Inhibitory Effect of Lactococcus lactis HY 449 on Cariogenic Biofilm

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Introduction

Streptococcus mutans plays a potent role in the induction of dental caries. This bacterium strongly produces lactic acid and tolerates a low acidic environment [10]. Furthermore, S. mutans produces glucan from sucrose by glucosyltransferases such as GtfB, GtfC, and GtfD [9, 14] and uses sucrose as a substrate for growth [8]. Oral biofilm consists of oral bacteria, glucan, and debris. Among these components, glucan is a key contributor to the development of biofilm via formation of a thick barrier [11]. The biofilm of a healthy person maintains a balanced composition of bacterial species. However, when the conditions of the oral biofilm are changed by a sugar-rich diet, low pH, and low saliva flow, the proportion of S. mutans in the oral biofilm increases compared with other streptococci [12]. Moreover, continuous production of glucan and acid by S. mutans reduces the pH level and leads to formation of mature biofilm that ultimately induces dental caries. Therefore, the glucosyltransferases (Gtfs) and acid production are virulence factors of S. mutans. Cariogenic biofilm as oral biofilm including S. mutans is considered to be a greater risk factor for induction of dental caries than planktonic S. mutans.

Dental caries is caused by cariogenic biofilm, an oral biofilm including Streptococcus mutans. Recently, the prevention of dental caries using various probiotics has been attempted. Lactococcus lactis HY 449 is a probiotic bacterium. The aim of this study was to investigate the effect of L. lactis HY 449 on cariogenic biofilm and to analyze its inhibitory mechanisms. Cariogenic biofilm was formed in the presence or absence of L. lactis HY 449 and L. lactis ATCC 19435, and analyzed with a confocal laser microscope. The formation of cariogenic biofilm was reduced in cultures spiked with both L. lactis strains, and L. lactis HY 449 exhibited more inhibitory effects than L. lactis ATCC 19435. In order to analyze and to compare the inhibitory mechanisms, the antibacterial activity of the spent culture medium from both L. lactis strains against S. mutans was investigated, and the expression of glucosyltransferases (gtfs) of S. mutans was then analyzed by real-time RT-PCR. In addition, the sucrose fermentation ability of both L. lactis strains was examined. Both L. lactis strains showed antibacterial activity and inhibited the expression of gtfs, and the difference between both strains did not show. In the case of sucrose-fermenting ability, L. lactis HY 449 fermented sucrose but L. lactis ATCC 19435 did not. L. lactis HY 449 inhibited the uptake of sucrose and the gtfs expression of S. mutans, whereby the development of cariogenic biofilm may be inhibited. In conclusion, L. lactis HY 449 may be a useful probiotic for the prevention of dental caries.

Keywords: Lactococcus lactis, probiotics, Streptococcus mutans, cariogenic biofilm, antibiofilm effects
bacteria [3, 17, 24]. Most studies of probiotics have been focused on the bacterial ecology of the gut and gut immune systems. Recently, probiotics have been investigated for their effects on oral health. Lactobacillus rhamnosus GG inhibits the growth and bioactivity of oral pathogens via the production of small-molecular antimicrobial substances, whereby it reduces the risks of dental caries and periodontitis [13, 16, 21]. Moreover, L. reuteri and Bifidobacterium animalis reduced the S. mutans amount in saliva [2, 20]. However, the prevention of dental caries by these probiotics remains controversial because they are aciduric bacteria, like S. mutans [18]. Lactococcus lactis is a probiotic that has antibacterial activity. It produces various bacteriocins such as nisin and lactococcin, and diacetin [1, 7]. For these reasons, L. lactis is widely used in fermented foods and dairy products. Studies of L. lactis in oral health have examined the inhibition of planktonic S. mutans growth and mucosal immune stimulation [22, 23]. L. lactis HY 449 was isolated from contaminated milk products and contained the same fatty acid profiles as L. lactis ATCC 19435 as a type strain. Moreover, their characteristics are to ferment sucrose and salicline, unlike L. lactis ATCC 19435 [6]. The growth of L. lactis is inhibited at below pH 5 [4, 19]. Therefore, the current study investigated the effect of L. lactis HY 449 on cariogenic biofilm and analyzed the inhibitory mechanism of L. lactis HY 449 on the formation of cariogenic biofilm.

Materials and Methods

Bacterial Strains and Culture Conditions

L. lactis HY449 was donated from Korea Yakult (Korea Yakult, Korea), and L. lactis ATCC 19435 and S. mutans ATCC 25175 were purchased from American Type Culture Collection (ATCC). L. lactis ATCC 19435 and HY 449 were cultured in brain heart infusion (BHI) broth (BD Bioscience, USA) at 37°C. S. mutans ATCC 25175 was cultivated in BHI broth including 1% sucrose at 37°C.

Preparation of Conditioning Plate for Biofilm Formation

Pooled saliva of six healthy donors was centrifuged at 7,000 ×g for 10 min at 4°C. The supernatant was filtered through a polyvinylidene fluoride (PVDF) membrane and diluted to 2-fold with phosphate-buffered saline (PBS, pH 7.2). The prepared saliva was added to wells on a 12-well polystyrene plate. The 12-well plate was dried at 40°C in a drying oven and sterilized in an UV sterilizer. These procedures were repeated three times.

Inhibitory Effect of L. lactis on Cariogenic Biofilm

Cariogenic biofilm was formed according to the method described by Lee and Kim [9]. Unstimulated saliva was collected from 10 healthy donors and pooled in equal proportions. The pooled saliva was mixed with BHI broth containing 1% sucrose and 1% mannose and centrifuged at 2,000 ×g for 10 min at 4°C to remove debris. The supernatant was transferred into new tubes, and S. mutans (1 × 10⁶ cells) was added to form the cariogenic biofilm. L. lactis HY 449 and L. lactis ATCC 19435 (5 × 10⁵ and 1 × 10⁶ cells) were inoculated into each tube containing salivary bacteria. The preparation was vortexed for 10 sec, and 1 ml or 400 µl of the mixtures was then inoculated into a conditioned 12-well plate or 8-well glass chamber, respectively. The plates were incubated at 37°C for 72 h, and the media were changed daily by replacing with fresh BHI containing 1% sucrose and 1% mannose. The biofilm-formed 12-well plate was washed three times with PBS to remove planktonic bacteria, and the biofilm was disrupted mechanically with a scraper. The suspensions were diluted serially and plated on BHI agar plate and mitis-salivarius bacitracin (MSB) agar plates to count total bacteria and S. mutans in the biofilm, respectively. For the analysis of biofilm formation, an 8-well glass chamber was washed three times with PBS and stained with SYTO 9 dye (Invitrogen, USA) according to the manufacturer’s instructions. The biofilm was visualized by a confocal laser scanning microscope (Carl-Zeiss, Germany) using z-stack scans from 0 to 30 µm.

Comparison of Antibacterial Activity of L. lactis against S. mutans

In order to analyze the inhibitory mechanism of L. lactis HY 449 on formation of cariogenic biofilm, a susceptibility assay of S. mutans for the spent culture medium of L. lactis was performed according to the methods recommended by the Clinical and Laboratory Standards Institute (CLSI) [15]. The spent culture medium of L. lactis HY 449 and L. lactis ATCC 19435 was collected by centrifugation at 7,000 ×g for 10 min at 4°C and filtered through a PVDF membrane with a pore size of 0.22 µm. The spent culture medium was dispensed from 20 to 180 µl into the column wells of a 96-well polystyrene plate (SPL Life Sciences, Korea), and fresh BHI broth was added to bring the final volume up to 180 µl in the dispensed well of the spent culture medium. S. mutans was counted in a Petroff-Hassner bacteria counting chamber (Hausser Scientific, USA) and diluted to 1 × 10⁶ cells/ml with BHI broth. The diluted suspension of S. mutans (20 µl) was inoculated into each well. The plate was incubated at 37°C for 36 h, and the optical density was measured at 600 nm by a spectrophotometer.

Real-Time RT-PCR for the Analysis of Glucan Expression of S. mutans

Glucan production by S. mutans was indirectly analyzed by comparison of the expression levels of gtf6. S. mutans was cultured in BHI including 1% sucrose with or without the spent medium of both L. lactis at a non-killing concentration (20%) for S. mutans for 6 h. Total RNA was extracted with a TRIzol Max bacterial RNA isolation kit (Invitrogen Life Tech, USA) according to the
manufacturer’s instruction. cDNA was synthesized by Maxime RT Premix (random primer; iNtRON, Korea) in a 20 µl reaction volume, and the mixture was incubated at 45°C for 1 h. cDNA was mixed with 10 µl of SYBR Premix Ex Taq and 0.4 µM of each primer pair in a 50 µl final volume and was then subjected to 40 PCR cycles (94°C for 15 sec, 60°C for 10 sec, and 72°C for 33 sec) by ABI 7500 real-time PCR (Applied Biosystems, USA). 16S rRNA, which is a housekeeping gene, was used as a reference to normalize the expression levels and to quantify changes in expression levels of gtfB between cultures that were untreated and treated with the spent culture medium. The sequences of primers for real-time RT-PCR were as follows: 5’-AGC AAT GCA GCC AAT CTA CAA AT-3’ and 5’-ACG AAC TTT GCC GTT ATT GTC A-3’ for the gtfB gene; 5’-CTC AAC CAA CCG CCA CTG TT-3’ and 5’-GGT TAA CGT CAA AAT TAG CTG TAT TAG C-3’ for the gtfC gene; 5’-CAC AGG CAA AAG CTG AAT TAA CA-3’ and 5’-AAT GCC CCG TAA GTC AAC AG-3’ for the gtfD gene; and 5’-GAA AGT GTG GAG TAA AAG GCT A-3’ and 5’-GTT AGC TCC GGC ACT AAG CC-3’ for the 16S rRNA gene.

**Analysis of Sucrose Fermentation by *L. lactis***

In order to investigate the sucrose-fermenting ability of *L. lactis* HY 449 and *L. lactis* ATCC 19435, the bacteria were harvested by centrifugation at 4,000 × g for 10 min at 4°C and washed with fresh BHI broth. The bacteria were resuspended with fresh BHI broth, and the concentration was adjusted to 1 × 10⁷ cells/ml by using a bacterial counting chamber. Ten milliliters of BHI was dispensed into new conical tubes with or without sucrose and prewarmed at 37°C until inoculation. The bacterial suspension (1 ml) was inoculated into 15 tubes and the level of pH was measured every hour.

**Fig. 1.** Inhibition of formation of cariogenic biofilm by *L. lactis*. The cariogenic biofilm was formed using salivary bacteria and *S. mutans* in the presence or absence of *L. lactis* HY 449 and *L. lactis* ATCC 19435. The biofilms were then washed with PBS, stained with SYTO 9, and analyzed by a confocal laser scanning microscope (A–C). To count bacteria in the biofilm, the biofilm was disrupted by a scraper and resuspended with BHI. After plating on BHI agar plates for total bacteria (D) and MSB agar plates for *S. mutans* (E), the agar plates were incubated for 36 and 48 h, respectively. The colonies on the agar plates were then counted. Data are represented as the mean ± SD from six total experiments. * Statistically significant difference compared with untreated *L. lactis* (p < 0.001).
Statistical Analysis
Statistically significant differences were analyzed by the Kruskal-Wallis and Mann-Whitney tests using IBM SPSS Statistics 21 software (IBM, USA). P-values less than 0.05 were considered statistically significant.

Results

Inhibitory Effect of L. lactis on Formation of Cariogenic Biofilm
Salivary biofilm containing S. mutans as cariogenic biofilm is more related to dental caries than S. mutans biofilm. Therefore, the effect of L. lactis on cariogenic biofilm was investigated. L. lactis HY 449 and L. lactis ATCC 19435 inhibited formation of cariogenic biofilm (Figs. 1A–1C). The level of total bacteria in cariogenic biofilm was decreased when the growth medium was spiked with L. lactis (Fig. 1D). The level of S. mutans in the biofilm was also decreased in the presence of L. lactis in a dose-dependent manner (Fig. 1E). The peculiar point is that the anti-biofilm activity of L. lactis HY 449 and L. lactis ATCC 19435 was different. L. lactis HY 449 showed more inhibitory effect on formation of cariogenic biofilm than did L. lactis ATCC 19435.

Antimicrobial Activity of L. lactis against S. mutans
In order to investigate the difference in the inhibitory effects, the antibacterial activity of both L. lactis strains was compared. Although the growth of S. mutans was significantly decreased in media containing a greater than 40% concentration of the spent culture medium of both L. lactis strains, there was no difference in the antimicrobial activity of L. lactis HY 449 and L. lactis ATCC 19435 (Fig. 2).

Reduction of Glucosyltransferase Expression by the Spent Culture Medium of L. lactis
The formation and development of S. mutans biofilm are related to the presence of soluble and insoluble glucan, and glucan is a key contributor to the development of biofilm [9]. When S. mutans was cultivated in the presence or absence of the spent culture medium of both L. lactis strains at a non-killing concentration for S. mutans, the spent medium of both L. lactis strains was found to reduce expression of the three gtf genes (Fig. 3). However, there was no difference in the inhibitory effect of L. lactis HY 449 and L. lactis ATCC 19435.

Sucrose Fermentation Ability of L. lactis
Sucrose is a substrate for synthesis of glucan, and the

Fig. 2. Susceptibility of S. mutans to the spent culture medium of L. lactis.
S. mutans was cultivated with or without the spent culture medium of L. lactis HY 449 or L. lactis ATCC 19435 at various concentrations on 96-well polystyrene plates. The growth of S. mutans was measured by a microplate reader at 600 nm. The experiments were carried out three times in duplicates, and data are presented as the mean ± SD. * Statistically significant difference compared with cultures not treated with the spent culture medium (p < 0.05).

Fig. 3. Effect of the spent culture medium of L. lactis on expression of glucosyltransferases of S. mutans.
S. mutans was cultivated in the presence or absence of the spent culture medium of L. lactis HY 449 or L. lactis ATCC 19435 at a non-killing concentration for S. mutans for 12 h. Total RNA was isolated, and cDNA was synthesized. The expression levels of gtfB, gtfC, and gtfD were analyzed by real-time PCR. Data are presented as the mean ± SD from six total experiments. * Statistically significant difference compared with cultures not treated with the spent culture medium (p < 0.001).
and induces caries lesions on the tooth surface. Therefore, and low saliva flow, the biofilm is called cariogenic biofilm changing oral conditions such as sugar-rich diet, low pH, biofilm increases compared with other streptococci by

Interestingly, L. rhamnosus HY 449 and L. lactis ATCC 19435 were cultivated in BHI broth with or without sucrose. Initial pH was measured immediately after inoculating both bacteria cultures, and the pH level of the culture medium was measured at hourly intervals. The experiments were carried out three times in duplicates. * Statistically significant difference compared with sucrose-free condition (*p < 0.05).

glucan synthesis of S. mutans depends on the concentration of sucrose [8]. Therefore, the sucrose-fermenting ability of L. lactis was investigated. The acid production of L. lactis HY 449 and L. lactis ATCC 19435 with or without sucrose was compared. The pH level of L. lactis culture media was determined to be near pH 5.5, even after 5 h in the presence or absence of sucrose. Interestingly, L. lactis HY 449 was found to produce a higher level of acid in the presence of sucrose than in the absence of sucrose, and the pH level of L. lactis ATCC 19435 culture media was not significantly different between BHI broth with or without sucrose (Fig. 4).

Discussion

Dental caries is associated with oral biofilm including S. mutans. When the proportion of S. mutans in the oral biofilm increases compared with other streptococci by changing oral conditions such as sugar-rich diet, low pH, and low saliva flow, the biofilm is called cariogenic biofilm and induces caries lesions on the tooth surface. Therefore, S. mutans is considered to be a greater risk factor for induction of dental caries than planktonic S. mutans. L. rhamnosus GG interferes with the growth and bioactivity of cariogenic bacteria, whereby it may reduce the risks of dental caries [13, 16]. Moreover, L. reuteri and B. animalis reduced the S. mutans amount in saliva [2, 20]. However, the prevention of dental caries by these probiotics remains controversial because they are aciduric bacteria, like S. mutans [18]. However, L. lactis is not an aciduric bacterium because its growth is inhibited at below pH 5 [4, 19]. Therefore, the current study investigated the effects of L. lactis HY 449 on cariogenic biofilm.

L. lactis significantly inhibited formation of cariogenic biofilm as well as the growth of total bacteria and S. mutans in biofilm. This report is the first to investigate the effect of L. lactis on a cariogenic biofilm model using salivary bacteria and S. mutans. The peculiar point is that L. lactis HY 449 strongly inhibited formation of cariogenic biofilm compared with L. lactis ATCC 19435. Therefore, the characteristics of both L. lactis strains were compared and analyzed. When the susceptibility test of S. mutans was examined by the spent culture medium of L. lactis HY 449 and L. lactis ATCC 19435, the growth of S. mutans was significantly decreased in media containing a greater than 40% concentration of the spent culture medium of both L. lactis strains. However, there were no differences of the antimicrobial activity between L. lactis HY 449 and L. lactis ATCC 19435. Therefore, the correlation between the antibacterial activity and inhibitory difference of the biofilm was excluded.

Next, since glucan plays a key role in the development and formation of cariogenic biofilm [8], we investigated the effect of the spent culture medium of both L. lactis strains on the expression of glucosyltransferases. When S. mutans was cultivated with the spent culture medium of both L. lactis strains at a non-killing concentration for S. mutans, the expression levels of the three glucosyltransferases gtfB, gtfC, and gtfD were significantly reduced compared with single-cultured S. mutans. However, this experiment did not exhibit the difference between L. lactis HY 449 and L. lactis ATCC 19435 like the antibacterial experiment.

Finally, sucrose as a biofilm formation-related factor was investigated. The difference between L. lactis HY 449 and L. lactis ATCC 19435 is in their sucrose-fermenting ability. L. lactis HY 449 ferments sucrose, unlike L. lactis ATCC 19435 [6]. Both L. lactis strains were cultivated in BHI broth with or without sucrose, and the pH level of the culture medium of each bacterium was measured. The pH level of culture medium of L. lactis HY 449 quickly decreased in the presence of sucrose compared with in the absence of sucrose. However, the decrease of pH level of L. lactis ATCC 19435 culture medium was not different in the two conditions. From the S. mutans standpoint, L. lactis HY 449 is a sucrose competitor. Kreth et al. [8] reported that when
S. mutans is with other streptococcal competitors during oral biofilm formation and uses sucrose for a substrate, the antimicrobial susceptibility of S. mutans decreases. Moreover, the glucan synthesis of S. mutans depends on the concentration of sucrose [8]. Tong et al. [22] showed an antagonizing effect of L. lactis on S. mutans biofilm through production of a bacteriocin, nisin. On the basis of these studies, L. lactis HY 449 consumes sucrose for glucan synthesis and metabolism, whereby S. mutans may be inhibited to take up sucrose for glucan synthesis, and the resistance of oral bacteria in cariogenic biofilm may decrease for the antimicrobial peptide of L. lactis HY 449.

Recently, various groups have studied to apply probiotics for oral health, and sought candidate probiotics. However, most proposed probiotics are controversial because of their aciduric characteristic. In the current study, the culture medium of L. lactis was determined to be near pH 5.5, like oral commensal bacteria. S. mutans is a key contributor to the development of biofilm via glucan synthesis. L. lactis HY 449 is a competitor to S. mutans in the uptake of sucrose and inhibited glucan synthesis by S. mutans. Therefore, L. lactis HY 449 inhibited the formation of cariogenic biofilm. Furthermore, the antibacterial activity of L. lactis HY 449 inhibited the growth of S. mutans in immature biofilm. Eventually, L. lactis HY 449 might effectively inhibit the formation of cariogenic biofilm compared with L. lactis ATCC 19435.

In this study, we showed that L. lactis has an inhibitory effect on the formation of cariogenic biofilm by antimicrobial activity, inhibition of expression of GtfS, and consumption of sucrose. Moreover, this bacterium is not aciduric. For these reasons, L. lactis HY 449 may be a potential probiotic for use in the prevention of dental caries.

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**References**


