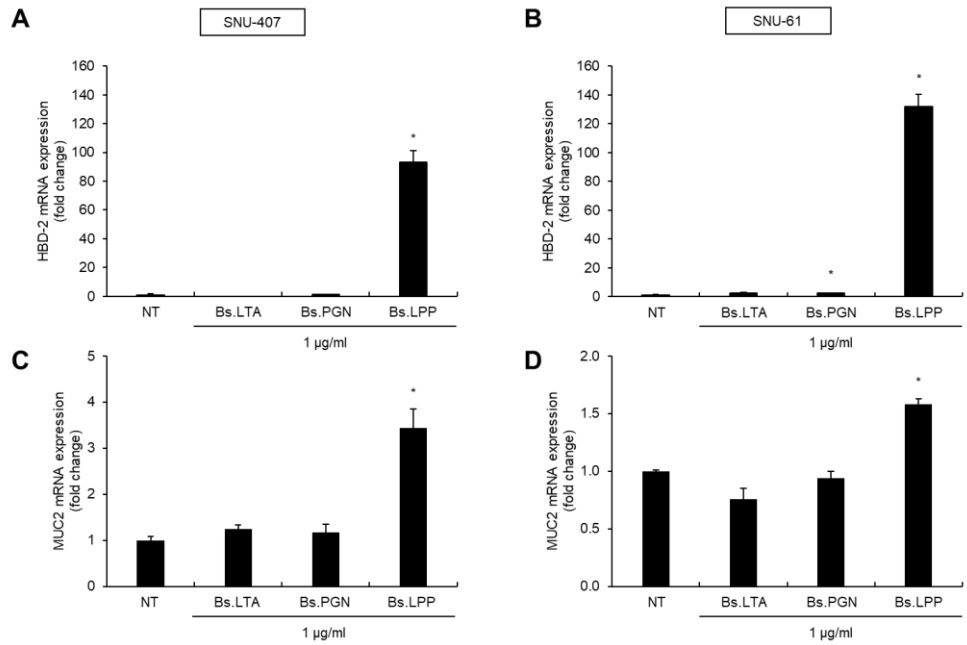

Figure 16. Lipoprotein from *B. subtilis* strongly induces HBD-2 and MUC2 mRNA expression in primary intestinal epithelial cells. (A, C) SNU407 cells (3×10^5 cells/well) and (B, D) SNU-61 cells (3×10^5 cells/well) were stimulated with 1 μg/ml of *B. subtilis* LTA, PGN, and LPP for 6 h. Total RNA was isolated, and the mRNA expression of (A, B) HBD-2 and (C, D) MUC2 was measured by real-time RT-PCR. All results were expressed as mean ± S.D. of triplicate samples. Asterisks (*) indicates statistical difference at $P<0.05$ compared with an appropriate control. NT, non-treatment.

3.15. Various *B. subtilis* lipoproteins can contribute to the induction of HBD-2 and MUC2 in human IECs

It has been well documented that *B. subtilis* possess at least 63 functionally distinct LPPs [62]. Therefore, to identify the LPP responsible for the induction of HBD-2 or MUC2, LPP extract from *B. subtilis* was separated by 10% SDS-PAGE gel and analyzed using linear ion trap quadrupole (LTQ)-orbitrap mass spectrometer. The information of identified proteins were obtained using Subtiwiki database (http://subtiwiki.uni-goettingen.de) and putative *B. subtilis* LPP candidates were selected by comparing with the known *B. subtilis* LPP sequence [63]. As shown in **Table 2**, more than 20 putative LPPs were identified in LPP extract and the molecular percentage of total LPPs was up to 15.63%. Interestingly, most LPPs were about 30 kDa except the OppA protein which was 61 kDa. This result is consistent with the previous data in which the strongest protein band was near 30 kDa (**Fig. 8A)**. In addition, methionine-binding lipoprotein occupied the highest molecular percentage among the identified LPPs. Collectively, these data show that the putative *B. subtilis* LPP candidates can contribute to the induction of HBD-2 or MUC2 in human IECs.

Table 2. Identification of *B. subtilis* LPP candidates

	Protein (Function)	*Mol (%)	Size (kDa)
1	Methionine-binding lipoprotein (Methionine ABC transporter)	8.1940	30.393
2	Foldase protein PrsA (Protein folding)	1.6382	32.547
3	Arginine-binding extracellular protein ArtP (Arginine ABC transporter)	1.1463	28.409
4	L-cystine-binding protein TcyA (Cystine and diaminopimelate ABC transporter)	1.0049	29.553
5	Manganese-binding lipoprotein MntA (Manganese ABC transporter)	0.7231	33.454
6	Fe (3^+)-citrate-binding protein YfmC (Iron/citrate ABC transporter)	0.3798	35.113
7	Iron-uptake system-binding protein (Iron ABC transporter)	0.3747	35.143
8	Putative carboxypeptidase YodJ (Cell wall synthesis)	0.3616	30.892
9	Probable siderophore-binding lipoprotein YfiY (Siderophore ABC transporter)	0.2222	36.339
10	Oligopeptide-binding protein OppA (Oligopeptide ABC transporter)	0.1666	61.543
11	Petrobactin-binding protein YclQ (Petrobactin ABC transporter)	0.1616	34.827
12	Quinol oxidase subunit 2 (Respiration)	0.1586	36.316
13	Ribose import binding protein RbsB (Ribose ABC transporter)	0.1111	32.264
14	Probable amino-acid-binding protein YxeM (S-(2-succino)cysteine ABC transporter)	0.1050	29.349
15	Putative ABC transporter substrate-binding lipoprotein YhfQ (Iron/citrate ABC transporter)	0.0808	35.524
16	Iron (3^+)-hydroxamate-binding protein YxeB (Hydroxamate siderophore ABC transporter)	0.0808	35.541
17	Putative lipoprotein YerB (Unknown)	0.0778	37.117
18	Probable ABC transporter extracellular-binding protein YckB (Unknown)	0.0697	31.757
19	Uncharacterized protein YtkA (Assembly of the CuA center in Cytochrome caa3)	0.0677	15.904
20	Iron (3^+)-hydroxamate-binding protein FhuD (Hydroxamate siderophore ABC transporter)	0.0626	34.462

* Mol (%) means molecule percentages in an extract add up to 100 mol percent.

IV. Discussion

B. subtilis is considered to be safe for use as food additives, and it is known to provide protective effects against GI diseases [64]. Since it has been reported that *B. subtilis* contributed to the upregulation of AMP in GI tracts [65], understanding the effect of *B. subtilis* and its components on AMP production of human IECs is important. In this study, *B. subtilis* potently increased the HBD-2 mRNA expression, and LPP, one of the major cell wall components of the bacteria, was identified as a key molecule responsible for the upregulation. LPP from *B. subtilis* induced HBD-2 expression through TLR2 mediated JNK/p38/NF-κB pathway and secreted HBD-2 by LPP stimulation efficiently inhibited the growth of bacterial pathogens. Moreover, LPPs of the *B. subtilis* also increased the MUC2 production in goblet-like cells. Collectively, this study identified *B. subtilis* LPP as a major factor which contributes to the upregulation of HBD-2 and MUC2 in human IECs.

The current study demonstrated that both live and heat-killed *B. subtilis* potently increased the mRNA expression of HBD-2 but not HBD-1 or HBD-3. It is consistent with the previous report saying that HBD-2 is promptly induced by bacterial stimulation while HBD-1 is constitutively expressed [66]. Notably, the study found that all other strains of *B. subtilis* were able to upregulate HBD-2 mRNA expression, but other bacteria, such as *L. plantarum*, *B. cereus*, and *S. aureus* failed to increase the HBD-2 expression. It has been widely reported that the administration of *B. subtilis* strengthens innate immune responses by promoting serum immunoglobulin

levels and tight junction proteins [67, 68]. The immunostimulatory effect of *B. subtilis* has also been reported in RAW264.7 cells and the middle intestine of grass carp [69, 70]. Therefore, considering that HBD-2 can contribute to the modulation of gut homeostasis via wound healing, angiogenesis, and clearance of pathogens [71], the study suggested that *B. subtilis* can be an effective therapeutic agent for improving GI diseases.

In this study, LPP was identified as a key molecule contributing to the production of HBD-2 rather than LTA or PGN. It has been well documented that LPP is a potent immunostimulatory molecule among cell wall components [50, 72]. Similarly, it has been reported that LPPs of *S. aureus* play a crucial role in enhancing IL-8 production in Caco-2 cells [73]. In addition, macrophage-activating lipopeptide-2 increased the HBD-2 mRNA expression in human keratinocytes, while PGN and LTA showed moderate effects [74]. These previous reports support the current study saying that LPP is the major molecule responsible for the upregulation of HBD-2 gene expression. Also, LPP treated with proteinase K, or lipase showed decreased HBD-2 induction than non-treated LPP, suggesting that both lipid moieties and protein moieties are important for the induction of HBD-2. This is consistent with a previous study identifying that lipase-treated synthetic triacylated lipopeptide Pam3CSK4 potently decreased the production of tumor necrosis factor (TNF)-α [75].

LTQ-orbitrap mass spectrometry data proposed 20 LPP candidates that potentially

leads to HBD-2 induction. Interestingly, most of the identified LPPs were ABC transporters. This is in accordance with the previous report that *B. subtilis* LPP predominantly function as a transporter [62]. Considering that ABC transporters such as MntA, YfmC, YclQ, or OppA are recognized by TLR2 receptors [76-78], it can be hypothesized that the identified LPPs upregulate HBD-2 expression presumably through TLR2 recognition. Nevertheless, further study is required to clarify the key LPP of *B. subtilis* that promotes the immune response.

The study found that purified LPP from four bacterial species increased the mRNA expression of HBD-2, but the induction rate differed depending on the species. As a reason, it can be hypothesized that structural differences of each LPP contributed to differential activities. Nguyen et al. reported that LPP with *N*-acetylation induced high immune responses, while LPP with *N*-long-chain acylation had low immunostimulatory effect [79]. Therefore, it can be postulated that *B. subtilis* potently induce HBD-2 mRNA expression because they are known to possess acetylated LPP. In contrast, *L. plantarum* and *B. cereus* hardly induced HBD-2 expression since they are known to have long-chain acylated LPP [62, 80]. However, it is still unclear why *S. aureus*, known to have long-chain acylated LPP, potently increased the HBD-2 mRNA expression. One possible mechanism is the involvement of protein moiety of LPP in HBD-2 expression. Previous study found that peptide side chain can also interact with TLR2 and initiate TLR2-mediated immune responses [81], suggesting that the protein of *S. aureus* LPP can participate in the HBD-2 induction. Further studies are needed to identify the molecules

involved in AMP induction in LPPs.

The present study found that LPP induced HBD-2 production through TLR2 mediated JNK/p38 and NF-κB pathways. In line with the current study, TLR2-mediated HBD-2 upregulation has been widely reported. For instance, S-layer proteins of *Lactobacillus* species induced HBD-2 production via TLR2 pathway [82]. Also, Pam3CSK4 induced HBD-2 gene expression through TLR2 pathway in human corneal epithelial cells and lung epithelial cells [83, 84]. These previous reports suggest that the TLR2 signaling pathway can cause the production of AMPs. Moreover, the current study figured out that activation of JNK and p38, but not ERK, were crucial for HBD-2 mRNA expression among MAP kinase signaling pathways. Similar to this result, JNK pathway was considered a crucial factor for inducing HBD-2 production, while ERK pathway seemed to be dispensable [85, 86]. According to previous reports, the importance of p38 pathway is still controversial [85, 87, 88]. However, other studies suggested that the expression of AMPs required ERK signaling pathway upon *Candida albicans* [89] or IL-17A stimulation [90]. Therefore, further study will be needed to elucidate the differential regulation of MAP kinase pathway on HBD-2 production.

The current study found that the recognition of LPP and secretion of HBD-2 occurred in the apical side of differentiated Caco-2 cells. In agreement with this result, it has been demonstrated that AMPs are secreted by apical release mediated by exocytosis

[91]. In addition, another study found that epithelial cells release AMP-containing exosomes to the apical side of cell monolayers [92]. Therefore, it can be hypothesized that apically secreted HBD-2 contributes to the efficient elimination of pathogens located in the lumen. Moreover, the present study elucidated that differentiated Caco-2 cells more potently induced the mRNA expression of various AMPs. Similarly, previous study reported that the mRNA expression of LL-37 was increased along with the Caco-2 cell differentiation [93]. Also, it has been reported that differentiated Caco-2 cells expressed increased mRNA levels in most inflammation-related genes, such as *tnfa*, *ccl20*, and *il1a* [94]. Pshezhetsky et al. reported that differentiated Caco-2 cells possess more proteins associated with metabolic enzymes, keeping cellular structures, and transmembrane transporters while proliferating cells express more proteins which are related to gene expression, protein synthesis, and folding [95, 96]. Given that polarized Caco-2 cells represent the properties of the small intestine, it can be suggested that LPP could induce higher AMP production in actual environments such as human intestinal epithelium.

In this study, HBD-2 induced by LPP-treated Caco-2 cells efficiently inhibited the growth of *S. aureus* and showed the moderate effect on that of *B. cereus*. Previous study revealed that HBD-2 and HBD-3 significantly inhibited the biofilm formation of *S. aureus* [97] and the effective concentrations (EC_{50}) value of HBD-2 for *S. aureus* was 7.6 μg/ml [98]. Furthermore, it has been known that *B. cereus* is more susceptible to HBD-2 than HBD-1 or HBD-3, and the EC_{50} value of HBD-2 for *B. cereus* was 22 μg/ml [99], concordant with the present result that LPP-induced HBD-

2 more efficiently attenuated the growth of *S. aureus*. Although Caco-2 cells released HBD-2 at 20 pg/ml concentration at maximum, which seemed to be extremely low for inducing antibacterial activities for both bacteria, culture supernatant efficiently inhibited the growth of *B. cereus* and *S. aureus*. Given that the concentration of HBD-2 can be upregulated by pro-inflammatory cytokines [100], LPP could upregulate enough HBD-2 protein for intestinal pathogens inhibition in the actual environment. Therefore, co-culture experiments of IECs with immune cells should be conducted to mimic the *in vivo* situations.

MUC2 has been considered an important component of the intestine, which provides a physical barrier against intestinal pathogens and retains AMPs in mucus layers [18, 57, 58]. Furthermore, it has been reported that MUC2 facilitated BD-2 production in the presence of pro-inflammatory cytokines and adenosine triphosphate [101], suggesting that MUC2 is important for both formation of mucus layer and production of AMPs. Although *B. subtilis* are known to increase the MUC2 expression [102], effector molecules responsible for the upregulation are poorly understood. In this study, *B. subtilis* LPP was identified as a critical molecule that significantly enhanced the production of MUC2. It is in agreement with the previous reports saying that the TLR2 ligand strongly induced the growth of MUC2 *in vivo* [103]. However, previous reports suggested that LTA from *Lactobacillus paracasei* D3-5 enhanced the MUC2 expression via TLR2/MAP kinase/NF-κB pathway [104]. Since the current study figured out that *B. subtilis* LPP had more substantial effects on MUC2 production than *B. subtilis* LTA, it can be suggested that LPP is a major component that can

promote MUC2 production in Caco-2 cells.

In summary, this study suggests that LPP is the key cell wall component of *B. subtilis* responsible for the induction of AMP and MUC2 expression in human IECs. It is well known that *B. subtilis* possess more than 60 LPPs which function in transportation, cell wall composition, germination, and miscellaneous functions [62]. Therefore, identifying specific LPP contributing to the upregulation of AMP and MUC2 is needed to develop a therapeutic agent. In addition, since HBD-2 can be upregulated by various pro-inflammatory cytokines [105], studies about the effect of *B. subtilis* LPP on AMP production *in vivo* would be a meaningful study for understanding the advantageous effects of LPP on IECs. In conclusion, this study identified the major cell wall component of *B. subtilis* which play important roles in protecting intestinal health.

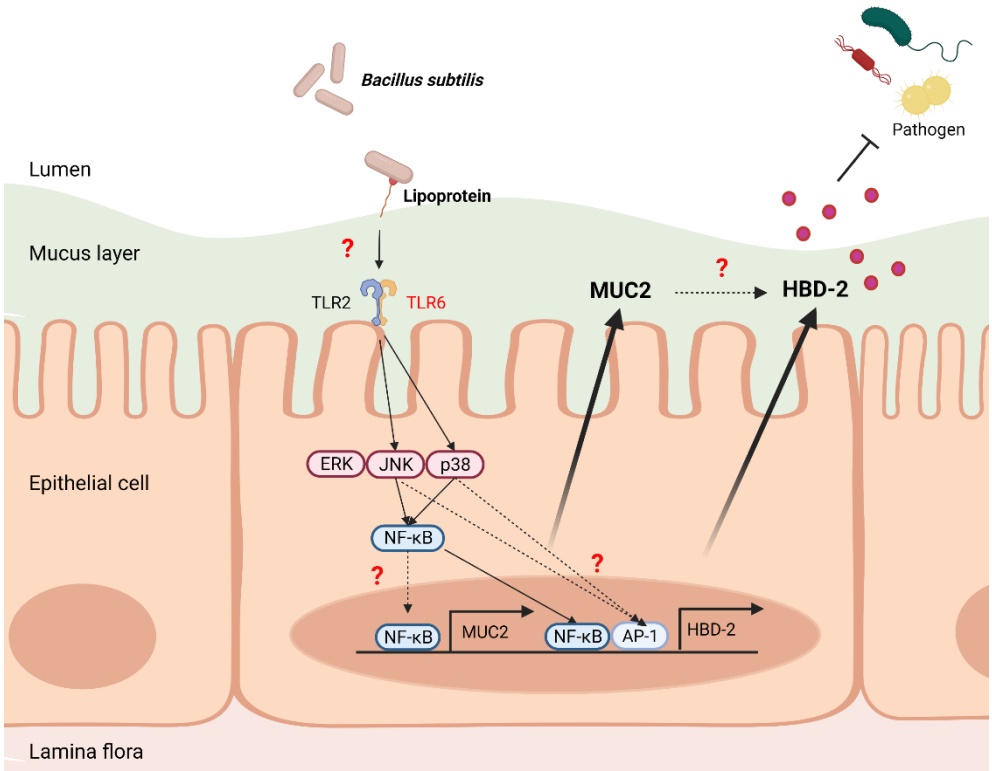

Figure 17. Schematic illustration of the proposed mechanism. In the gut, intestinal epithelial cells recognize *B. subtilis* LPP, which is released from the bacteria. TLR2 expressed in the intestinal epithelial cell membrane recognizes the LPP and triggers the JNK/p38 and NF-κB pathway to induce the mRNA expression of HBD-2 and MUC2. Secreted HBD-2 efficiently inhibits the growth of bacterial pathogens, and MUC2 forms a mucus layer outside of cells, protecting intestinal epithelium from pathogens.

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국문초록

인간 장 상피세포에서 고초균 지질단백질에 의한 인간 베타 디펜신-2의 유도

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1. 목적

사람의 장 상피세포는 장내의 항상성을 유지하는데 중요한 역할을 수행한다. 이들은 항균펩타이들을 만들어내어 장내의 유해균을 제거하거나 뮤신을 생성하여 뮤신 장벽을 만들어 장벽을 보호한다. 고초균은 사람의 장에서 발견되는 균으로 과거 연구를 통해 안전성이 입증되어 다양한 식품 및 제약산업 등에 응용되는 균이다. 이 균은 유해균에 대한 보호 또는 장과 연관된 질병의 개선에 도움을 주는 등 소화기관의 장벽보호에 다양한 긍정적인 효과를 가져올 수 있는 것으로 알려져 왔다. 하지만 이러한 긍정적 효과를 유도함에 있어 어떤 물질이 주요한지에 대해서는 많이 밝혀져 있지

않다. 따라서 본 연구에서는 (i) 고초균이 인간 장 상피세포에서 항균펩타이드 생성에 미치는 영향 규명, 그리고 (ii) 해당 반응을 유도하는 주요 물질 및 작용기전을 밝히는 것을 목표로 하고 있다.

2. 방법

고초균을 열처리하여 사멸시킨 균과 살아있는 균을 인간 장 상피세포인 Caco-2 세포에 처리하여 여러 항균펩타이드의 유전자 발현을 real-time RT-PCR 방법을 통해 확인하였다. 고초균으로부터 리포테이코익산, 펩티도글리칸 및 지질단백질 등 다양한 세포벽 구성요소들을 분리 후 HBD-2 항균펩타이드의 유전자 발현 및 생성을 확인하였다. 이후 다양한 균주들의 열처리 균 및 지질단백질의 HBD-2 유도 능력의 차이를 비교하였다. 전사억제 및 번역 억제제를 처리하여 유전자의 발현과 단백질 수준에서의 분비 수준의 연관성을 확인하였다. 분리한 지질단백질을 열, DNase I, proteinase K, lipase 등을 처리하고 염색하여 항균펩타이드의 유도에 중요한 요소를 확인하였다. Caco-2 세포를 분화시켜 HBD-2 생성을 ELISA를 통해 확인하였다. 지질단백질에 의해 유도되는 HBD-2의 메커니즘을 확인하기 위해 TLR2의 발현을 유세포분석기를 통해 확인하고 TLR2, MAP kinase, NF-κB 전사인자 각각의 억제제를 처리하였다. 지질단백질이 처리된 세포로부터 얻은 상등액을 병원성 균에 처리하여 병원성 균의 성장 저해를 확인하였다. 또한 LS174T 세포주에 고초균의 세포벽 구성성분들을 처리하여 점액층의 구성요소인 MUC2의 유전자 발현 및 단백질 발현을 PCR 및 단백질 면역 블롯, 공초점 레이저 현미경을 통해 확인하였다. 해당 현상이 보편적인 현상임을 확인하기 위해 SNU-61 및 SNU-407과 같은 인간 장세포에 세포벽 구성성분들을 처리하여 HBD-2 및 MUC2의 발현량을 확인하였다.

3. 결과

인간 장 상피세포인 Caco-2 세포에 살아있는 혹은 열로 사멸시킨 고초균을 처리하여 여러 HBD의 발현량을 확인하였을 때 HBD-2가 시간 및 농도 의존적으로 발현이 증가함을 확인하였다. HBD-2의 증가에 기여한 물질을 규명하기 위해 고초균의 상등액을 처리하였을 때 상등액에 의해 HBD-2의 발현이 증가하였고 따라서 세포 벽 성분들을 분리하여 Caco-2 세포에 처리하였다. 리포에티코익산, 펩티도글리칸, 지질단백질을 처리하였을 때 지질단백질에서 HBD-2의 발현 및 분비가 눈에 띄게 증가하였다. 해당 현상이 모든 고초균의 균주에서 보편적임을 확인하기 위해 다양한 균주들의 열처리균과 지질단백질을 처리하였을 때 모든 균주들에서 HBD-2가 유의적으로 유도되었다. 또한 다른 종의 균주들과 비교하였을 때 고초균이 가장 HBD-2를 높게 유도하였으며 고초균의 지질단백질 또한 높은 수준으로 유도하였다. 전사억제제 및 번역억제제의 처리를 통해 HBD-2의 유전자 수준과 단백질 수준이 비례함을 확인하였고 지질단백질이 농도 및 시간의존적으로 HBD-2의 발현을 유도함을 확인하였다. 지질단백질을 다양한 방법으로 변형시키고 Caco-2 세포에 처리한 결과 지질 및 단백질 부분이 HBD-2의 유도에 있어 중요함을 확인하였다. 분화된 Caco-2 세포를 통해 지질단백질의 인지 및 HBD-2의 분비가 apical 부분으로 이루어짐을 확인하고 분화된 세포가 더 높은 수준의 항균펩타이드를 발현함을 확인하였다. 지질단백질에 의한 HBD-2의 유도는 TLR2에 의해 매개되는 JNK/p38 및 NF-κB 경로를 통해 일어남을 억제제의 처리를 통해 일어났으며 분비된 HBD-2에 의해 병원성 세균의 성장이 억제됨을 확인하였다. 지질단백질은 또한 LS174T 세포주에서 MUC2를 가장 높게 발현시켰으며 이는 농도의존적으로 발현이 증가하였다. 지질단백질에 의해 항균펩타이드 및 MUC2의 발현이 증가하는 양상은 다른 인간 장세포에서도 동일하게 나타났으며, 이는 해당 현상이 보편적인 현상임을 제시한다.

4. 결론

위의 결과들을 종합하여 보면, 고초균은 인간 장 상피세포에서 항균펩타이드 및 뮤신을 높은 수준으로 유도해낼 수 있으며 해당 현상에 기여하는 물질은 고초균의 세포벽 구성 성분인 지질단백질임을 시사한다. 지질단백질은 TLR2에 의해 매개되는 JNK/p38 및 NF-κB 경로에 의해 인간 베타 디펜신-2를 유도하여 장내 유해균의 성장 억제에 기여하였다.

주요어: 고초균, 지질단백질, 장 상피세포, 인간 베타 디펜신-2, 뮤신
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