



Whey fermented by *Enterococcus faecalis* M157 exhibits antiinflammatory and antibiofilm activities against oral pathogenic bacteria

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ABSTRACT

The aim of the present study was to investigate the antiinflammatory and antibiofilm effects of whey fermented by *Enterococcus faecalis* M157 (M157-W) against oral pathogenic bacteria. The M157-W significantly inhibited IL-1 β , IL-6, and nitric oxide induced by the lipopolysaccharide of *Porphyromonas gingivalis* in RAW 264.7 cells. The M157-W also inhibited the production of IL-1 β and IL-8 in human periodontal ligament cells. Treatment with M157-W suppressed the phosphorylation of mitogen-activated protein kinases as well as the activation of nuclear factor- κ B in RAW 264.7 cells stimulated by *P. gingivalis* lipopolysaccharide. Furthermore, M157-W dose-dependently inhibited *Streptococcus mutans* biofilm, whereas unfermented whey did not inhibit the biofilm. Treatment with M157-W significantly suppressed *gtfB*, *gtfC*, and *gtfD* gene expression in *S. mutans* compared with the control (0 μ g/mL), indicating that M157-W inhibits *S. mutans* biofilm formation by reducing the synthesis of extracellular polymeric substances. Collectively, these results suggest that M157-W has antiinflammatory and antibiofilm activities against oral pathogenic bacteria.

Key words: whey, fermentation, *Enterococcus faecalis*, oral disease

INTRODUCTION

Milk predominantly consists of 2 major proteins, casein and whey, which account for approximately 80 and 20% of the total protein in milk, respectively (Zhou et al., 2015). Although whey was first considered a by-product of cheese production, it is now recognized as a potential source of bioactive components, such as α -LA, β -LG, glycomacropeptide, immunoglobulins, lac-

toferrin, and serum albumin (Madureira et al., 2007). Whey contains several bioactive constituents that promote health; therefore, it is used in various commercial food and dairy products (Minj and Anand, 2020). Peptides derived from whey have been reported to exhibit antioxidant, antibacterial, immunomodulatory, and antihypertensive effects (Madureira et al., 2010). Furthermore, whey protein hydrolysates have been shown to exhibit potential antiinflammatory activity (Tavares et al., 2013). Although whey itself exhibits several beneficial effects on human health, fermentation of whey by bacteria, such as lactic acid bacteria, has also been reported to enhance its biological functions. For instance, whey fermented by various lactic acid bacteria including *Pediococcus*, *Lactobacillus*, and *Weissella* suppresses preadipocyte differentiation (Lee et al., 2021a,b). However, no studies are available about the effects of whey fermented by *Enterococcus faecalis* on oral pathogenic bacteria-mediated inflammation and biofilm as well as the associated underlying mechanisms.

Oral diseases have serious health and economic burdens (Peres et al., 2019). The most common oral diseases, periodontitis and dental caries, are crucially involved as a major cause of tooth loss in adults (Strauss et al., 2019). Periodontitis is a chronic inflammation caused by multiple pathogenic bacteria, including *Porphyromonas gingivalis*, which is considered the main pathogen involved in the development of chronic periodontitis. Lipopolysaccharide of *P. gingivalis* is a major virulence factor for chronic periodontitis because it has been shown to induce the release of inflammatory mediators, such as IL-1 β , IL-6, and nitric oxide (NO) (Nativel et al., 2017; Zhang et al., 2018). Dental caries is readily caused by formation of *Streptococcus mutans* biofilm accompanied with acidification and demineralization (Pitts et al., 2017). To enhance biofilm formation on tooth surfaces, *S. mutans* synthesizes glucosyltransferases that produce extracellular polymeric substances (EPS) by mixing insoluble and soluble glucans using carbohydrates such as sucrose (Bowen and Koo, 2011). The extracellular glucan matrix is the most

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important component that enhances *S. mutans* biofilm by adhering to the tooth surface (Schilling and Bowen, 1992). Moreover, EPS in biofilm can provide *S. mutans* with microenvironments for the bacterial growth, metabolism, and survival to be more resistant to harsh environmental conditions (Simón-Soro and Mira, 2015).

Lactic acid bacteria have been used as a starter for fermented foods. During fermentation, enzymatic reactions are associated with the production of metabolites such as acetaldehyde, hydrogen peroxide, organic acids, and peptides (García-Cano et al., 2019). Moreover, many lactic acid bacteria function as probiotics exhibiting several health benefits, such as intestinal health improvement, immune response enhancement, and cancer prevention (Kechagia et al., 2013). Some lactic acid bacteria are also beneficial in preventing and treating oral diseases. *Lactobacillus paracasei* and *L. salivarius* have been shown to exhibit antibacterial activities against *P. gingivalis* and *S. mutans* (Lai et al., 2021). Furthermore, probiotic strains of *Lactobacillus* and *Bifidobacterium* have been reported to reduce the adhesion and invasion of *P. gingivalis* to gingival epithelial cells, thereby preventing biofilm formation (Albuquerque-Souza et al., 2019; Ishikawa et al., 2020). Mendi et al. (2016) also showed that *L. rhamnosus* inhibited *P. gingivalis*-induced IL-8 expression.

Although biotransformation of whey by lactic acid bacteria has been reported to demonstrate inhibitory effects on the differentiation of 3T3-L1 preadipocytes in vitro (Lee et al., 2021a,b), the biological activities of biotransformed whey against pathological microorganisms have not been widely documented. Among the lactic acid bacteria, enterococci including *E. faecalis* have been extensively studied as potential candidates of probiotics (Hanchi et al., 2018). In addition, they are also involved in the fermentation activity of dairy products (Hanchi et al., 2014). Since it is known that *E. faecalis* has beneficial effects on improving human health during fermentation, we investigated the inhibition *P. gingivalis* LPS-induced inflammatory responses and biofilm formation by *S. mutans* using whey fermented by *E. faecalis* M157.

MATERIALS AND METHODS

Fermentation of Whey by *E. Faecalis*

Enterococcus faecalis M157 was grown in de Man, Rogosa, and Sharpe broth (Neogen) at 37°C for 24 h. Whey powder was obtained from Samik Dairy and Food Co. Ltd., and 3 g of whey powder was suspended in 27 mL of distilled water. To avoid bacterial contamination, the whey suspension was immersed in a

water bath at 80°C for 1 min. During pasteurization, the whey suspension was gently rotated several times. Bacterial contamination was determined by plating on nutrient agar. The whey suspension was then inoculated with *E. faecalis* M157 and incubated at 37°C for 24 h. After the incubation, the whey suspension was filtered to remove bacteria and the cell-free supernatants were obtained, evaporated, and dried using a spray dryer (B-290, Buchi). The whey fermented by *E. faecalis* M157 (**M157-W**; 1 mg) was resuspended in 1 mL of distilled water, centrifuged, and the supernatant was collected and adjusted to pH 6.5 with 1 N NaOH. M157-W was filtered using a 0.2- μ m syringe filter and stored at -80°C until used.

Cell Culture

The RAW 264.7 murine macrophage cells were purchased from American Type Cell Culture (ATCC) and maintained in Dulbecco's modified Eagle's medium (Welgene) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μ g/mL streptomycin (HyClone) at 37°C in a 5% CO₂-humidified incubator. Human periodontal ligament (PDL) cells were kindly provided by Seung Hyun Han (Seoul National University, Seoul, Korea) and grown in minimal essential medium, α modification (Welgene) containing 10% fetal bovine serum and the aforementioned antibiotics. The RAW 264.7 cells and PDL cells were treated with appropriate concentrations of M157-W (0, 0.1, 1, 10, or 100 μ g/mL and 100 μ g/mL, respectively) with or without 1 μ g/mL of *P. gingivalis* LPS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay did not show any significant difference in the viability of RAW 264.7 cells and PDL cells (data not shown). After treatment, the expression of IL-1 β , IL-6, and NO in RAW 264.7 cells and IL-1 β , IL-6, and IL-8 in PDL cells was determined. In addition, RAW 264.7 cells were treated with unfermented whey (0, 0.1, 1, 10, and 100 μ g/mL) with or without 1 μ g/mL of *P. gingivalis* LPS for 3 h.

Quantitative Reverse-Transcription PCR to Determine the Expression Levels of Inflammatory Mediators

To examine whether M157-W suppresses *P. gingivalis* LPS-induced inflammatory responses, RAW 264.7 cells were co-treated with *P. gingivalis* LPS (1 μ g/mL) and M157-W (0, 0.1, 1, 10, or 100 μ g/mL) for 3 h. Total RNA was then extracted from the cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Subsequently, the total RNA was

reverse transcribed to cDNA using random hexamers and reverse transcriptase (Promega). Samples were amplified by quantitative reverse-transcription PCR (qRT-PCR; Applied Biosystems) using the SYBR Green Real Time PCR master mix (Toyobo), according to the manufacturer's instructions. Expression levels of specific genes responsible for inflammatory responses, such as *IL1B*, *IL6*, and inducible nitric oxide synthase (*iNOS*, encoded by *NOS2*) were amplified under the following PCR conditions: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 55°C for 31 s. The primer sequences for *IL1B*, *IL6*, and *NOS2* were as follows: *IL1B*, forward, 5'-CTCACAAGCAGAGCACAAGC-3' and reverse, 5'-TCTTGGCCGAGGACTAAGGA-3'; *IL6*, forward, 5'-TCCTACCCCAATTTCCAATGCT-3' and reverse, 5'-TCTGACCACAGTGAGGAATGTC-3'; *NOS2*, forward, 5'-CATTGATCTCCGTGACAGCC-3' and reverse, 5'-CATGCTACTGGAGGTGGGTG-3'; *ACTB*, forward, 5'-TACAGCTTACCACCACAGC-3' and reverse, 5'-GGAAAAGAGCCTCAGGGCAT-3'.

qRT-PCR to Determine Expression Levels of *S. mutans* Biofilm-Associated Genes

To investigate the inhibitory effect of M157-W on biofilm formation by *S. mutans*, gene expression levels of glucosyltransferases (*gtf*) were determined by qRT-PCR. *Streptococcus mutans* (1×10^7 cfu/mL) was cultured with and without M157-W (0.001, 0.01, 0.1, or 1 mg/mL) in brain heart infusion (BHI) broth (Becton Dickinson) containing 0.05% sucrose at 37°C for 24 h. For the control, *S. mutans* was also cultured with unfermented whey (1 mg/mL) under the same conditions. After centrifugation, bacterial pellets were resuspended in TRIzol reagent (Invitrogen) and transferred to tubes containing High Impact Zirconium Beads (Benchmark Scientific) and homogenized using a BeadBug microtube homogenizer (Benchmark Scientific) at 4°C for 100 s to lyse bacteria. Subsequently, total RNA was extracted from bacteria according to the manufacturer's instructions, and reverse transcribed into cDNA as described above. Amplifications of *gtfB*, *gtfC*, and *gtfD* were conducted under the same PCR conditions as those described above. The relative expression levels of *gtf* genes were analyzed using the $2^{-\Delta\Delta C_t}$ method with normalization against the 16S rRNA. The primer sequences of *gtf* genes were as follows: *gtfB*, forward, 5'-AGCAATGCAGC-CAATCTACAAAT-3' and reverse, 5'-ACGAACCTT-GCCGTTATTGTCA-3'; *gtfC*, forward, 5'-CTCAAC-CAACCGCCACTGTT-3' and reverse, 5'-GGTTAAC-GTCAAATTAGCTGTATTAGC-3'; *gtfD*, forward, 5'-CACAGGCAAAAGCTGAATTACA-3' and reverse, 5'-GATGGCCGCTAAGTCAACAG-3'; 16S rRNA,

forward, 5'-CCTACGGGAGGCAGCAGTAG-3' and reverse, 5'-CAACAGAGCTTTACGATCCGAAA-3'.

Determination of Cytokine and Nitric Oxide Production

The RAW 264.7 cells (5×10^5 cells/mL) or PDL cells (2.5×10^5 cells/mL) were plated onto 48-well culture plates and treated with various concentrations of M157-W (0, 0.1, 1, 10, or 100 µg/mL or 100 µg/mL, respectively) or unfermented whey (100 µg/mL) in the presence or absence of *P. gingivalis* LPS (1 µg/mL) for the indicated time periods. Subsequently, culture supernatants were collected and IL-1β, IL-6, and IL-8 secretions were determined using a commercial ELISA kit (R&D Systems) according to the manufacturer's instructions. To determine nitric oxide production, the culture supernatants were mixed with the same volume of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 5 min. Then, the optical density was measured at 540 nm using a microtiter plate reader (Allsheng) with NaNO₂ as the standard.

Western Blot Analysis for the Assessment of MAPK and NF-κB

To examine whether mitogen-activated protein kinase (MAPK) and NF-κB are involved in the inhibitory effect of M157-W on *P. gingivalis* LPS-induced inflammatory responses, RAW 264.7 cells (5×10^5 cells/mL) were treated with M157-W in the presence or absence of *P. gingivalis* LPS (1 µg/mL) for 30 min. Cell lysates were obtained using a lysis buffer (1 M HEPES, 1 M NaCl, 1% IGEPAL-CA 630, 0.75% sodium deoxycholate, 10% glycerol) containing proteinase inhibitors. Protein samples (20 µg) were separated by SDS-PAGE on a 10% gel and then electro-transferred onto a PVDF membrane (Millipore). After blocking the membrane with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated with specific primary antibodies against p38 kinase, phospho-p38 kinase, extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun-N-terminal kinase (JNK), phospho-JNK, or IκBα (Cell Signaling Technology) at 4°C overnight. The membrane was then washed with Tris-buffered saline containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody at room temperature for 1 h. Immunoreactive bands were detected using an enhanced chemiluminescence reagent (Dyne Bio) and visualized using the C-DiGit blot scanner (Li-Cor Bioscience).

Determination of *S. mutans* Biofilm

The overnight culture of *S. mutans* was diluted to 1×10^7 cfu/mL in BHI broth containing 0.05% sucrose, and 100 μ L of *S. mutans* suspension was then transferred to 96-well microtiter plates in various concentrations of M157-W or unfermented whey (0, 0.001, 0.01, 0.1 and 1 mg/mL; 100 μ L). To evaluate the inhibitory effect of M157-W on *S. mutans* biofilm, the bacteria were incubated at 37°C for 24 h. Subsequently, the bacteria were gently washed with PBS to remove unattached bacteria and then stained with 0.1% crystal violet for 30 min. After excess stain was removed by gently washing with PBS, the stained *S. mutans* biofilm was dissolved in 0.1% acetic acid and 95% ethanol and its absorbance was measured at 595 nm using a microtiter plate reader (Allsheng).

Confocal Laser Scanning Microscopy for *S. mutans* Biofilm in the Presence of M157-W

Streptococcus mutans (1×10^7 cfu/mL) was cultured on a confocal dish (SPL Life Science) in BHI broth containing 0.05% sucrose to investigate biofilm formation with or without M157-W (1 mg/mL) at 37°C for 24 h. Subsequently, the supernatants were discarded and the unattached bacteria were removed by gently washing with PBS. *Streptococcus mutans* biofilm was stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes) according to the manufacturer's instructions. The stained biofilm was visualized using a confocal laser scanning microscope (Eclipse Ti-E, Nikon).

Statistical Analysis

Data presented in this study are expressed as mean \pm standard deviation of triplicate samples from 3 independent experiments. Statistical significance between groups was determined by either an unpaired 2-tailed *t*-test using GraphPad Prism 5 (GraphPad Software Inc.) or one-way ANOVA performed using the IBM SPSS Statistics 23 software (IBM Corp.).

RESULTS AND DISCUSSION

Fermentation of Whey by *E. faecalis* M157 Suppresses *P. gingivalis* LPS-Induced Inflammatory Responses

The M157-W significantly ($P < 0.05$) inhibited the mRNA expression of *P. gingivalis* LPS-induced *IL1B* (Figure 1A), *IL6* (Figure 1C), and *NOS2* (Figure 1E) in a dose-dependent manner. The highest concentration

of M157-W (100 μ g/mL) most effectively inhibited the mRNA expression of *IL1B*, *IL6*, and *NOS2* by approximately 80, 32, and 57%, respectively. Therefore, 100 μ g/mL of M157-W was used for further experiments. Treatment of RAW 264.7 cells with *P. gingivalis* LPS in the presence or absence of M157-W (100 μ g/mL) for various time periods (1, 3, 6, 12, and 24 h) revealed that M157-W significantly ($P < 0.05$) inhibited the mRNA expression of *IL1B* (Figure 1B), *IL6* (Figure 1D), and *NOS2* (Figure 1F) with 3 and 6 h of incubation. However, M157-W did not inhibit the mRNA expression of *IL1B*, *IL6*, and *NOS2* after 12 and 24 h of incubation, suggesting that M157-W effectively inhibits expression of genes responsible for inflammatory responses at early time points in RAW 264.7 cells. The inhibition of inflammatory responses by M157-W was confirmed at the protein level by treating RAW 264.7 cells with *P. gingivalis* LPS in either the presence or absence of M157-W and determining the amounts of IL-1 β , IL-6, and NO production. Consistent with the results of mRNA expression, the secretion of IL-1 β and IL-6 was significantly ($P < 0.05$) reduced by M157-W treatment (Figures 2A and 2C, respectively). The NO production was dose-dependently inhibited by M157-W treatment (Figure 2E). The inhibition was temporally investigated by treating RAW 264.7 cells with *P. gingivalis* LPS or M157-W (100 μ g/mL), or both, for 1, 3, 6, 12, and 24 h. The secretion of IL-1 β was significantly ($P < 0.05$) inhibited in the presence of M157-W at 12 and 24 h (Figure 2B), whereas that of IL-6 was significantly ($P < 0.05$) reduced at 24 h, but not at 12 h (Figure 2D). Nitric oxide production also decreased at 12 and 24 h in the presence of M157-W (Figure 2F). These results suggest that M157-W most effectively reduced the protein levels of IL-1 β , IL-6, and NO at 24 h of incubation. During an inflammatory process, several inflammatory mediators are generated, among which IL-1 β has been demonstrated to be one of the most important inflammatory cytokines, produced by macrophages, that are stimulated by LPS and lead to cell and tissue damage (Won et al., 2006). Interleukin-6 has the multiple effects on the immune system, such as hematopoiesis (Van Snick, 1990). Synthesized by iNOS, NO is a common molecule that is crucially involved in the pathogenesis of LPS-induced inflammation (Won et al., 2006). The excessive production of IL-1 β can lead to the acceleration of periodontal tissues (Cheng et al., 2020). In addition, IL-1 β and IL-6 induce matrix-metalloproteinase-1 expression, resulting in periodontal tissue destruction (Sawada et al., 2013). Nitric oxide mediates the pathological effects of bacterial LPS and inflammatory cytokines, such as IL-1 β , indicating that NO may be involved in the development of periodontitis (Wang et al., 2019). Our findings showed that

M157-W effectively suppresses inflammatory mediators, thereby relieving inflammation. To evaluate the whey fermented by *E. faecalis* M157, the antiinflammatory activity of unfermented whey was also examined. As shown in Figure 3, unfermented whey appears to

inhibit the mRNA expression of *IL1B*, *IL6*, and *NOS2* in RAW 264.7 cells stimulated with *P. gingivalis* LPS. However, the inhibitory effects of unfermented whey (12, 23, and 22% inhibition, respectively) at 100 $\mu\text{g}/\text{mL}$ on *IL1B* (Figure 3A), *IL6* (Figure 3B), and *NOS2* (Fig-

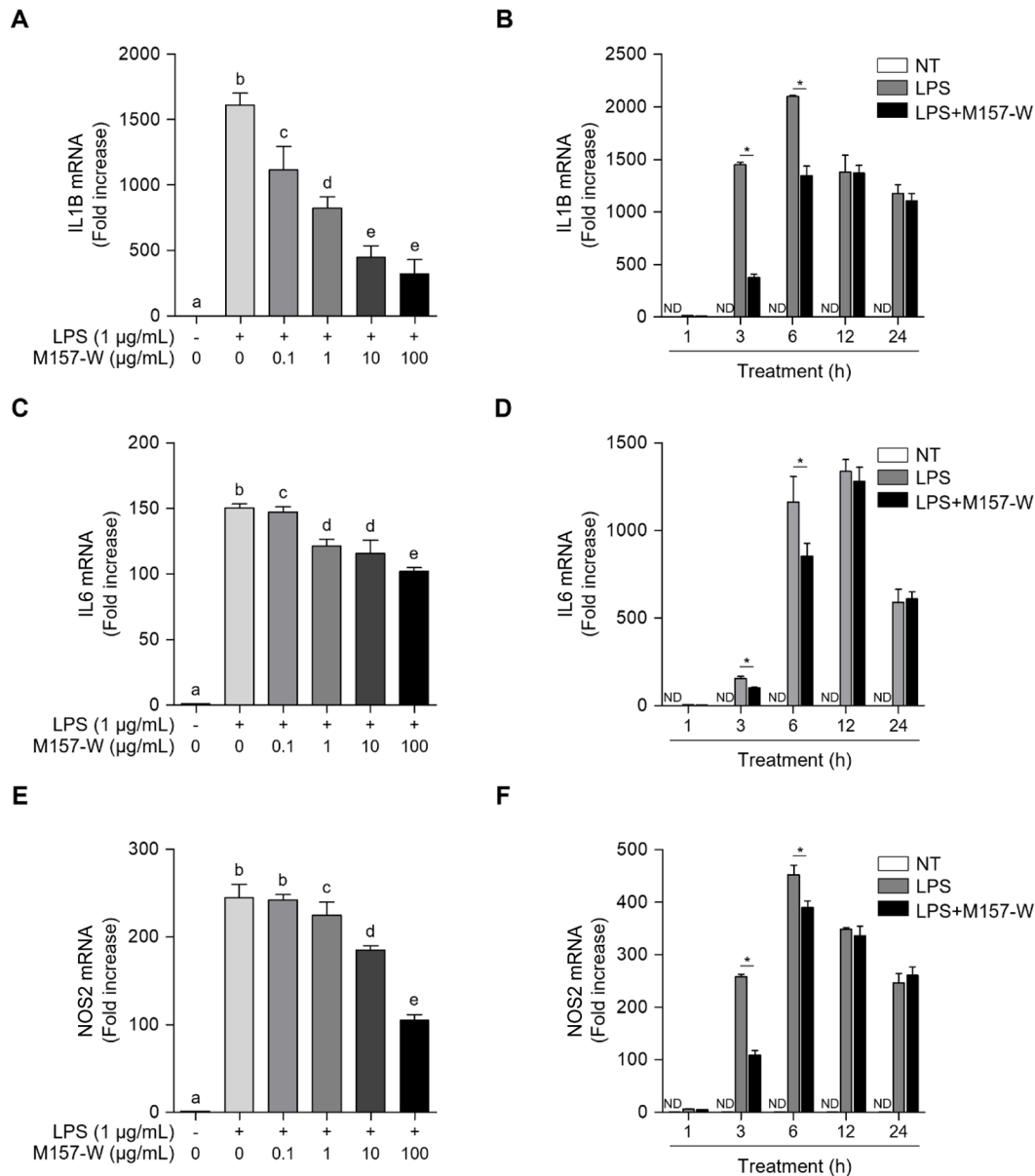


Figure 1. Inhibitory effects of whey fermented by *Enterococcus faecalis* M157 (M157-W) on the mRNA expression levels of *Porphyromonas gingivalis* LPS-induced proinflammatory mediators in RAW 264.7 cells. The RAW 264.7 cells were co-incubated with *P. gingivalis* LPS (1 $\mu\text{g}/\text{mL}$) and various concentrations of M157-W. After 3 h of incubation, mRNA expression of *IL1B* (A), *IL6* (C), and inducible nitric oxide synthase (*NOS2*; E) was determined using quantitative reverse-transcription PCR (qRT-PCR). The control was the treatment with *P. gingivalis* LPS only (1 $\mu\text{g}/\text{mL}$). After RAW 264.7 cells were co-incubated with *P. gingivalis* LPS (1 $\mu\text{g}/\text{mL}$) and M157-W (100 $\mu\text{g}/\text{mL}$) for 1, 3, 6, 12, and 24 h, mRNA expression levels of *IL1B* (B), *IL6* (D), and *NOS2* (F) were determined using qRT-PCR. The control was the treatment with *P. gingivalis* LPS only (1 $\mu\text{g}/\text{mL}$) at each time point. NT and ND denote not treated and not detected, respectively. Significance between groups was determined by one-way ANOVA (A, C, and E) and 2-tailed *t*-test (B, D, and F). The results are presented as mean \pm standard deviation. Different lowercase letters (a–e) or an asterisk indicate a significant difference with $P < 0.05$.

ure 3C) mRNA expression were much lower than those of M157-W. Moreover, unfermented whey (100 µg/mL) slightly inhibited IL-1β and NO production (Figure

3D and 3F, respectively), whereas IL-6 production was not inhibited by the treatment with unfermented whey (Figure 3E). Therefore, fermented whey exhibits more

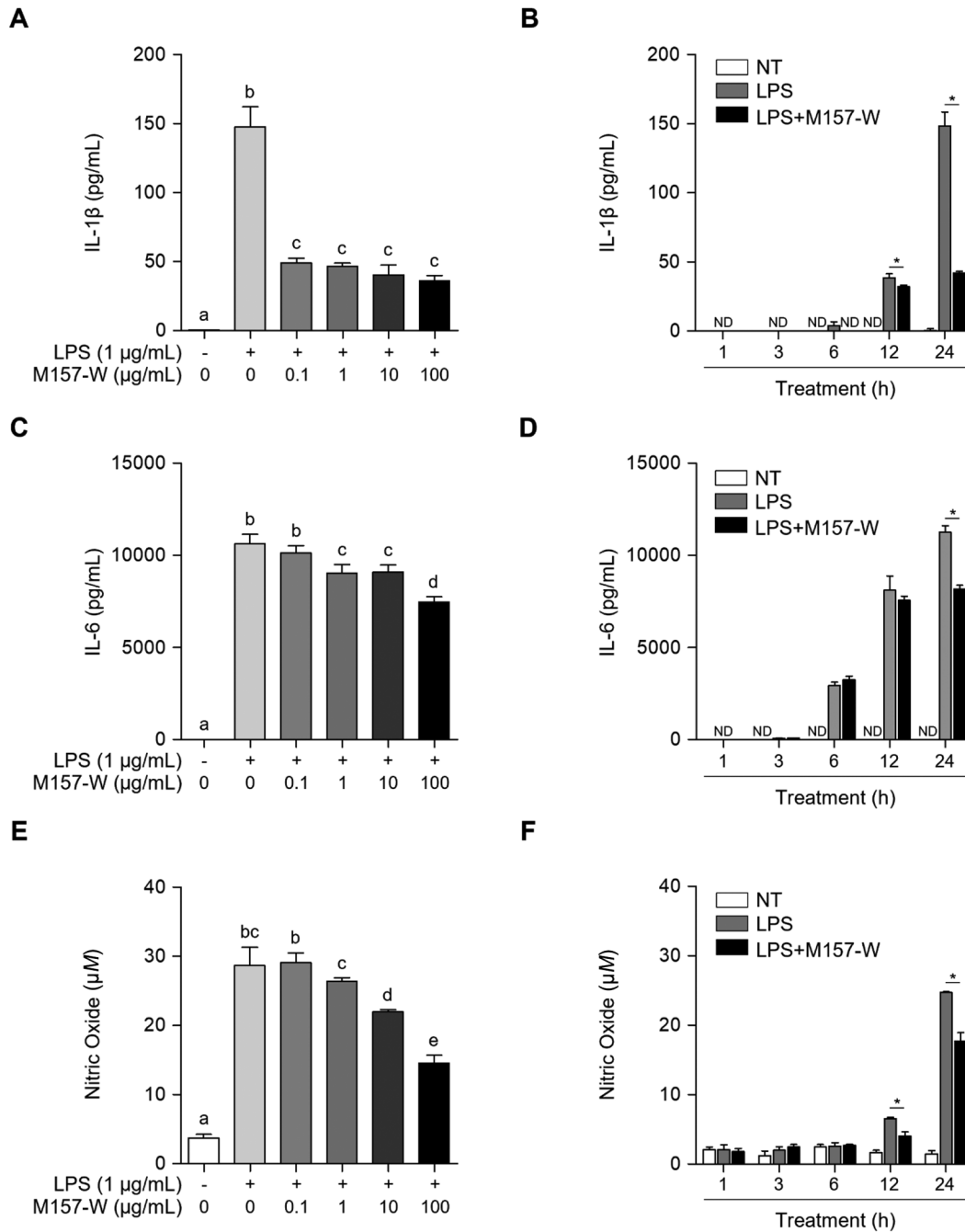


Figure 2. Inhibitory effects of whey fermented by *Enterococcus faecalis* M157 (M157-W) on the production of *Porphyromonas gingivalis* LPS-induced proinflammatory mediators in RAW 264.7 cells. RAW 264.7 cells were co-incubated with *P. gingivalis* LPS (1 µg/mL) and various concentrations of M157-W. After 24 h of incubation, protein levels of IL-1β (A) and IL-6 (C) were determined using ELISA, and NO production (E) was measured using the Griess reagent. The control was the treatment with *P. gingivalis* LPS only (1 µg/mL). After RAW 264.7 cells were co-incubated with *P. gingivalis* LPS (1 µg/mL) and M157-W (100 µg/mL) for 1, 3, 6, 12, and 24 h, protein levels of IL-1β (B) and IL-6 (D) were determined using ELISA, and NO production (F) was measured using the Griess reagent. The control was the treatment with *P. gingivalis* LPS only (1 µg/mL) at each time point. NT and ND denote not treated and not detected, respectively. Significance between groups was determined by one-way ANOVA (A, C, and E) and 2-tailed *t*-test (B, D, and F). The results are presented as mean ± standard deviation. Different lowercase letters (a-e) and an asterisk indicate a significant difference with *P* < 0.05.

effective antiinflammatory activities against *P. gingivalis* LPS. In fact, peptides derived from whey have been shown to exhibit antiinflammatory potential. Two purified peptides, Asp-Tyr-Lys-Lys-Tyr and Asp-Gln-Trp-Leu, have been reported to exhibit strong inhibitory effects on the mRNA expression of *IL1B*, tumor necrosis factor- α , and cyclooxygenase-2 in RAW 264.7 cells stimulated by *Escherichia coli* O111:B4 LPS (Ma et al., 2016). It has been established that *E. coli* LPS exclusively activates toll-like receptor (TLR)4 on the cell surface, leading to inflammatory responses (Netea et al., 2002). In contrast, *P. gingivalis* LPS predominantly activates TLR2 and poorly activates TLR4 (Martin et al., 2001), suggesting that inflammatory consequences may be different by the exposure to *E. coli* LPS and *P. gingivalis* LPS. Furthermore, unlike the previous study by Ma et al. (2016) that identified 2 peptides with an-

tiinflammatory potential derived from whey, our study has not identified specific peptides responsible for the inhibition of *P. gingivalis* LPS-induced inflammatory responses. However, it can be deduced that peptides released from whey during fermentation by *E. faecalis* M157 could be associated with the inhibition of *P. gingivalis* LPS-induced inflammatory responses.

Fermentation of Whey by *E. faecalis* M157 Suppresses MAPK Phosphorylation and NF- κ B Activation

As shown in Figure 4A, *P. gingivalis* LPS induced the phosphorylation of MAPK such as p38, ERK, and JNK in RAW 264.7 cells; in contrast, M157-W treatment inhibited the phosphorylation of p38, ERK, and JNK, suggesting that *P. gingivalis* LPS-mediated inflamma-

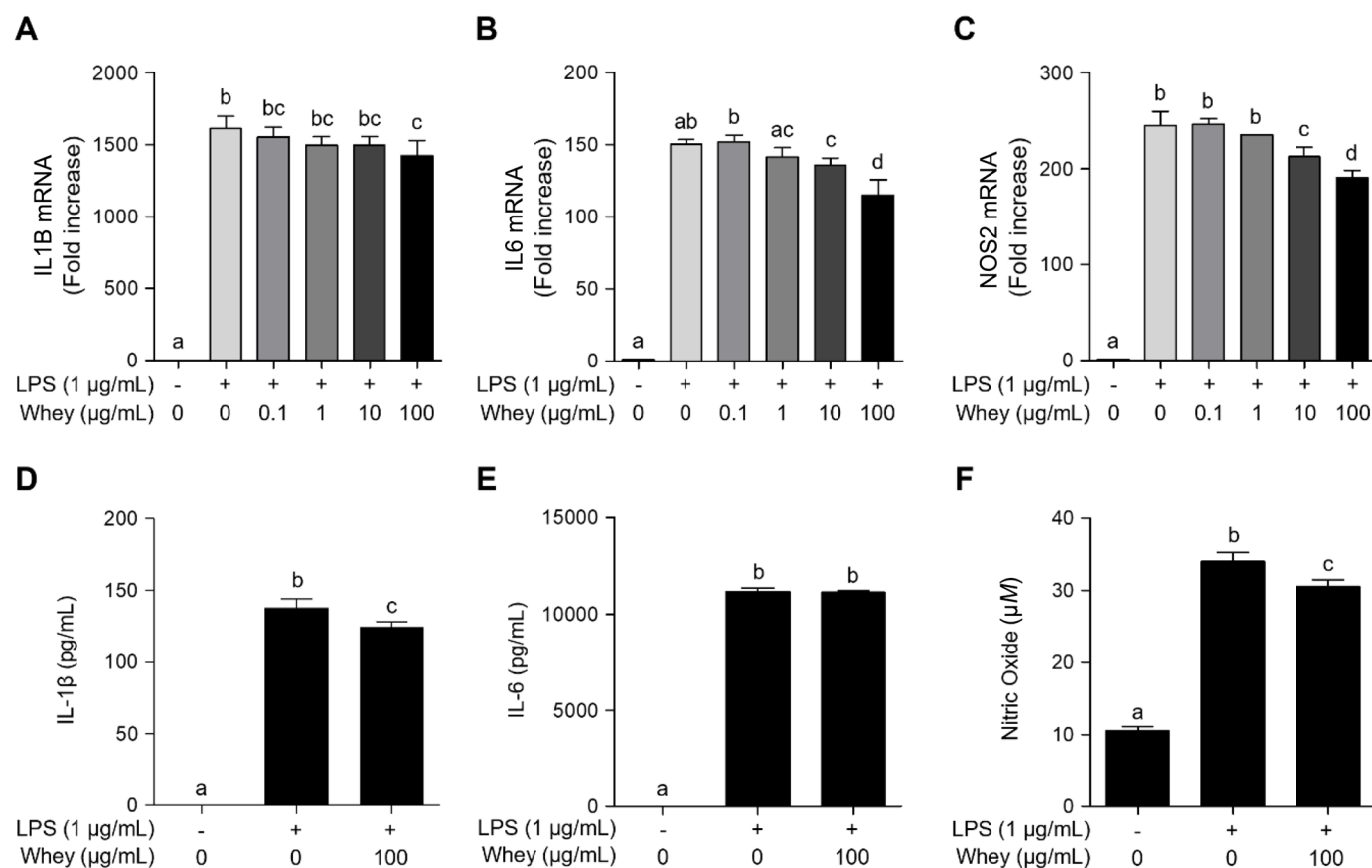


Figure 3. Effects of unfermented whey on the inhibition of *Porphyromonas gingivalis* LPS-induced inflammatory mediators in RAW 264.7 cells. The RAW 264.7 cells were co-incubated with *P. gingivalis* LPS (1 μ g/mL) with various concentrations of unfermented whey for 3 h. mRNA expression levels of *IL1B* (A), *IL6* (B), and inducible nitric oxide synthase (*NOS2*; C) were determined using quantitative reverse-transcription PCR (qRT-PCR). After RAW 264.7 cells were co-incubated with *P. gingivalis* LPS (1 μ g/mL) and unfermented whey (100 μ g/mL) for 24 h, protein levels of IL-1 β (D) and IL-6 (E) were determined using ELISA, and NO production (F) was measured using the Griess reagent. The control was the treatment with *P. gingivalis* LPS only (1 μ g/mL). Significance between groups was determined by one-way ANOVA. The results are presented as mean \pm standard deviation. Different lowercase letters (a-d) indicate a significant difference with $P < 0.05$.

tory responses are reduced by decreasing the phosphorylation of MAPK. Furthermore, M157-W restored *P. gingivalis* LPS-induced $\text{I}\kappa\text{B}\alpha$ degradation in a dose-dependent manner (Figure 4B). These results indicate that M157-W suppresses *P. gingivalis* LPS-induced activation of MAPK and NF- κB , thereby attenuating the inflammatory responses in RAW 264.7 cells. The stimulation of cells with *P. gingivalis* LPS increased the production of proinflammatory cytokines, such as IL-1 β and IL-6, through the upregulation of MAPK and

NF- κB (Sun et al., 2020). NF- κB is crucially involved in inflammatory reactions; the inhibitors of NF- κB , including $\text{I}\kappa\text{B}\alpha$, are important in downregulating NF- κB activation (Wang et al., 2020). Several studies have confirmed that MAPK and NF- κB signaling pathways are involved in periodontitis (Liang et al., 2014; Kim et al., 2018). Ginsenoside Rb3 has been reported to attenuate periodontitis by downregulating MAPK and NF- κB signaling pathways (Sun et al., 2020). Moreover, panduratin A isolated from *Boesenbergia pandurata* has

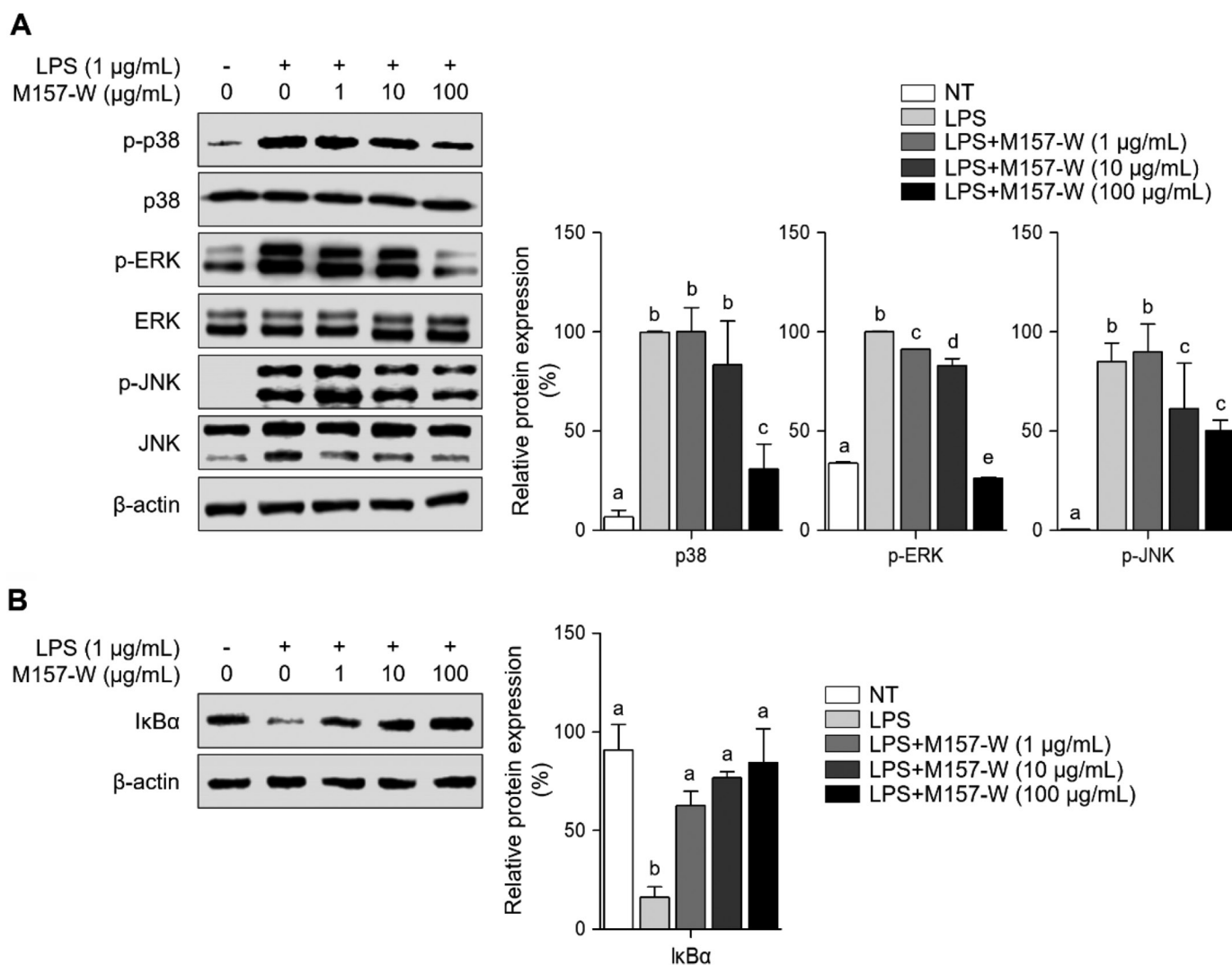


Figure 4. Inhibitory effects of whey fermented by *Enterococcus faecalis* M157 (M157-W) on mitogen-activated protein kinase (MAPK) phosphorylation and NF- κB activation in RAW 264.7 cells. The RAW 264.7 cells were co-incubated with *Porphyromonas gingivalis* LPS (1 $\mu\text{g}/\text{mL}$) and various concentrations of M157-W for 30 min. The MAPK phosphorylation, p38 kinase, phospho-p38 kinase, extracellular signal-regulated kinase (ERK), phospho-ERK, c-Jun-N-terminal kinase (JNK), and phospho-JNK (A) and $\text{I}\kappa\text{B}\alpha$ degradation (B) were determined using western blot analysis. The control was the treatment with *P. gingivalis* LPS only (1 $\mu\text{g}/\text{mL}$). The relative expression levels of each MAPK and $\text{I}\kappa\text{B}\alpha$ are represented as mean \pm standard deviation obtained from 3 independent experiments. Significance between groups was determined by one-way ANOVA. NT denotes not treated. The results are presented as mean \pm standard deviation. Different lowercase letters (a–e) indicate a significant difference with $P < 0.05$.

been shown to suppress MAPK signaling pathways in periodontitis-associated inflammatory responses (Kim et al., 2018). Our study also suggests that M157-W can demonstrate an antagonistic effect on *P. gingivalis* LPS-induced inflammatory responses by inhibiting MAPK and NF- κ B signaling pathways.

Whey Fermented by *E. faecalis* M157 Inhibits *P. gingivalis* LPS-Induced Inflammatory Responses in PDL Cells

To evaluate the inhibition of *P. gingivalis* LPS-induced inflammatory responses in PDL cells by M157-W, the cells were co-treated with M157-W and *P. gingivalis* LPS, and the levels of IL-1 β , IL-6, and IL-8 secretion were measured. As shown in Figure 5A, *P. gingivalis* LPS significantly ($P < 0.05$) induced IL-1 β secretion, which was moderately decreased in the presence of M157-W. However, unfermented whey failed to inhibit *P. gingivalis* LPS-induced IL-1 β secretion. *Porphyromonas gingivalis* LPS induced IL-6 secretion, whereas M157-W and unfermented whey significantly ($P < 0.05$) inhibited IL-6 secretion (Figure 5B). Moreover, *P. gingivalis* LPS induced IL-8 secretion in PDL cells, whereas M157-W treatment significantly ($P < 0.05$) inhibited this increased IL-8 secretion, unlike unfermented whey (Figure 5C). These results indicate that M157-W partially attenuates inflammatory responses in PDL cells. In addition to *P. gingivalis*, several bacteria including *Streptococcus gordonii*, a particularly harmful pathogen, are involved in the development of periodontitis. Kim et al. (2017) showed that *S. gordo-*

nii lipoproteins increase IL-8 production in PDL cells, which crucially contributes to inflammatory periodontitis. Our results revealed that *P. gingivalis* LPS-induced proinflammatory mediators were downregulated by M157-W, suggesting that M157-W can decrease inflammatory periodontitis by partially inhibiting the secretion of IL-1 β and IL-8 in human PDL cells.

Whey Fermented by *E. faecalis* M157 Suppresses *S. mutans* Biofilm

The effect of M157-W on the biofilm formation by *S. mutans* was investigated using crystal violet staining. Our results showed that M157-W dose-dependently inhibits *S. mutans* biofilm formation (Figure 6A). In contrast, unfermented whey did not inhibit *S. mutans* biofilm (Figure 6B). As shown in Figure 6C, confocal laser scanning microscopy confirmed that *S. mutans* biofilm was less dense in the presence of M157-W than in its absence. A potential probiotic strain, *L. salivarius*, reduces the biofilm formation by *S. mutans* (Wu et al., 2015). More recently, *L. plantarum* showed an inhibitory effect on the biofilm formation and the growth of *S. mutans* (Zhang et al., 2020). Although the previous studies have reported that probiotics exhibit antibiofilm activity against *S. mutans*, whey fermentation by lactic acid bacteria has not elucidated the antibiofilm activity against *S. mutans*. Our results suggested that M157-W inhibits *S. mutans* biofilm. In addition, the genes *gtfB*, *gtfC*, and *gtfD* are associated with EPS synthesis (Koo et al., 2010). For instance, deleting *gtfB* and *gtfC* in *S. mutans* has been shown to result in the disruption of

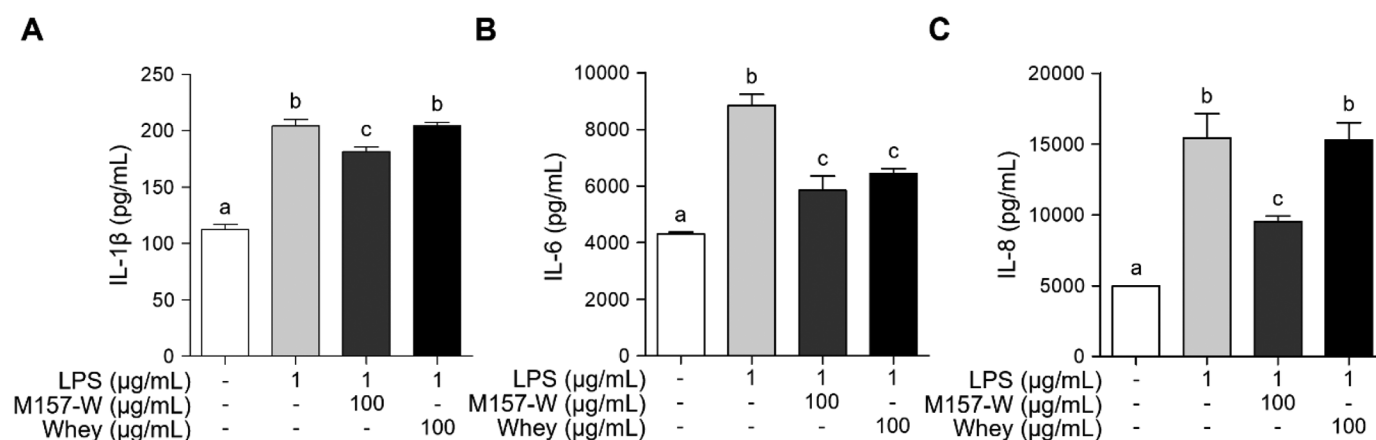


Figure 5. Effects of whey fermented by *Enterococcus faecalis* M157 (M157-W) on *Porphyromonas gingivalis* LPS-induced proinflammatory cytokines in PDL cells. PDL cells were treated with either M157-W (100 μ g/mL) or unfermented whey (100 μ g/mL) in the presence of *P. gingivalis* LPS (1 μ g/mL) for 24 h. The protein levels of IL-1 β (A), IL-6 (B), and IL-8 (C) were determined using ELISA. The control was the treatment with *P. gingivalis* LPS only (1 μ g/mL). Significance between groups was determined by one-way ANOVA. The results are presented as mean \pm standard deviation. Different lowercase letters (a–c) indicate a significant difference with $P < 0.05$.

microcolony and biofilm formation (Fears et al., 2015). Therefore, *gtf* genes are essential for the development of *S. mutans* biofilm. Our results showed that M157-W dose-dependently inhibits the expression levels of *gtfB* (Figure 7A), *gtfC* (Figure 7B), and *gtfD* (Figure 7C). In contrast, unfermented whey (1 mg/mL) did not inhibit the expression levels of *gtfB* (Figure 7D), *gtfC* (Figure 7E), and *gtfD* (Figure 7F). Several studies have shown that antibiofilm compounds prevent *S. mutans* biofilm by regulating the expression levels of biofilm-

associated genes including *gtf* genes (Koo et al., 2006; Salles Branco-de-Almeida et al., 2011; Subramenium et al., 2015). Interpreted together with previous results, our results indicate that M157-W suppresses *S. mutans* biofilm formation by downregulating *gtf* genes.

CONCLUSIONS

In this study, we demonstrated that M157-W effectively inhibits *P. gingivalis* LPS-induced inflammatory

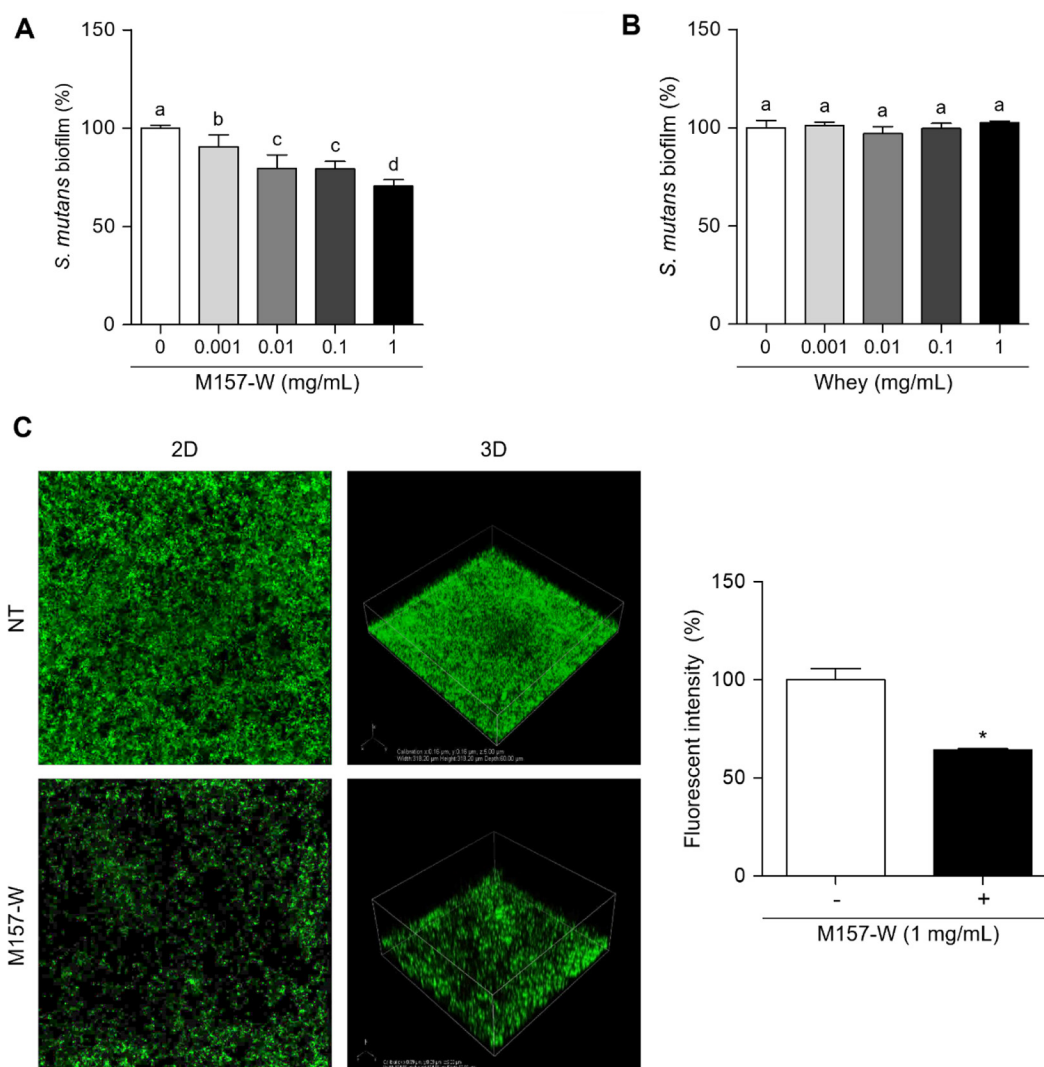


Figure 6. Antibiofilm activity of whey fermented by *Enterococcus faecalis* M157 (M157-W) against *Streptococcus mutans*. *Streptococcus mutans* was incubated with either different concentrations of M157-W (A) or unfermented whey (B; 0, 0.001, 0.01, 0.1, and 1 mg/mL) in 96-well culture plates for 24 h. *Streptococcus mutans* biofilm was examined using crystal violet staining. The control was the treatment without M157-W or unfermented whey. Significance between groups were determined by one-way ANOVA. (C) *S. mutans* biofilm in either the presence or absence of M157-W (1 mg/mL) was assessed using confocal laser scanning microscopy. Images displayed were selected from 1 of 3 similar results. The fluorescent intensity of images from *S. mutans* biofilm treated without M157-W (1 mg/mL) was assigned to 100%. Significance between groups was determined by 2-tailed *t*-test. NT denotes not treated. The results are presented as mean \pm standard deviation. Different lowercase letters (a–d) and an asterisk indicate a significant difference with $P < 0.05$.

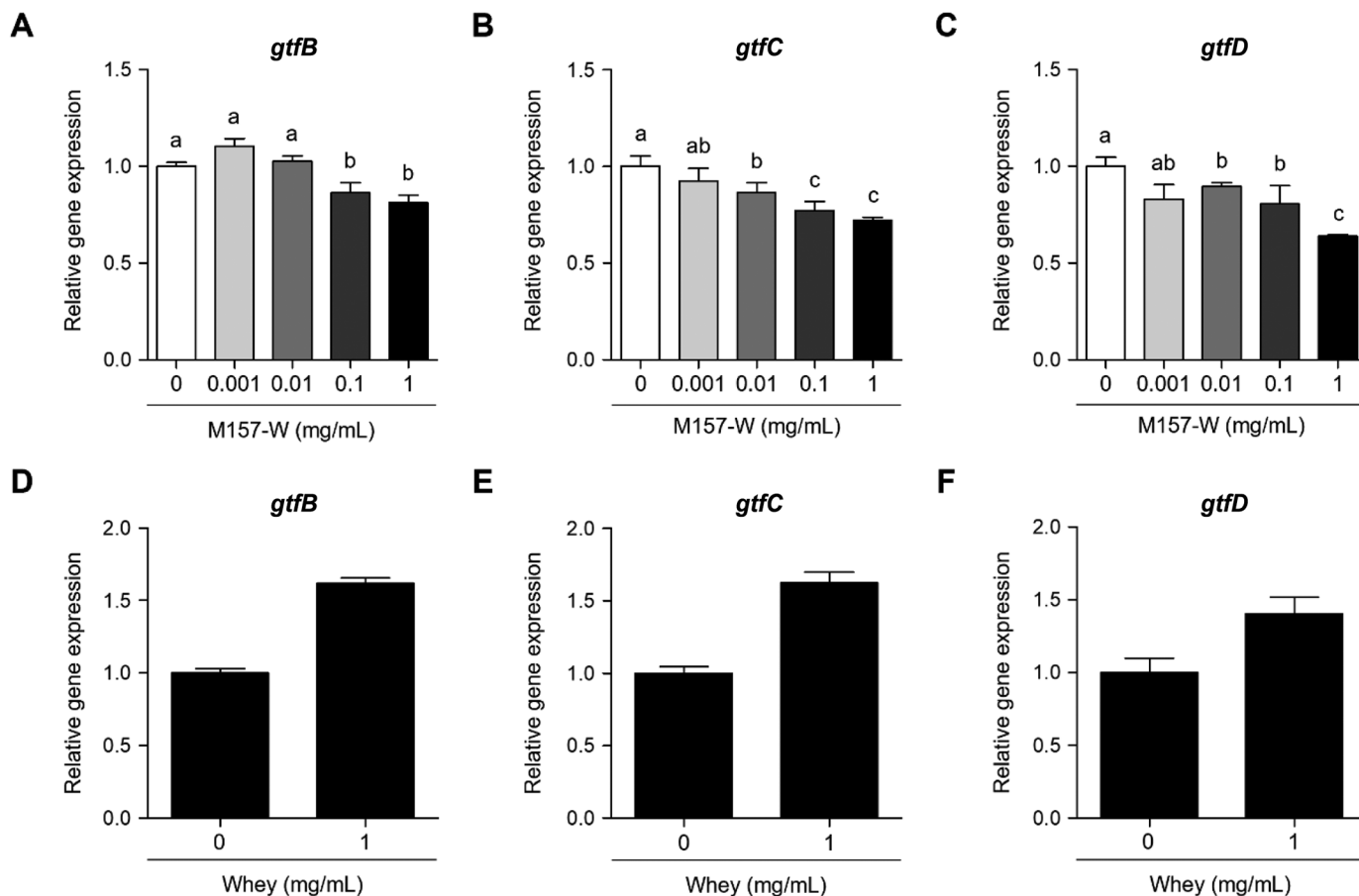


Figure 7. Effects of whey fermented by *Enterococcus faecalis* M157 on the expression levels of genes responsible for *Streptococcus mutans* biofilm formation. Expression levels of *gtfB* (A), *gtfC* (B), and *gtfD* (C) in *S. mutans* treated with the different concentrations of M157-W (0, 0.001, 0.01, 0.1, and 1 mg/mL) were determined using quantitative reverse-transcription PCR (qRT-PCR). Expression levels of *gtfB* (D), *gtfC* (E), and *gtfD* (F) in *S. mutans* treated with unfermented whey (1 mg/mL) were determined using qRT-PCR. The control was the treatment without M157-W or unfermented whey. Statistical significance between groups was determined by one-way ANOVA. The results are presented as mean \pm standard deviation. Different lowercase letters (a–e) indicate a significant difference with $P < 0.05$.

responses. In addition, M157-W moderately suppressed *S. mutans* biofilm formation that may be associated with the downregulation of genes responsible for EPS synthesis. Although the antiinflammatory effects of whey have been reported previously, the antiinflammatory and antibiofilm activities of fermented whey against oral pathogenic bacteria were demonstrated here for the first time. Although further studies are required to identify specific compounds in fermented whey that are involved in these antiinflammatory and antibiofilm activities, this study highlights the promising preventive and therapeutic potential of M157-W in the treatment of periodontitis and dental caries.

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