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

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**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
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 (H₂S), methyl mercaptan (CH₃SH), and dimethyl sulfide ((CH₃)₂S) 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% H₂, 10% CO₂, and 85% N₂). F. nucleatum ATCC 25586 and P. gingivalis ATCC 33277 were cultured anaerobically with BHI broth supplemented with hemin (1 µg/ml) and vitamin K (0.2 µg/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 × 10⁷ 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 × 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 µm). After adding human and bacterial components, 20–180 µl of BHI broth containing hemin (1 µg/ml) and vitamin K (0.2 µg/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 the mixtures and vortexing until the bacteria pellet disappeared, 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 milliliter 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 × g for 10 min at 4°C and then washed with cold phosphate buffered saline (PBS; pH 7.0). 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 × g for 15 min at 4°C. The supernatant was then transferred to a new tube. The solution was incubated with endonuclease (100 µg/ml) for 1 h at 37°C and then with proteinase K (250 µg/ml) at 55°C for 1 h.
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 monocytic 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.

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Fig. 1. Antibacterial activity of *L. lactis* against periodontopathogens.

*F. nucleatum, P. gingivalis, T. forsythia,* and *T. denticola* were incubated in each of specified media in the presence or absence of the SCM or proteinase K-treated SCM of *L. lactis* at various concentrations. The control group was treated with fresh preparations of each of the specified media. Bacterial growth was measured by a spectrophotometer at 600 nm. The experiments were performed three times in duplicate, and data are presented as means ± S.Ds. * Statistically significant difference compared with untreated control bacteria (*P* < 0.05).
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 mercapatan (CH₃SH). 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 mercapatan, 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-α, 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-α 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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\section*{Inhibitory effect of \textit{Lactococcus lactis} on the bioactivity of periodontopathogens}

\textbf{Fig. 3.} Inhibitory effect of the spent culture medium of \textit{L. lactis} on gaseous VSCs.

The SCM of the periodontopathogens were mixed with \textit{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.

\section*{Inhibitory effect of UV-killed \textit{L. lactis} on the induction of cytokine expression by periodontopathogens}

\textbf{Fig. 4.} Inhibitory effect of UV-killed \textit{L. lactis} on the induction of cytokine expression by periodontopathogens.

THP-1 cells were treated with LPS extracted from \textit{F. nucleatum}, \textit{P. gingivalis}, and \textit{T. forsythia} in the presence or absence of UV-killed \textit{L. lactis}. After collection of the conditioned media, the levels of IL-6 and TNF-\(\alpha\) were measured by ELISA. Each of the experiments was performed three times in duplicate, and data are presented as means \(\pm\) S.D.s * 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 \textit{L. lactis} ($P < 0.05$).
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 TYGVS 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 (Andrukhov 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 pathogenes, 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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