Inhibitory effect of *Lactococcus lactis* on the bioactivity of periodontopathogens

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*Lactococcus lactis* is a probiotic bacterium that produces various bacteriocins. Periodontopathogens induce inflammation and halitosis through the actions of lipopolysaccharide (LPS) and trypsin-like enzymes. The purpose of this study was to investigate the inhibitory effects of *L. lactis* on the bioactivity of periodontopathogens. To investigate the antimicrobial peptide of *L. lactis*, the spent culture medium (SCM) of *L. lactis* was treated with or without proteinase K after collection by centrifugation, and the antibacterial activity of SCM against periodontopathogens was assessed. To evaluate the neutralizing effect of *L. lactis* on halitosis, SCM of periodontopathogens was mixed with an *L. lactis* suspension, and the levels of volatile sulfur compounds (VSCs) were measured by gas chromatography. LPS from the periodontopathogens was extracted by an LPS extraction kit with little modification, and THP-1 cells as a monocytic cell line were treated with the extracted LPS in the presence or absence of UV-killed *L. lactis*. The production of inflammatory cytokines was analyzed by ELISA. The SCM of *L. lactis* exhibited antimicrobial activity against the periodontopathogens, whereas the proteinase K-treated SCM showed little antimicrobial activity. In addition, the *L. lactis* suspension had a neutralizing effect on the VSCs produced by periodontopathogens, and UV-killed *L. lactis* inhibited the production of IL-6 and TNF-α induced by the LPS. These results suggest that *L. lactis* may be a useful probiotic to prevent and treat periodontitis and halitosis.

Key Words: antibacterial activity; *L. lactis*; probiotics; neutralizing activity; periodontopathogens

Introduction

*Lactococcus lactis* is a Gram-positive and spherical-shaped facultative anaerobic bacterium (Brooijmans et al., 2007). This bacterium is widely used in the production of fermented dairy foods such as cheese, yogurt, and sour cream as a probiotic bacterium (Karpinski and Szkaradkiewicz, 2013; Kimoto-Nira et al., 2014; van Hylckama Vlieg et al., 2006). Also, *L. lactis* produces various bacteriocins including diacetin, lactococcin and nisin (Ali et al., 1995; Dussault et al., 2016; Holo et al., 1991), and these bacteriocins exhibit bactericidal or bacteriostatic effects on Gram-positive and Gram-negative pathogens (Ali et al., 1995; Arques et al., 2015). Furthermore, *L. lactis* antagonizes pathogenic bacteria in the host gut through its antimicrobial metabolites, such as hydrogen peroxide, acetaldehyde, and ethanol organic acids (Enan et al., 2013). This bacterium improves human health by providing nutritional benefits and helping to balance cholesterol and bile salts (Enan et al., 2013; Tanaka et al., 1999) and is therefore considered a beneficial bacterium.

Periodontitis is a chronic inflammatory condition of the gingiva with a polymicrobial etiology. *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* are directly associated with this condition and are therefore referred to as periodontopathogens or red complex bacteria (Socransky and Haffajee, 2002; Socransky et al., 1998). These Gram-negative obligate anaerobic bacteria exist within a biofilm that forms in subgingival pockets, with *Fusobacterium nucleatum* serving as a bridge bacterium to the supragingival biofilm, which consists mostly

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of streptococci (Socransky and Haffajee, 2005). The lipopolysaccharide (LPS) of these bacteria acts as an immune stimulator by inducing gingival inflammation and activating osteoclasts via Toll-like receptors (TLR2 or TLR4) that trigger the expression of various cytokines, which in turn causes alveolar bone resorption (Kim and Lee, 2014; Lee, 2015; Lee and Baek, 2013; Socransky et al., 1998). Another characteristic of periodontopathogens is their ability to induce halitosis, an oral malodor. Periodontopathogens secrete trypsin-like enzymes that produce volatile sulfur compounds (VSCs) such as hydrogen sulfide (H\(_2\)S), methyl mercaptan (CH\(_3\)SH), and dimethyl sulfide ((CH\(_3\))\(_2\)S) in the presence of methionine and cysteine in human serum protein (Lee and Baek, 2014). VSCs are responsible for halitosis.

This study investigated the effects of \(L.\) lactis on the periodontopathogens, \(F.\) nucleatum, \(P.\) gingivalis, \(T.\) forsythia, and \(T.\) denticola by focusing on their bioactivity; namely, their growth, induction of inflammation, and production of VSCs.

**Materials and Methods**

**Bacterial strain and culture conditions.** \(L.\) lactis HY449 was gratefully received from Yakult (Korea Yakult Com, Gyeonggi, Korea) and was cultivated in brain heart infusion (BHI) broth (BD Bioscience, San Jose, CA, USA) at 37°C under anaerobic conditions (5% H\(_2\), 10% CO\(_2\), and 85% N\(_2\)). \(F.\) nucleatum ATCC 25586 and \(P.\) gingivalis ATCC 33277 were cultured anaerobically with BHI broth supplemented with hemin (1 \(\mu\)g/ml) and vitamin K (0.2 \(\mu\)g/ml) at 37°C, anaerobically. \(T.\) forsythia ATCC 43037 and \(T.\) denticola ATCC 35405 were cultured in modified new oral spirochete (mNOS) broth (Lee et al., 2010) and tryptone-yeast extract-gelatin-volatile fatty acid-serum (TYGVS) broth respectively, at 37°C (Ohta et al., 1986) in an anaerobic atmosphere.

**Antibacterial activity of \(L.\) lactis against periodontopathogens.** The antimicrobial susceptibility of periodontopathogens to \(L.\) lactis was determined by a minimum inhibitory concentration assay in a microplate, according to methods recommended by Clinical and Laboratory Standards Institute (CLSI) (Hecht et al., 2007). Five milliliters of \(L.\) lactis (1 \(\times\) 10\(^7\) cells/ml) was inoculated into 50 ml fresh BHI broth, and the bacteria were cultivated for 24 h under aerobic conditions. The bacterial suspension was centrifuged at 7,000 \(\times\) g, and the supernatant (spent culture medium) was transferred into a new tube and then filtrated through a polyvinylidene fluoride filter (pore size 0.22 \(\mu\)m). To investigate susceptibility, 20–180 \(\mu\)l of BHI broth containing hemin (1 \(\mu\)g/ml) and vitamin K (0.2 \(\mu\)g/ml) was dispensed into each well (three rows) from the 10th column to the 1st column in a 96-well polystyrene plate (SPL Life Sciences, Gyeonggi, Korea) using a multi-channel pipette. After adding hemin and mandelone to the spent culture medium (SCM) of the probiotics, 20–160 \(\mu\)l of the SCM was added into wells containing fresh medium from the 2nd column to the 10th column. In addition, to investigate whether the antibacterial activity of the SCM against the periodontopathogens was by the antibacterial peptide or by an acid condition, the SCM of \(L.\) lactis was treated with proteinase K (100 \(\mu\)g/ml) at 55°C for 30 min and heated to inactivate proteinase K at 100°C for 10 min. Proteinase K-treated SCM was assayed as described above.

All four periodontopathogens were counted with a bacterial counting chamber (Marienfeld, Lauda-Könighofen, Germany) and then diluted to 3 \(\times\) 10\(^6\) cells/ml in the respective media. Twenty microliters of the periodontopathogen suspensions was inoculated into the prepared wells. The plates were incubated at 37°C in an anaerobic chamber for 36 h, and optical densities were measured at 600 nm using a microplate reader (BioTek, Winooski, VT, USA).

**Co-cultivation of \(L.\) lactis and periodontopathogens.** To investigate the effect of \(L.\) lactis on periodontitis when they co-existed, co-cultivation of \(L.\) lactis and \(F.\) nucleatum, \(P.\) gingivalis, \(T.\) forsythia, or \(T.\) denticola was performed. \(L.\) lactis and periodontopathogens were co-cultivated by Millicell® cell culture insert (Millipore, Billerica, MA, USA). In the cases of \(F.\) nucleatum and \(P.\) gingivalis, BHI broth supplemented with hemin and vitamin K was used, and in the cases of \(T.\) forsythia and \(T.\) denticola, a mixture of BHI and an equal volume of a specific medium was used. The prepared media were dispensed into two new tubes, and \(L.\) lactis and periodontopathogens were inoculated into each tube. Culture inserts were placed into the wells of a 12-well plate. The suspension of periodontopathogens and \(L.\) lactis were dispensed into the inside and basolateral side, respectively, of the insert, with the recommended volumes. The plates were incubated at 37°C in an anaerobic chamber for 36 h, and the periodontopathogens were counted by a bacterial counting chamber (Marienfeld).

**Measurement of VSCs.** VSC levels were measured in gas from the SCM from the periodontopathogen cultures mixed or unmixed with various volumes of \(L.\) lactis suspension. After cultivation for 36 h, the SCM (1 ml) of periodontopathogens were transferred to 50-ml conical tubes to which 1, 2, or 3 ml of \(L.\) lactis suspension was added. The mixtures were filled to 5 ml with fresh BHI media, and the control group was filled with 4 ml of fresh BHI medium. The preparations were vortexed for 30 s. VSC gas was collected above the mixed solution using a 10 ml syringe, and one millilitre of VSC gas was injected into Oral Chroma® gas chromatograph (FIS Inc., Itami, Hyogo, Japan), and the level of VSCs was measured.

**Lipopolysaccharide extraction.** LPS was extracted from \(F.\) nucleatum, \(P.\) gingivalis, \(T.\) forsythia, and \(T.\) denticola by an LPS extraction kit with little modification as described by Lee (2015). After cultivation in the respective media, the periodontopathogens were harvested by centrifugation at 6,500 \(\times\) g for 10 min at 4°C and then washed with cold phosphate buffered saline (PBS; pH 7.0). Then, the periodontopathogens were mixed with lysis buffer and vortexed until the bacteria pellet disappeared. Chloroform was added, and the mixture was vortexed for 10 s and centrifuged at 13,000 \(\times\) g for 15 min at 4°C. The supernatant was then transferred to a new tube. The solution was incubated with endonuclease (100 \(\mu\)g/ml) for 1 h at 37°C and then with proteinase K (250 \(\mu\)g/ml) at 55°C for 1 h.
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Lysis buffer was added to the solution, following the protocol described above, and the solution was incubated with a purification buffer for 30 min at –20°C. After centrifugation at 13,000 × g for 15 min, the supernatant was removed. The pellet was then washed with 1 ml of 70% ethyl alcohol using endotoxin-free water, air-dried and dissolved in endotoxin-free water. After lyophilization, the dry weight of LPS was measured. LPS from the periodontopathogens was dissolved in endotoxin-free water at a concentration of 1 mg/ml. To verify LPS purity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide gel) and agarose gel electrophoresis were performed to detect proteins and nucleic acid, respectively, and the gels were stained with Coomassie blue (for protein staining) or ethidium bromide (for nucleotide staining), respectively.

**Cell culture and treatment.** A monotypic cell line, THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate) at 37°C in a 5% CO2 incubator. The cells were washed and re-suspended with serum-free RPMI-1640. The cells (1 × 106 cells/ml) were plated in 12-well plates with 1% human serum (Sigma-Aldrich Co., St Louis, MO, USA) providing soluble CD14 and LPS-binding proteins. The cells were then treated with the LPS (500 ng/ml) from the periodontopathogens in the presence or absence of UV-killed *L. lactis* at concentrations of 1 × 105 and 1 × 106 cells for 8 h at 37°C in a CO2 incubator. The conditioned media were collected to measure the production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) by enzyme-linked immunosorbent assay (ELISA).

**ELISA.** The conditioned media of the cells treated with the LPS in the presence or absence of UV-killed *L. lactis* were harvested by centrifugation at 4,000 × g for 10 min at 4°C. Supernatants were collected every 3 days in two different experiments and then stored at –80°C until the ELISA. The supernatants were analyzed for IL-8 and TNF-α levels using a BD OptEIA Human ELISA kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s protocol.
Statistical analysis. Statistical analyses were performed with Kruskal-Wallis and Mann-Whitney tests using IBM SPSS Statistics 21 software (IBM, Armonk, NY, USA). P-values less than 0.05 were considered statistically significant.

Results

Antibacterial activity of L. lactis against periodontopathogens

When the SCM of L. lactis at various concentrations was examined, significant antibacterial activity against F. nucleatum was observed at a concentration of 50% (Fig. 1A). The growth of both P. gingivalis and T. forsythia was also significantly reduced in the media containing 50–90% concentrations of L. lactis SCM (Figs. 1B and C). The growth of T. denticola was decreased in the TYGVS medium with a SCM concentration of 60% (Fig. 1D). The proteinase K-treated SCM of L. lactis weakly inhibited the growth of the periodontopathogens. In evaluating the experiment of the antibacterial activity of L. lactis co-existing with the periodontopathogens, the growth of F. nucleatum, P. gingivalis, and T. forsythia was inhibited by L. lactis at the bacterial concentration of 5 fold or more (Figs. 2A, B, and C), and the growth of T. denticola was decreased by L. lactis at a bacterial concentration of 10 fold (Fig. 2D).

Neutralizing effect of L. lactis on VSCs from periodontopathogens

P. gingivalis produced the most total VSCs of all of the periodontopathogens, whereas F. nucleatum hardly produced any methyl mercaptan (CH\textsubscript{3}SH). The L. lactis suspension significantly reduced the total VSCs produced by the periodontopathogens relative to that in the fresh L. lactis medium as a control. In addition, the L. lactis suspension significantly decreased each of the individual VSCs (i.e., hydrogen sulfide, methyl mercaptan, and dimethyl sulfide) in a dose-dependent manner (Fig. 3).

Inhibition of LPS bioactivity of periodontopathogens

Periodontopathogen LPS is a virulence factor associated with inflammation and bone resorption that induces the expression of various cytokines. Thus, to test the inhibitory effect of L. lactis on the bioactivity of LPS from the periodontopathogens, THP-1 cells were treated with LPS derived from each periodontopathogen in the presence or absence of UV-killed L. lactis. The LPS from F. nucleatum, P. gingivalis, and T. forsythia induced the production of IL-6 and TNF-\alpha, and UV-killed L. lactis significantly and dose-dependently reduced the levels of these cytokines (p < 0.05) (Fig. 4). However, LPS from T. denticola did not significantly induce the production of IL-6 or TNF-\alpha compared with that in the control group (data not shown).

Discussion

The microorganism L. lactis is used as an artisanal starter for fermented foods or dairy products because it is recognized to be safe for consumption. Such organisms are called probiotic bacteria, and their roles and characteristics have been studied for their potential health and nutri-
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The inhibitory effect of *Lactococcus lactis* on the bioactivity of periodontopathogens was investigated. The SCM of the periodontopathogens were mixed with *L. lactis* suspension at various concentrations, or with a fresh BHI medium (control), and then vortexed. VSCs were collected using a syringe held above the bacterial suspensions. The levels of VSCs were measured by gas chromatography (Oral Chroma). * denotes a statistically significant difference compared to the unmixed control group (*P* < 0.05). SCM, spent culture medium.

Fig. 3. Inhibitory effect of the spent culture medium of *L. lactis* on gaseous VSCs.

Fig. 4. Inhibitory effect of UV-killed *L. lactis* on the induction of cytokine expression by periodontopathogens.

THP-1 cells were treated with LPS extracted from *F. nucleatum, P. gingivalis, and T. forsythia* in the presence or absence of UV-killed *L. lactis*. After collection of the conditioned media, the levels of IL-6 and TNF-α were measured by ELISA. Each of the experiments was performed three times in duplicate, and data are presented as means ± S.D. * denotes a statistically significant difference compared with untreated control cells (*P* < 0.05). # denotes a statistically significant difference compared with LPS-treated cells without UV-killed *L. lactis* (*P* < 0.05).

Fig. 3. Inhibitory effect of UV-killed *L. lactis* on the induction of cytokine expression by periodontopathogens.

THP-1 cells were treated with LPS extracted from *F. nucleatum, P. gingivalis, and T. forsythia* in the presence or absence of UV-killed *L. lactis*. After collection of the conditioned media, the levels of IL-6 and TNF-α were measured by ELISA. Each of the experiments was performed three times in duplicate, and data are presented as means ± S.D. * denotes a statistically significant difference compared with untreated control cells (*P* < 0.05). # denotes a statistically significant difference compared with LPS-treated cells without UV-killed *L. lactis* (*P* < 0.05).

Periodontitis is associated with multiple-species of bacteria, and epidemiological studies have shown that *P. gingivalis, T. forsythia, and T. denticola*, are the most relevant to periodontitis (Socransky et al., 1998). Therefore, they are referred to as periodontopathogens or red complex bacteria. In addition, *F. nucleatum* is essential for the formation of biofilms that contain periodontopathogens (Marcotte and Lavoie, 1998; Socransky and Haffajee, 2002). We therefore examined whether *L. lactis* exhibited antibacterial activity against these periodontitis-related...
pathogens. Using the SCM of *L. lactis* the growth of *F. nucleatum*, *P. gingivalis*, and *T. forsythia*, was significantly inhibited at a concentration of 50%. Interestingly, *T. denticola* was found to be more resistant to the SCM of *L. lactis* than the other three periodontopathogens. This phenomenon might have been related to the presence of proteins or enzymes from rabbit serum used as a supplement in TGYVS medium. The SCM is to contain metabolites produced by *L. lactis* that consumes various nutrients in BHI media. Therefore, the SCM has lactic acid and various bacteriocins. The protease K-treated SCM of *L. lactis* weakly inhibited the growth of periodontopathogens. Because the bacteriocins of *L. lactis* are heat-stable peptides (Enan et al., 2013; Lee et al., 1999), we used protease K to inactivate or remove the secreted bacteriocins from *L. lactis*. Therefore, periodontopathogen growth may have been affected by *L. lactis* bacteriocins.

Periodontopathogens also produce VSCs, which cause halitosis that is typical in patients with periodontitis. Suspensions of *L. lactis* were mixed with the SCM of each periodontopathogen after independent cultivation in the specified media, and *L. lactis* suspensions were found to significantly neutralize VSCs produced by the periodontopathogens. In a previous study, we showed that both whole bacteria of *Streptococcus thermophilus* HY9012 and their SCM neutralized VSCs produced by *P. gingivalis* (Lee and Baek, 2014). The difference between *L. lactis* and *S. thermophilus* is that whole *S. thermophilus* inhibits or reduces the level of gaseous VSCs, whereas whole *L. lactis* does not.

Finally, we evaluated the effect of *L. lactis* on inflammatory cytokines induced by LPS as a virulence factor of periodontopathogens. Because BHI broth, which was used for *L. lactis* culture, induces expression of inflammatory cytokines in THP-1 cells, the SCM of *L. lactis* was not used in this study. Instead, we investigated the effects of UV-killed *L. lactis* on the induction of inflammatory cytokines by LPS from the periodontopathogens. UV-killed *L. lactis* inhibited, in a dose-dependent manner, IL-6 and TNF-α production induced by the LPS. IL-6 and TNF-α participate in inflammatory signaling (Lee et al., 2015). Moreover, IL-6 production is an important mechanism for controlling alveolar bone resorption (Graves et al., 2011; Shimizu et al., 1992). LPS is composed of three domains as lipid A, core-oligosaccharide and O-antigen chain. O-antigen chain binds to LPS-binding protein (LBP), is transported to CD14, and binds to TLR2 or TLR4 (Kim and Lee, 2014; Raetz and Whitfield, 2002). *F. nucleatum* LPS and *T. forsythia* LPS stimulate TLR4, and *P. gingivalis* LPS activates TLR2 (Andrukov et al., 2015; Asai et al., 2007; Kim and Lee, 2014). The stimulation of TLR2 or TLR4 initiates inflammatory signalings pathway and induces inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Algate et al., 2016; Liu and Ding, 2016). Also, these inflammatory cytokines induce alveolar bone resorption. *L. lactis* HY 449 may inhibit the induction of inflammatory cytokines by blocking the LPSs binding to TLR2 or TLR4 through LPS attachment to its surface molecules. Therefore, *L. lactis* may prevent not only inflammation but also alveolar bone resorption caused by periodontopathogen LPSs.

In this study, we showed that *L. lactis* has antimicrobial activity against periodontopathogens, such as *F. nucleatum*, *P. gingivalis*, *T. forsythia*, and *T. denticola*. Furthermore, *L. lactis* neutralized and inhibited VSCS produced by these pathogens, as well as inflammatory cytokines induced by LPS derived from these pathogens. These data suggest that *L. lactis* may be an effective probiotic for the prevention and treatment of periodontitis and halitosis.

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