TITLE PAGE

- Food Science of Animal Resources -

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title	Inhibitory effect of genomic DNA extracted from *Pediococcus acidilactici* on *Porphyromonas gingivalis* lipopolysaccharide-induced inflammatory responses
Running Title (within 10 words)	*P. acidilactici* gDNA inhibits *P. gingivalis* LPS-induced inflammatory responses
Author	Young Hyeon Choi, Bong Sun Kim, Seok-Seong
Affiliation	Department of Food Science and Biotechnology, College of Life Science and Biotechnology, Dongguk University, Goyang, 10326, Korea
Special remarks – if authors have additional information to inform the editorial office	None
ORCID (All authors must have ORCID) https://orcid.org	Young Hyeon Choi (http://orcid.org/0000-0002-4954-7766) Bong Sun Kim (https://orcid.org/0000-0001-7676-6238) Seok-Seong Kang (https://orcid.org/0000-0001-7029-9122)
Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (NRF-2020R1A2C1010010).
Author contributions (This field may be published.)	Conceptualization: Choi YH, Kim BS, Kang SS. Data curation: Choi YH, Kim BS. Formal analysis: Choi YH, Kim BS. Methodology: Choi YH, Kim BS. Software: Choi YH, Kim BS. Validation: Choi YH, Kim BS. Investigation: Choi YH, Kim BS. Writing - original draft: Choi YH. Writing - review & editing: Choi YH, Kang SS.
Ethics approval (IRB/IACUC) (This field may be published.)	This article does not require IRB/IACUC approval because there are no human and animal participants.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Seok-Seong Kang
Email address – this is where your proofs will be sent	sskang@dgu.ac.kr
Secondary Email address	Kangelo72@gmail.com
Postal address	Department of Food Science and Biotechnology, Dongguk University, 32 Dongguk-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do, 10326, Korea
Cell phone number	+82-10-3669-6409
Office phone number	
Fax number	

Abstract

This study aimed to assess whether genomic DNA (gDNA) extracted from *Pediococcus acidilactici* inhibits *Porphyromonas gingivalis* lipopolysaccharide (LPS)-induced inflammatory responses in RAW 264.7 cells. Pretreatment with gDNA of *P. acidilactici* K10 or *P. acidilactici* HW01 for 15 h effectively inhibited *P. gingivalis* LPS-induced mRNA expression of interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein (MCP)-1. Although both gDNAs did not dose-dependently inhibit *P. gingivalis* LPS-induced mRNA expression of IL-6 and MCP-1, they inhibited IL-1β mRNA expression in a dose-dependent manner. Moreover, pretreatment with both gDNAs inhibited the secretion of IL-1β, IL-6, and MCP-1. When RAW 264.7 cells were stimulated with *P. gingivalis* LPS alone, the phosphorylation of mitogen-activated protein kinases (MAPKs) was increased. However, the phosphorylation of MAPKs was reduced in the presence of gDNAs. Furthermore, both gDNAs restored IκBα degradation induced by *P. gingivalis* LPS, indicating that both gDNAs suppressed the activation of nuclear factor-κB (NF-κB). In summary, *P. acidilactici* gDNA could inhibit *P. gingivalis* LPS-induced inflammatory responses through the suppression of MAPKs and NF-κB, suggesting that *P. acidilactici* gDNA could be effective in preventing periodontitis.

Keywords: *Pediococcus acidilactici*; genomic DNA; *Porphyromonas gingivalis*; Periodontitis; Inflammation

Introduction

Probiotics, which mostly consist of lactic acid bacteria, are well known to confer beneficial effects (Chugh and Kamal-Eldin, 2020). Their physiological functions are beneficial to human health by inhibiting pathogen adhesion, enhancing the functions of the intestinal epithelial barrier, and modulating host immune responses (Lebeer et al., 2010). Moreover, the use of probiotics is a promising therapeutic approach against many inflammatory diseases (Maekawa and Hajishengallis, 2014). These effects of probiotics can be attributed to the production of bioactive molecules, such as cell wall components, bacteriocins, short-chain fatty acids, exopolysaccharides, enzymes, and amino acids (Chugh and Kamal-Eldin, 2020; Lebeer et al., 2010).

In addition to the bioactive molecules of probiotics, their genomic DNA (gDNA) has been proven to exert probiotic effects. For instance, gDNA extracted from *Lactobacillus plantarum* inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α secretion by suppressing the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) in THP-1 cells (Hee Kim et al., 2012). Moreover, oligodeoxynucleotides from *L. casei* gDNA inhibited inflammatory responses in human intestinal epithelial cells *in vitro* as well as in a dextran sulfate sodium-induced murine colitis model (Hiramatsu et al., 2014). More recently, gDNA extracted from *L. rhamnosus* GG was found to inhibit LPS-induced interleukin (IL)-6 production in RAW 264.7 cells (Qi et al., 2020).

Periodontitis is defined as a chronic inflammatory oral disease caused by multiple microbial infections, resulting in the gradual destruction of tissues supporting teeth and the alveolar bone (Saini et al., 2009). Moreover, periodontitis is known to be associated with systemic diseases, such as cardiovascular and respiratory diseases, rheumatoid arthritis, and metabolic syndrome

(Winning and Linden, 2015). *Porphyromonas gingivalis* is a Gram-negative anaerobic bacterium that is considered to be the main pathogen responsible for periodontitis by producing several virulence factors, such as extracellular proteases and LPS (Bostanci and Belibasakis, 2012). Among the bacteria associated with the pathogenesis of periodontitis, *P. gingivalis* has been extensively studied because of its unique ability to penetrate the gingivae and cause tissue destruction by inducing inflammation (How et al., 2016).

The findings of *in vitro* and *in vivo* analyses have confirmed that probiotics can alleviate periodontal diseases (Nguyen et al., 2021). However, probiotics need to be viable to perform their biological functions. Moreover, the use of probiotics may cause some adverse effects, such as antibiotic resistance and systemic infections in immunocompromised patients (Doron and Snydman, 2015). Therefore, molecules with functional bioactivities, also known as postbiotics, are considered promising alternatives to probiotics. Although the health benefits of probiotics, particularly lactobacilli, are well known, pediococci are also exhibit certain probiotic effects. Bioactive molecules, such as exopolysaccharides and bacteriocins, of *Pediococcus acidilactici* exhibit probiotic effects, including antioxidant, lipid-lowering, antimicrobial, and anti-inflammatory effects (Moon et al., 2014; Seo and Kang, 2020; Song et al., 2021). In addition, our studies have shown the beneficial effects of postbiotics derived from *P. acidilactici* K10 and *P. acidilactici* HW01, such as bacteriocin and bacterial lysates. Bacteriocins of *P. acidilactici* K10 and *P. acidilactici* HW01 inhibit biofilm formation of foodborne pathogenic bacteria, including *Enterococcus faecalis* and *Pseudomonas aeruginosa* (Lee et al., 2020; Yoon and Kang, 2020). More recently, bacterial lysates of both *P. acidilactici* K10 and *P. acidilactici* HW01 exert antibiofim, anti-adhesive and anti-invasive activities against *Listeria monocytogenes* (Lee et al., 2022). Therefore, this study aimed to assess the

anti-inflammatory effect of gDNA extracted from *P. acidilactici* K10 and *P. acidilactici* HW01 against *P. gingivalis* LPS-induced inflammatory responses.

Materials and methods

Bacterial culture and gDNA purification

P. acidilactici K10 and *P. acidilactici* HW01, which were isolated from kimchi and malt (Ahn et al., 2017; Kwon et al., 2002), were maintained in Man–Rogosa–Sharpe (MRS) medium (Neogen, Lansing, MI, USA) at 37°C. The gDNAs of *P. acidilactici* K10 and *P. acidilactici* HW01 (K10 gDNA and HW01 gDNA, respectively) were extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, with some modifications. In brief, bacterial pellets were harvested by centrifugation at 13,000 × g for 10 min and extensively washed with phosphate-buffered saline (PBS). The bacterial pellets were resuspended in 480 μL of 50 mM EDTA and 120 μL of lysozyme (10 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 30 min. After centrifuging at 13,000 × g for 10 min, nuclei lysis solution (600 μL) was added to the pellets and incubated at 80°C for 5 min to lyse the cell membrane. RNase solution (3 μL) was then added to the lysates, which were mixed by inverting the tubes five times and then incubated at 37°C for 40 min. Protein precipitation solution (200 μL) was added to the mixture and incubated on ice for 5 min to purify the gDNA. After centrifuging at 13,000 × g for 3 min, the supernatants were transferred to a clean microtube and gently mixed with 600 μL of isopropanol. After centrifuging at 13,000 × g for 3 min, the supernatants were removed and the remaining pellets were washed with 70% ethanol. Finally, the ethanol was discarded and the

pellets containing gDNA were rehydrated by adding DNA rehydration solution. The concentration and purity of gDNA were determined using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Hampton, NH, USA).

Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan, Korea) containing 10% heat-inactivated fetal bovine serum (Gibco, Burlington, ON, Canada), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone, Logan, UT, USA) at 37°C in a 5% CO_2-humidified incubator.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RAW 264.7 cells (1×10^5 cells/mL) were plated on a 12-well culture plate and incubated at 37°C for 24 h. The cells were pretreated with K10 gDNA or HW01 gDNA (1 μg/mL) for 3, 9, or 15 h and stimulated with 1 μg/mL of *P. gingivalis* LPS for 3 h. In a separate experiment, RAW 264.7 cells (1×10^5 cells/mL) were plated on a 12-well culture plate and incubated at 37°C for 24 h. The cells were pretreated with various concentrations of K10 gDNA or HW01 gDNA (0.1, 1, and 10 μg/mL) for 15 h and stimulated with 1 μg/mL of *P. gingivalis* LPS for 3 h. Subsequently, spent culture supernatants were discarded and total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA was reversed transcribed to complementary DNA (cDNA) using random hexamers and reverse transcriptase (Promega). cDNA was amplified by

qPCR with SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Specific genes responsible for inflammatory responses, namely those encoding interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein (MCP)-1, were amplified under the following conditions: denaturation at 95°C for 10 s, amplification for 40 cycles at 95°C for 5 s and 60°C for 31 s. The sequences of specific primers were as follows: IL-1β, forward, 5′-CTCACAAGCAGAGCACAAGC-3′ and reverse, 5′-TCTTGGCCGAGGACTAAGGA-3′; IL-6, forward, 5′-TCCTACCCCAATTTCCAATGCT-3′ and reverse, 5′-TCTGACCACAGTGAGGAATGTC-3′; MCP-1, forward, 5′-AGCCAACTCTCACTGAAGCC-3′ and reverse, 5′-TCTCCAGCCTACTCATTGGGA-3′; and β-actin, forward, 5′-TACAGCTTCACCACCACAGC-3′ and reverse, 5′-GGAAAAGAGCCTCAGGGCAT-3′. The relative mRNA expression levels of IL-1β, IL-6, and MCP-1 were normalized to that of β-actin using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells (1×10^5 cells/mL) were plated on a 96-well culture plate and incubated at 37°C for 24 h. The cells were pretreated with 1 μg/mL of K10 gDNA or HW01 gDNA at 37°C for 15 h and stimulated with *P. gingivalis* LPS (1 μg/mL) at 37°C for further 24 h. Subsequently, spent culture supernatants were collected and the secretion levels of IL-1β, IL-6, and MCP-1 were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Western blot analysis

RAW 264.7 cells (1×10^5 cells/mL) were plated on a 6-well culture plate and incubated at 37°C for 24 h. The cells were pretreated with 1 μg/mL of K10 gDNA or HW01 gDNA at 37°C for 15 h and stimulated with *P. gingivalis* LPS (1 μg/mL) at 37°C for 30 min. The cells were then washed with PBS and lysed with a lysis buffer (1 M HEPES, 1 M NaCl, 1% IGEPAL®-CA 630, 0.75% sodium deoxycholate, and 10% glycerol) containing protease and phosphatase inhibitors. Equal amounts of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with primary antibodies specific to extracellular signal-regulated kinase (ERK), phosphorylated ERK, p38 kinase, phosphorylated p38 kinase, c-Jun-N-terminal kinase (JNK), phosphorylated JNK, IκBα (Cell Signaling Technology, Danvers, MA, USA), or β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C for 15 h. The membranes were washed with TBST and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) at room temperature for 1 h. Immunoreactive proteins were detected using an enhanced chemiluminescence reagent (Dyne Bio, Seongnam, Korea) and visualized using a C-DiGit blot scanner (Li-Cor Bioscience, Lincoln, NE, USA).

Statistical analysis

All experiments were performed thrice. The results are expressed as the means ± standard deviations. Statistical differences between the appropriate control and gDNA-treated groups were assessed by performing unpaired two-tailed *t*-tests using GraphPad Prism 5 software

(GraphPad Software Inc., La Jolla, CA, USA).

Results

K10 gDNA and HW01 gDNA inhibit *P. gingivalis* LPS-induced mRNA expression of IL-1β, IL-6, and MCP-1

When the cells were pretreated with K10 gDNA and HW01 gDNA for 3 h, the gDNAs did not inhibit *P. gingivalis*-induced mRNA expression of IL-1β (Fig. 1A), IL-6 (Fig. 1B), and MCP-1 (Fig. 1C). However, when the cells were pretreated with K10 gDNA and HW01 gDNA for 9 h, the gDNAs partially suppressed inflammatory responses. As shown in Fig. 1D, *P. gingivalis* LPS-induced IL-1β mRNA expression was significantly downregulated in the presence of K10 gDNA and HW01 gDNA ($P < 0.05$), whereas IL-6 and MCP-1 mRNA expression was not inhibited by these gDNAs (Figs. 1E and 1F, respectively). When RAW 264.7 cells were pretreated with gDNAs for 15 h, the gDNAs significantly inhibited the mRNA expression of IL-1β (Fig. 1G), IL-6 (Fig. 1H), and MCP-1 (Fig. 1I) ($P < 0.05$). As 15 h-pretreatment with gDNAs effectively inhibited *P. gingivalis* LPS-induced inflammatory responses, all subsequent experiments were performed using cells pretreated with gDNAs for 15 h.

K10 gDNA and HW01 gDNA dose-dependently inhibit *P. gingivalis* LPS-induced mRNA expression of IL-1β but not that of IL-6 and MCP-1

To assess the inhibitory effect of different concentrations of gDNAs on *P. gingivalis* LPS-induced inflammatory responses in RAW 264.7 cells, the cells were pretreated with gDNAs

(0.1, 1, and 10 μg/mL) for 15 h and then stimulated with *P. gingivalis* LPS (1 μg/mL) for 3 h. As expected, *P. gingivalis* LPS alone induced IL-1β mRNA expression. However, both K10 gDNA and HW01 gDNA dose-dependently inhibited *P. gingivalis* LPS-induced IL-1β mRNA expression (Figs. 2A and 2D, respectively). Pretreatment with 0.1 and 1 μg/mL of K10 gDNA and HW01 gDNA significantly inhibited *P. gingivalis* LPS-induced IL-6 mRNA expression ($P < 0.05$) (Figs. 2B and 2E, respectively). However, 10 μg/mL of both gDNAs failed to inhibit *P. gingivalis* LPS-induced IL-6 mRNA expression (Figs. 2B and 2E). Similarly, 0.1 and 1 μg/mL of K10 gDNA significantly inhibited *P. gingivalis* LPS-induced MCP-1 mRNA expression, whereas 10 μg/mL did not (Fig. 2C). Moreover, 1 μg/mL of HW01 gDNA inhibited *P. gingivalis* LPS-induced MCP-1 expression, whereas 0.1 and 10 μg/mL did not (Fig. 2F). Therefore, we decided that the optimal concentration of gDNAs to inhibit *P. gingivalis* LPS-induced inflammatory responses was 1 μg/mL and used this concentration for further analysis.

K10 gDNA and HW01 gDNA inhibit *P. gingivalis* LPS-induced secretion of IL-1β, IL-6, and MCP-1

To further confirm the inhibition of inflammatory responses at the protein level, RAW 264.7 cells were pretreated with gDNAs (1 μg/mL) for 15 h and then stimulated with *P. gingivalis* LPS (1 μg/mL) for 24 h. Consistent with the results of mRNA expression, both gDNAs significantly inhibited IL-1β (Fig. 3A), IL-6 (Fig.3B), and MCP-1 (Fig. 3C) secretion induced by *P. gingivalis* LPS ($P < 0.05$). These results indicate that K10 gDNA and HW01 gDNA effectively inhibit *P. gingivalis* LPS-induced inflammatory responses.

K10 gDNA and HW01 gDNA alleviate MAPK phosphorylation and NF-κB activation

MAPK pathways, such as ERK 1/2, p38 kinase, JNK, and NF-κB pathways, are known to be common inflammatory signaling pathways that cause the release of proinflammatory cytokines, such as IL-1β, and consequently lead to inflammation (Jayakumar et al., 2021). Therefore, the activation of MAPK and NF-κB was assessed in RAW 264.7 cells pretreated with or without gDNAs. As shown in Fig. 4, the phosphorylation of ERK 1/2, p38 kinase, and JNK was higher in RAW 264.7 cells stimulated with *P. gingivalis* LPS than in those not treated with *P. gingivalis* LPS. However, pretreatment with K10 gDNA and HW01 gDNA abrogated the phosphorylation of ERK 1/2, p38 kinase, and JNK, indicating that ERK 1/2, p38 kinase, and JNK signaling pathways were blocked on pretreatment with both these gDNAs and consequently inhibited *P. gingivalis* LPS-induced inflammatory responses. Moreover, inhibitors of NF-κB, such as IκBα, are associated with the downregulation of NF-κB (Wang et al., 2020). *P. gingivalis* LPS alone inhibited IκBα degradation, indicating that the inflammatory responses were augmented by NF-κB activation. However, pretreatment of RAW 264.7 cells with K10 gDNA or HW01 gDNA (Fig. 4) restored IκBα degradation, suggesting that both these gDNAs attenuated NF-κB activation, which was associated with *P. gingivalis* LPS-induced inflammatory responses.

Discussion

Periodontitis is the most prevalent oral disease that is characterized by gingival inflammation and the loss of connective tissue attachment, resulting in alveolar bone resorption and tooth loss (Xu et al., 2020). Although the role of probiotics in modulating periodontal diseases is not fully understood, several studies have reported the protective effects of probiotics, particularly

Lactobacillus spp., on periodontal diseases based on *in vitro* and *in vivo* analyses (Gatej et al., 2018; Nguyen et al., 2021). *P. acidilactici* is frequently found in fermented vegetables and meat-based products and is considered a potential probiotic with antimicrobial, antioxidant, and immunomodulatory effects (Feng et al., 2016; Papagianni and Anastasiadou, 2009; Song et al., 2021). Furthermore, the present study demonstrated that K10 gDNA and HW01 gDNA markedly inhibited *P. gingivalis* LPS-induced inflammatory responses by suppressing MAPK and NF-κB signaling pathways in RAW 264.7 cells, suggesting that *P. acidilactici* could also be a potential probiotic that could be beneficial to oral health.

Several lactobacilli have been reported to inhibit inflammatory responses induced by periodontal pathogenic bacteria, such as *P. gingivalis*. Co-culture with *P. gingivalis* and *L. acidophilus* significantly decreased the mRNA and protein expression levels of IL-1β, IL-6, and IL-8 in gingival epithelial cells in a dose-dependent manner (Zhao et al., 2012). More recently, mono-infection of gingival epithelial cells with *P. gingivalis* was found to increase the expression of IL-1β and TNF-α. However, treatment with *L. rhamnosus* Lr-32 or *L. acidophilus* LA-5 markedly decreased the expression of IL-1β and TNF-α (Albuquerque-Souza et al., 2019). In fact, *P. acidilactici* has displayed anti-inflammatory activities against various pathogenic bacteria. We previously found that bacteriocins isolated from *P. acidilactici* K10 and HW01 suppressed IL-8 production in the human intestinal epithelial cell line Caco-2 in response to *Enterococcus faecalis* infection (Yoon and Kang, 2020). Cell-free supernatants of *P. acidilactici* TMAB26 effectively inhibited *Klebsiella pneumoniae* LPS-induced IL-6 and TNF-α mRNA expression in HT-29 cells and peripheral blood mononuclear cells (Barigela and Bhukya, 2021). However, the antagonistic effect of *P. acidilactici* or its component(s) on *P. gingivalis*-induced inflammatory responses has not been elucidated. In addition, gDNA derived

from *Lactobacillus* spp. has been found to have anti-inflammatory properties. For example, high-frequency oligodeoxynucleotides of *L. casei* gDNA inhibited IL-8 production in Caco-2 cells *in vitro* and alleviated dextran sulfate sodium-induced murine colitis (Hiramatsu et al., 2014). Consistent with the present findings, pretreatment with *L. rhamnosus* GG gDNA was found to alleviate mRNA expression levels of inflammatory cytokines in RAW 264.7 cells upon LPS challenge (Qi et al., 2020). However, whether gDNAs of probiotic strains exhibit anti-inflammatory effects against oral pathogenic bacteria remains unclear. We found that *P. acidilactici* gDNA can inhibit *P. gingivalis* LPS-induced inflammatory responses.

During the development of periodontitis, periodontal pathogens trigger innate immune responses by releasing various cytokines and chemokines, which play a pivotal role in the progression of inflammation (Ramadan et al., 2020). Excessive IL-6 production may be associated with periodontal tissue destruction by inducing matrix metalloproteinase (MMP)-1 secretion during the progression of periodontitis (Sundararaj et al., 2009). In addition, IL-1β is involved in the production of MMP-1, leading to tissue damage (Cheng et al., 2020). MCP-1 is produced by macrophages and fibroblasts and acts as a chemoattractant for monocytes and neutrophils (Strecker et al., 2011). Excessive neutrophil activation can result in the production of toxic compounds and reactive oxygen species that can also affect aggressive periodontitis (Ramadan et al., 2020). We found that both K10 gDNA and HW01 gDNA significantly suppressed these cytokines/chemokines by regulating MAPK and NF-κB signaling pathways, suggesting that they could relieve inflammation and consequently prevent periodontitis. Although the precise mechanisms by which *P. acidilactici* gDNAs inhibit the production of cytokines/chemokines responsible for periodontitis are not fully understood, it can be speculated that cell signaling mediated by toll-like receptor 9 (TLR9) negatively regulates

inflammatory responses. TLR9 is a critical receptor for recognizing bacterial DNA (Chockalingam et al., 2012). Li et al. (2017) reported that TLR9 deficiency enhanced the activation of NF-κB, resulting in increased IL-1β expression in intestinal epithelial cells. Moreover, the use of CpG oligodeoxynucleotides as a TLR9 ligand inhibited MCP-1 expression in adipocytes (Thomalla et al., 2019). Although TLR signaling pathways mainly induce inflammatory responses, TLR9 signaling promotes the accumulation of IκBα in the cytoplasm, thereby suppressing NF-κB activation (Li et al., 2017). Therefore, TLR9 activation by *P. acidilactici* gDNAs may negatively regulate intracellular signaling pathways, such as MAPK and NF-κB pathways, thereby attenuating inflammatory responses.

Conclusion

In conclusion, the present study demonstrated the inhibitory effect of *P. acidilactici* gDNA on *P. gingivalis* LPS-induced inflammatory responses. Several probiotic lactobacilli have shown anti-inflammatory activities against *P. gingivalis*. Our results provide important insights into the inhibition of periodontal inflammation using *P. acidilactici* gDNA. Thus, *P. acidilactici* gDNA could be a potential therapeutic option against periodontitis.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded

298 by the Korean government (MSIT) (NRF-2020R1A2C1010010). This work was also supported
299 by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and
300 Forestry (IPET) through Technology Commercialization Support Program, funded by Ministry
301 of Agriculture, Food and Rural Affairs (MAFRA) (821028-03-2-HD040).

302

303 **Author contributions**

304 Conceptualization: Choi YH, Kim BS, Kang SS. Data curation: Choi YH, Kim BS. Formal
305 analysis: Choi YH, Kim BS. Methodology: Choi YH, Kim BS. Software: Choi YH, Kim BS.
306 Validation: Choi YH, Kim BS. Investigation: Choi YH, Kim BS. Writing - original draft: Choi
307 YH. Writing - review & editing: Choi YH, Kang SS.

308

309 **Ethics Approval**

310 This article does not require IRB/IACUC approval because there are no human and animal
311 participants.

312

313 **References**

314 Ahn HW, Kim JS, Kim WJ. 2017. Isolation and characterization of bacteriocin-producing
315 *Pediococcus acidilactici* HW01 from malt and its potential to control beer spoilage
316 lactic acid bacteria. Food Control 80:59-66.
317 Albuquerque-Souza E, Balzarini D, Ando-Suguimoto ES, Ishikawa KH, Simionato MR,
318 Holzhausen M, Mayer MP. 2019. Probiotics alter the immune response of gingival
319 epithelial cells challenged by porphyromonas gingivalis. J Periodontal Res 54:115-127.

Barigela A, Bhukya B. 2021. Probiotic *Pediococcus acidilactici* strain from tomato pickle displays anti-cancer activity and alleviates gut inflammation *in vitro*. 3 Biotech 11:23.

Bostanci N, Belibasakis GN. 2012. *Porphyromonas gingivalis*: An invasive and evasive opportunistic oral pathogen. FEMS Microbiol Lett 333:1-9.

Cheng R, Wu Z, Li M, Shao M, Hu T. 2020. Interleukin-1β is a potential therapeutic target for periodontitis: A narrative review. Int J Oral Sci 12:2.

Chockalingam A, Rose WA, Hasan M, Ju C-H, Leifer CA. 2012. Cutting edge: A TLR9 cytoplasmic tyrosine motif is selectively required for proinflammatory cytokine production. J Immunol 188:527-530.

Chugh B, Kamal-Eldin A. 2020. Bioactive compounds produced by probiotics in food products. Curr Opin Food Sci 32:76-82.

Doron S, Snydman DR. 2015. Risk and safety of probiotics. Clin Infect Dis 60 Suppl 2:S129-S134.

Feng J, Wang L, Zhou L, Yang X, Zhao X. 2016. Using *in vitro* immunomodulatory properties of lactic acid bacteria for selection of probiotics against *Salmonella* infection in broiler chicks. PLoS One 11:e0147630.

Gatej SM, Marino V, Bright R, Fitzsimmons TR, Gully N, Zilm P, Gibson RJ, Edwards S, Bartold PM. 2018. Probiotic *Lactobacillus rhamnosus* gg prevents alveolar bone loss in a mouse model of experimental periodontitis. J Clin Periodontol 45:204-212.

Kim CH, Kim HG, Kim JY, Kim NR, Jung BJ, Jeong JH, Chung DK. 2012. Probiotic genomic DNA reduces the production of pro-inflammatory cytokine tumor necrosis factor-alpha. FEMS Microbiol Lett 328:13-19.

Hiramatsu Y, Satho T, Hyakutake M, Irie K, Mishima K, Miake F, Kashige N. 2014. The anti-inflammatory effects of a high-frequency oligodeoxynucleotide from the genomic DNA

of *Lactobacillus casei*. Int Immunopharmacol 23:139-147.

How KY, Song KP, Chan KG. 2016. *Porphyromonas gingivalis*: An overview of periodontopathic pathogen below the gum line. Front Microbiol 7:53.

Jayakumar T, Lin KC, Chang CC, Hsia CW, Manubolu M, Huang WC, Sheu JR, Hsia CH. 2021. Targeting MAPK/NF-κB pathways in anti-inflammatory potential of rutaecarpine: Impact on Src/FAK-mediated macrophage migration. Int J Mol Sci 23:92.

Kwon DY, Koo MS, Ryoo CR, Kang CH, Min KH, Kim WJ. 2002. Bacteriocin produced by *Pediococcus* sp. In Kimchi and its characteristics. J Microbiol Biotechnol 12:96-105.

Lebeer S, Vanderleyden J, De Keersmaecker SC. 2010. Host interactions of probiotic bacterial surface molecules: Comparison with commensals and pathogens. Nat Rev Microbiol 8:171-184.

Lee DH, Kim BS, Kang SS. 2020. Bacteriocin of *Pediococcus acidilactici* HW01 inhibits biofilm formation and virulence factor production by *Pseudomonas aeruginosa*. Probiotics Antimicrob Proteins 12:73-81.

Lee HB, Kim KH, Kang GA, Lee KG, Kang SS. 2022. Antibiofilm, antiadhesive and anti-invasive activities of bacterial lysates extracted from *Pediococcus acidilactici* against *Listeria monocytogenes*. Foods 11:2948.

Li Y, Liu M, Zuo Z, Liu J, Yu X, Guan Y, Zhan R, Han Q, Zhang J, Zhou R, Sun R, Tian Z, Zhang C. 2017. TLR9 regulates the NF-κB-NLRP3-IL-1β pathway negatively in *Salmonella*-induced NKG2D-mediated intestinal inflammation. J Immunol 199:761-773.

Moon Y-J, Baik S-H, Cha Y-S. 2014. Lipid-lowering effects of *Pediococcus acidilactici* M76 isolated from Korean traditional makgeolli in high fat diet-induced obese mice. Nutrients 6:1016-1028.

Nguyen T, Brody H, Radaic A, Kapila Y. 2021. Probiotics for periodontal health-current molecular findings. Periodontol 2000 87:254-267.

Papagianni M, Anastasiadou S. 2009. Pediocins: The bacteriocins of pediococci. Sources, production, properties and applications. Microb Cell Fact 8:3.

Qi SR, Cui YJ, Liu JX, Luo X, Wang HF. 2020. *Lactobacillus rhamnosus* GG components, SLP, gDNA and CpG, exert protective effects on mouse macrophages upon lipopolysaccharide challenge. Lett Appl Microbiol 70:118-127.

Ramadan DE, Hariyani N, Indrawati R, Ridwan RD, Diyatri I. 2020. Cytokines and chemokines in periodontitis. Eur J Dent 14:483-495.

Saini R, Marawar PP, Shete S, Saini S. 2009. Periodontitis, a true infection. J Glob Infect Dis 1:149-150.

Seo HJ, Kang SS. 2020. Inhibitory effect of bacteriocin produced by *Pediococcus acidilactici* on the biofilm formation of *Salmonella* Typhimurium. Food Control 117:107361.

Song YR, Lee CM, Lee SH, Baik SH. 2021. Evaluation of probiotic properties of *Pediococcus acidilactici* M76 producing functional exopolysaccharides and its lactic acid fermentation of black raspberry extract. Microorganisms 9:1364.

Strecker JK, Minnerup J, Gess B, Ringelstein EB, Schabitz WR, Schilling M. 2011. Monocyte chemoattractant protein-1-deficiency impairs the expression of IL-6, IL-1β and G-CSF after transient focal ischemia in mice. PLoS One 6:e25863.

Sundararaj KP, Samuvel DJ, Li Y, Sanders JJ, Lopes-Virella MF, Huang Y. 2009. Interleukin-6 released from fibroblasts is essential for up-regulation of matrix metalloproteinase-1 expression by U937 macrophages in coculture: Cross-talking between fibroblasts and u937 macrophages exposed to high glucose. J Biol Chem 284:13714-13724.

Thomalla M, Schmid A, Neumann E, Pfefferle PI, Muller-Ladner U, Schaffler A, Karrasch T.

2019. Evidence of an anti-inflammatory toll-like receptor 9 (TLR 9) pathway in adipocytes. J Endocrinol 240:325-343.

Wang X, Peng H, Huang Y, Kong W, Cui Q, Du J, Jin H. 2020. Post-translational modifications of IκBα: The state of the art. Front Cell Dev Biol 8:574706.

Winning L, Linden GJ. 2015. Periodontitis and systemic disease. BDJ Team 2:15163.

Xu W, Zhou W, Wang H, Liang S. 2020. Roles of *Porphyromonas gingivalis* and its virulence factors in periodontitis. Adv Protein Chem Struct Biol 120:45-84.

Yoon JW, Kang SS. 2020. *In vitro* antibiofilm and anti-inflammatory properties of bacteriocins produced by *Pediococcus acidilactici* against *Enterococcus faecalis*. Foodborne Pathog Dis 17:764-771.

Zhao J-J, Feng X-P, Zhang X-L, Le K-Y. 2012. Effect of *Porphyromonas gingivalis* and *Lactobacillus acidophilus* on secretion of IL1β, IL6, and IL8 by gingival epithelial cells. Inflammation 35:1330-1337.

Figure Legends

Fig. 1. Inhibition of mRNA expression of inflammatory mediators by *P. acidilactici* gDNAs in RAW 264.7 cells stimulated with *P. gingivalis* LPS. RAW 264.7 cells were pretreated with K10 gDNA or HW01 gDNA (1 μg/mL) for 3, 6, or 15 h and then stimulated with *P. gingivalis* LPS (1 μg/mL) for 3 h. The mRNA expression levels of IL-1β, IL-6, and MCP-1 were determined using RT-qPCR. The results are expressed as the means ± standard deviations from three independent experiments. The asterisk (*) indicates a statistically significant difference ($P < 0.05$) compared with the control. LPS: *P. gingivalis* LPS; K10: K10 gDNA; HW01: HW01 gDNA.

Fig. 2. Inhibition of mRNA expression of inflammatory mediators by various concentrations of *P. acidilactici* gDNAs in RAW 264.7 cells stimulated with *P. gingivalis* LPS. RAW 264.7 cells were pretreated with K10 gDNA or HW01 gDNA (0.1, 1, and 10 μg/mL) for 15 h and then stimulated with *P. gingivalis* LPS (1 μg/mL) for 3 h. The mRNA expression levels of IL-1β, IL-6, and MCP-1 were determined using RT-qPCR. The results are expressed as the means ± standard deviations from three independent experiments. The asterisk (*) indicates a statistically significant difference ($P < 0.05$) compared with the control. LPS: *P. gingivalis* LPS; K10: K10 gDNA; HW01: HW01 gDNA.

Fig. 3. Inhibition of protein expression of inflammatory mediators by *P. acidilactici* gDNAs in RAW 264.7 cells stimulated with *P. gingivalis* LPS. RAW 264.7 cells were pretreated with K10 gDNA or HW01 gDNA (1 μg/mL) for 15 h and then stimulated with *P. gingivalis* LPS (1 μg/mL)

435 for 24 h. The secretion levels of IL-1β, IL-6, and MCP-1 were determined using ELISA. The
436 results are expressed as the means ± standard deviations from three independent experiments.
437 The asterisk (*) indicates a statistically significant difference ($P < 0.05$) compared with the
438 control. LPS: *P. gingivalis* LPS; K10: K10 gDNA; HW01: HW01 gDNA.

439

440 **Fig. 4.** Inhibition of MAPK phosphorylation and NF-κB activation by *P. acidilactici* gDNAs
441 in RAW 264.7 cells stimulated with *P. gingivalis* LPS. RAW 264.7 cells were pretreated with
442 K10 gDNA or HW01 gDNA (1 μg/mL) for 15 h and then stimulated with *P. gingivalis* LPS (1
443 μg/mL) for 30 min. The phosphorylation of ERK 1/2, p38, and JNK and the degradation of
444 IκBα were assessed using western blot analysis.

445

446

447

448

449

450

451

452

453

454

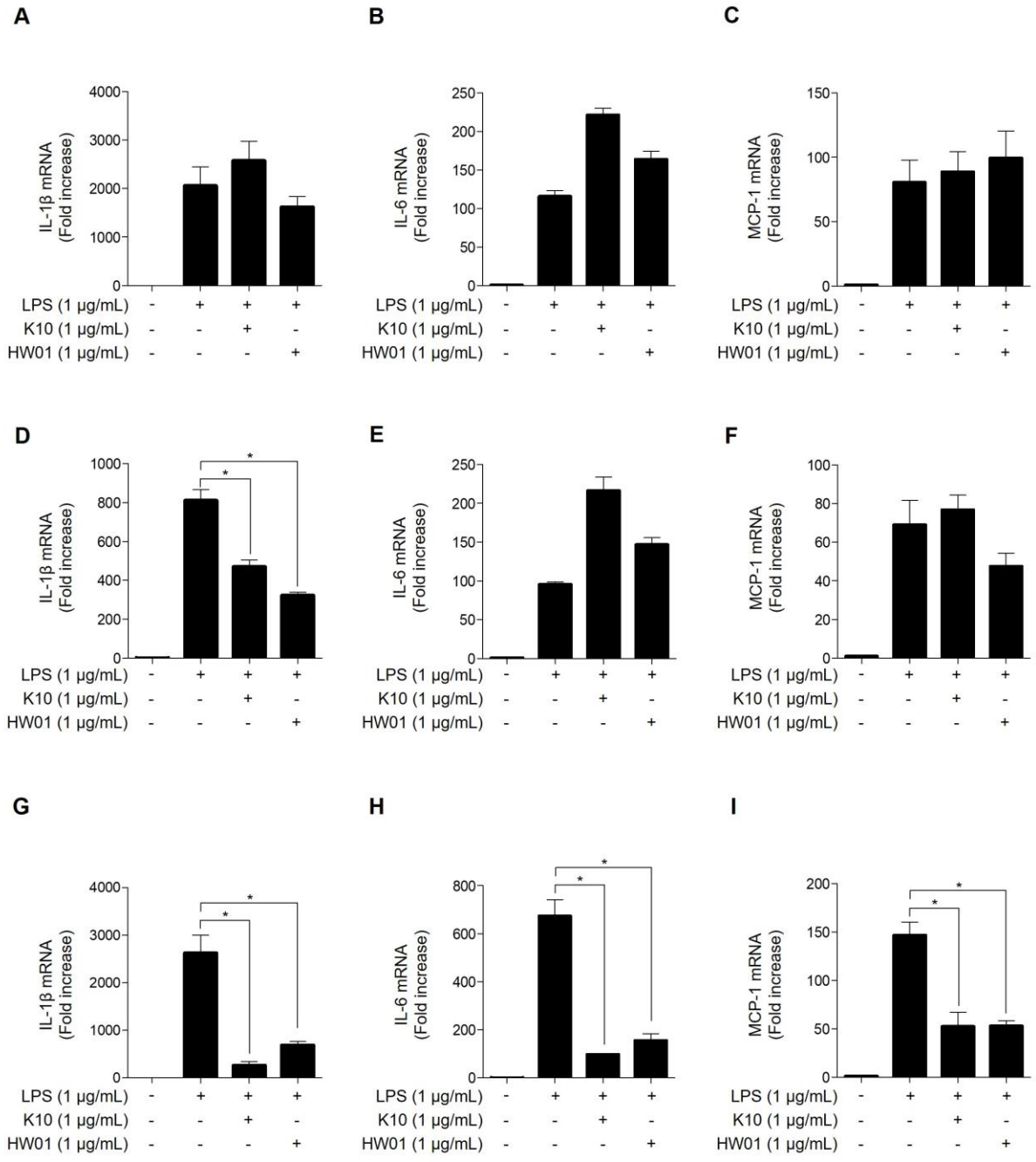
A
B
C
D
E
F
G
H
I
IL-1β mRNA (Fold increase)
IL-6 mRNA (Fold increase)
MCP-1 mRNA (Fold increase)
LPS (1 μg/mL)
K10 (1 μg/mL)
HW01 (1 μg/mL)
*

Figure 1

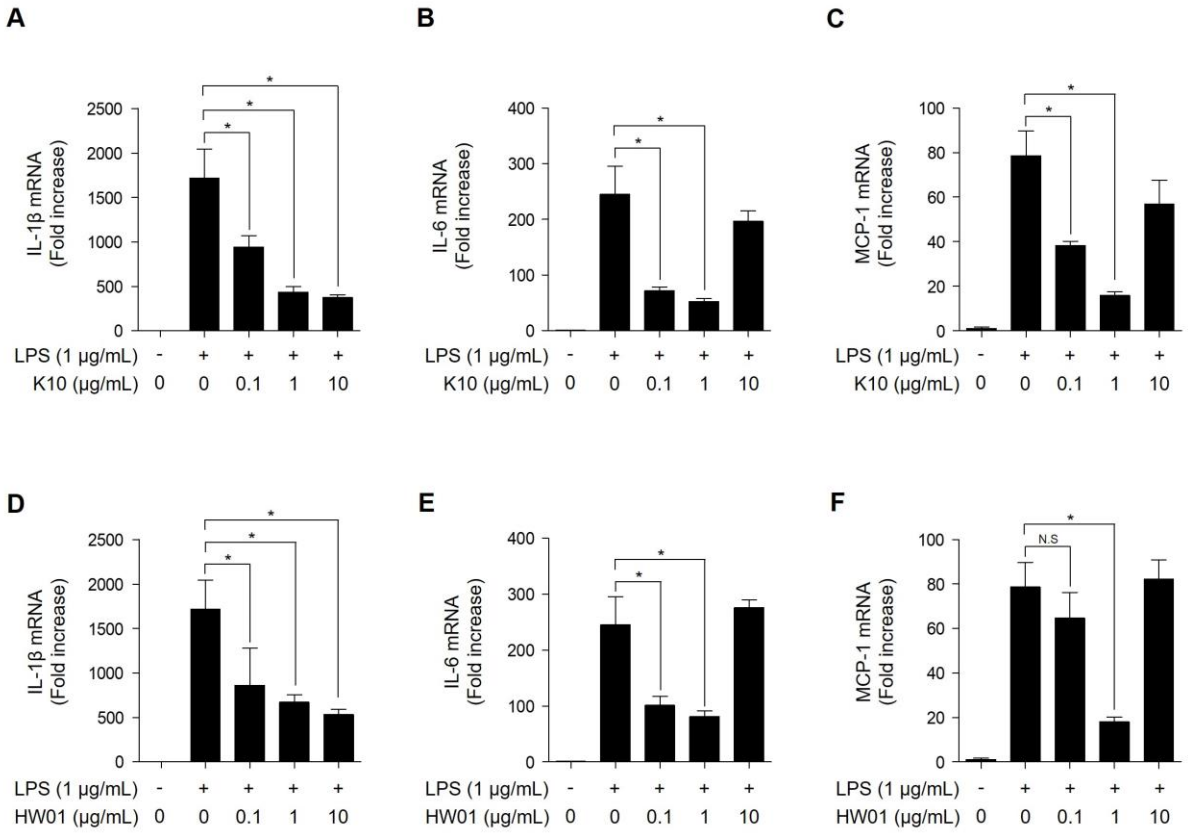

Figure 2

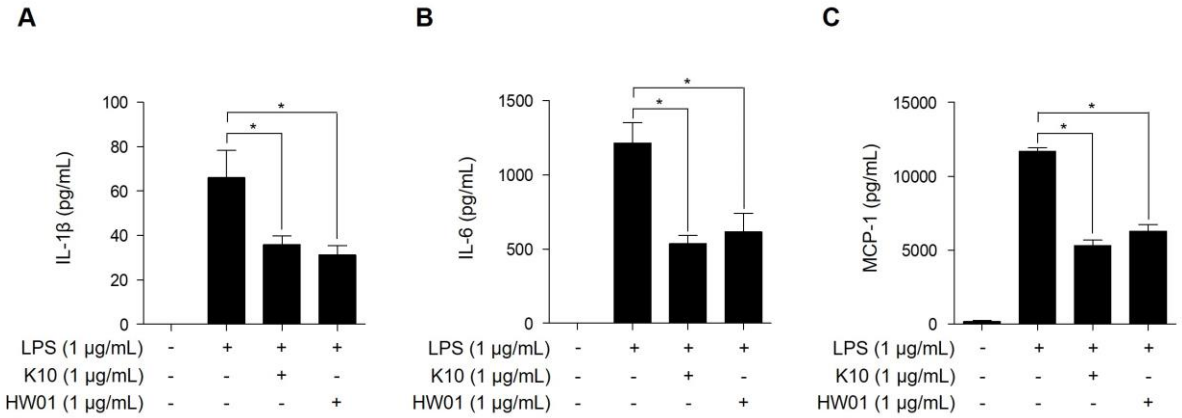

Figure 3

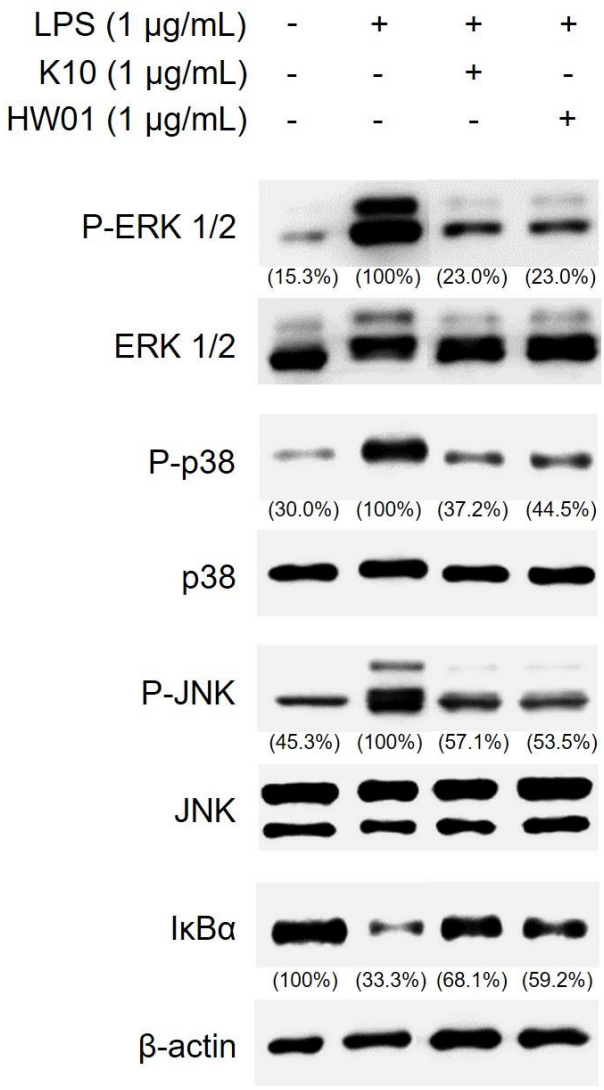
LPS (1 μg/mL) - + + +
K10 (1 μg/mL) - - + -
HW01 (1 μg/mL) - - - +
P-ERK 1/2
(15.3%) (100%) (23.0%) (23.0%)
ERK 1/2
P-p38
(30.0%) (100%) (37.2%) (44.5%)
p38
P-JNK
(45.3%) (100%) (57.1%) (53.5%)
JNK
IκBα
(100%) (33.3%) (68.1%) (59.2%)
β-actin

Figure 4