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Use of D-galactose to regulate biofilm growth of oral streptococci

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ABSTRACT

In the oral microbial community, commensals can compete with pathogens and reduce their colonization in the oral cavity. A substance that can inhibit harmful bacteria and enrich beneficial bacteria is required to maintain oral health. The purpose of this study was to examine the effect of D-galactose on the biofilm formation of the cariogenic bacteria *Streptococcus mutans* and oral commensal streptococci and to evaluate their use in solution and in paste form. Biofilms of *S. mutans*, *Streptococcus oralis*, and *Streptococcus mitis* were formed on saliva-coated glass slips in the absence or presence of D-galactose and evaluated by staining with 1 % crystal violet. D-Galactose significantly inhibited the biofilm formation of *S. mutans* at concentrations ranging from 2 μM to 200 mM but increased the biofilm formation of *S. oralis* and *S. mitis* at concentrations of 2–200 mM. D-Galactose significantly inhibited three glucosyltransferase genes, *gtfB*, *gtfC*, and *gtfD*. The effect of D-galactose in the form of solution and paste was evaluated using bovine teeth. Pretreatment with 100 mM D-galactose on bovine teeth resulted in significantly reduced *S. mutans* biofilm formation. Our results suggest that D-galactose can be a candidate substance for the development of oral hygiene products to prevent caries by inhibiting the biofilm formation of *S. mutans* and simultaneously increasing the biofilm formation of commensal oral streptococci.

1. Introduction

Dental caries is a representative oral disease. As acids produced from the bacterial glycolytic pathway develop carious lesions, there have been many trials to selectively target cariogenic bacteria or to neutralize biofilm pH. Pheromone (CSP)-guided antimicrobial peptides have been developed to deliver antimicrobial peptides specifically to *Streptococcus mutans* and eliminate them from biofilms composed of multiple species (Eckert et al., 2006). Xylitol exhausts *S. mutans* energy metabolism (Trahan, 1995), and arginine metabolism contributes to neutralizing biofilm acidic pH (Nascimento, 2018). Fluoride has an antimicrobial effect (Randall, Seow, & Walsh, 2015) and reduces acid production by acidogenic bacteria, including *S. mutans* and lactobacilli, in addition to reducing the solubility of calcium hydroxyapatite. An *S. mutans* strain lacking *gcrR,* which regulates sucrose-dependent adherence of the bacterium, has been suggested to have the ability to replacement therapy of dental caries (Pan et al., 2013). In addition to the removal of cariogenic bacteria, the maintenance of beneficial bacteria in the oral cavity plays an important role in competing pathogens; thus, probiotics are marketed to benefit oral health (Zahradnik et al., 2009).

As *S. mutans* is a prominent cariogenic bacterium that forms a biofilm on the tooth surface, biofilm-inhibiting substances may be candidates for anti-cariogenic agents. Pathogenic bacteria use lectins on their surface to bind carbohydrates on host cells or bacteria to establish biofilms. Galactose is a monosaccharide that is involved in lectin binding. L-Galactose binds to lectin from *Pseudomonas aeruginosa,* which specifically binds to cultured cystic fibrosis airway cells (Sabin et al., 2006). D-Galactose inhibited the interaction between a lectin on the surface of *Fusobacterium nucleatum* and salivary glycoproteins (Murray, Kern, & Winkler, 1988). Previously, we reported that D-galactose functions as a quorum sensing (QS) inhibitor, inhibiting biofilm formation of periodontopathogens induced by autoinducer 2 (AI-2), a QS molecule (Ryu, Sim, Sim, Lee, & Choi, 2016). Bacterial QS is associated with diverse biological functions, including biofilm formation and virulence expression. Recently, QS pathways are attractive drug targets that inhibit biofilm formation and virulence expression (Jiang, Chen, Yang, Yin, & Yao, 2019; Kaur, Rajesh, & Princy, 2015). Oral streptococci belonging to the mitis and sanguinis groups, including *S. oralis*, *S. mitis*, *S. gordonii*, and *S. sanguinis,* are primary colonizers and are considered commensals that compete with colonization of pathogenic streptococci, such as *S. mutans*, on the tooth surface (Abranches et al.,

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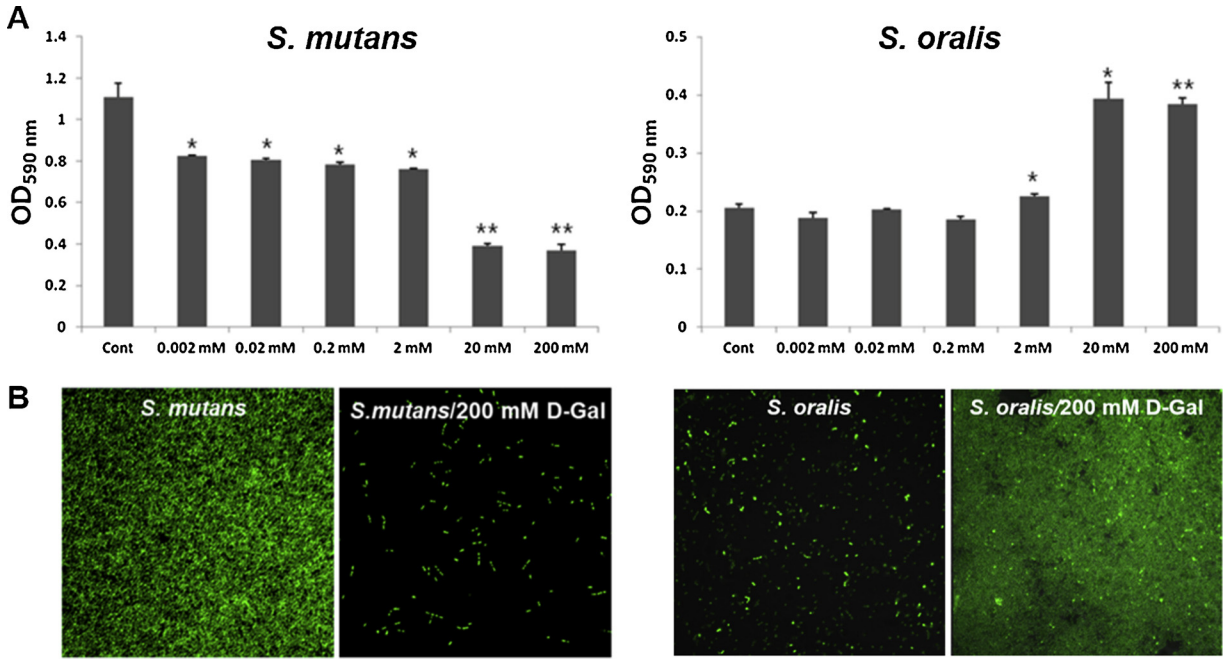

Fig. 1. Biofilm growth of *S. mutans* and *S. oralis* exposed to D-galactose. (A) *S. mutans* and *S. oralis* were grown in the absence or presence of D-galactose on saliva-coated glass slips for 24 h. The biofilms formed were stained with 1 % crystal violet for 10 min. The staining was eluted with 1 ml of acetone-alcohol solution. The optical density at 590 nm of the destaining solution containing crystal violet was measured using a microplate reader. Cont: only bacteria-treated group. The data represent the mean ± SD of three independent experiments. $*p < 0.05$ and $**p < 0.001$ compared to the control group. (B) Biofilms formed on glass slips were stained using a Live/Dead-BacLight bacterial viability kit and observed using a confocal laser scanning microscope at a magnification of 1,000×.

2018).

In this study, we evaluated the effect of D-galactose on the biofilm formation of the cariogenic bacteria *S. mutans* and the oral commensals *S. oralis* and *S. mitis*. D-Galactose significantly inhibited the expression of glucosyltransferases, which play an essential role in biofilm formation of *S. mutans* on tooth. In addition, we evaluated the effect of D-galactose in solution and paste form to inhibit the biofilm growth of *S. mutans*. D-Galactose appears to be an ideal substance to inhibit biofilm formation of cariogenic bacteria and to enrich commensal oral bacteria.

2. Materials and methods

2.1. Bacterial culture

S. mutans (ATCC 25175), *S. oralis* (ATCC 9811) and two strains of *S. mitis* (KCOM 1285, KCOM 1350) were cultured in Trypticase soy broth under aerobic conditions at 37 ˚C.

2.2. Biofilm formation assays

To assess bacterial biofilm formation, 2 ml of culture (2×10^7 bacteria/ml) of *S. mutans*, *S. oralis*, and *S. mitis* was placed onto saliva-coated glass slips (round, 12-mm radius) in 24-well plates and cultured with D- or L-galactose at concentrations ranging from 0.002–200 mM for biofilm formation for 24 h. The biofilms formed on the cover slips were stained with 1 % crystal violet [Tris(4-(dimethylamino)phenyl)methylium chloride] for 10 min, washed three times with PBS, and destained with 1 ml of acetone-alcohol (20:80, vol/vol). The optical density at 590 nm of the destaining solution containing crystal violet was measured using a microplate reader (a Wallac Victor3 microtiter, PerkinElmer Life Sciences, Waltham, MA, USA).

The biofilms of each bacteria formed on the glass slips were stained using the Live/Dead-BacLight bacterial viability kit (Invitrogen, Carlsbad, CA), observed using a confocal laser scanning microscope (Carl Zeiss LSM 700, Jena, Germany) at a magnification of 1,000 × . For multispecies biofilm formation assay, *S. oralis* and *S. mitis* were labelled with SYTO9 (Invitrogen) while *S. mutans* was labelled with pHrodo (Invitrogen) for 1 h. Then, *S. mutans*, *S. oralis*, and *S. mitis* (2×10^6 bacteria/ml, each) were co-cultured on saliva-coated glass slips in 24-well plates in the absence or presence of D-galactose (200 mM). After 15 h incubation, the biofilms formed on the glass slips were washed with PBS three times and quantified by measuring the fluorescence mean intensity of biofilm using Carl Zeiss LSM 700 program.

To evaluate the effect of D-galactose on bacterial growth, *S. mutans*, *S. oralis*, and *S. mitis* were grown in the absence or presence of D-galactose (2–200 mM) as described above, and the optical density at 600 nm of the culture was monitored for 30 h using a spectrophotometer.

2.3. Lactic acid assay

Two milliliter of coculture (*S. mutans, S. oralis* and *S. mitis*, 2×10^6 bacteria/ml each) were incubated on saliva-coated glass slips in 24-well plates for 15 h in the absence or presence of D-galactose under aerobic condition at 37 ˚C. After removing culture supernatants, biofilms on glass slips were washed with PBS to remove planktonic bacteria and incubated in fresh TSB medium for 6 h. The level of L(+)-lactate in the culture supernatants were determined using a Lactate Assay Kit (Sigma-Aldrich, St. Louis, MO, USA).

2.4. Evaluation of *S. mutans* biofilm formation by pretreatment with D-galactose in a solution and a paste form using bovine teeth

Sterilized bovine teeth were polished in the order of a stone, a rubber, and a polishing brush to smooth tooth surface. Teeth were further sterilized with 70 % ethanol followed by UV irradiation for 10 min. Each of the outer surfaces of the bovine teeth was covered with a coating paper with a circular opening with a diameter of approximately 7 mm to provide identical biofilm formation areas (Fig. 1).

For the application of galactose in a solution form (galactose in PBS), the bovine bone teeth were placed into a PBS solution containing 100 mM of D-galactose for 3 min. After removal of galactose solution, the teeth were placed in 2 ml (2×10^7/ml) of *S. mutans* culture and

then cultured for 24 h. For application of galactose in the form of toothpaste, bovine teeth were treated for 3 min with a toothpaste gel formed by mixing 100 mM of D-galactose with 2 % carboxymethyl cellulose sodium (CMC) followed by washing with a saline solution. The biofilm formed on the tooth surface was stained with 1 % crystal violet [Tris(4-(dimethylamino)phenyl)methylium chloride], as described above. The optical density at 590 nm of the destaining solution containing crystal violet was measured using a microplate reader.

2.5. qPCR

The expression of three glucosyltransferase genes of *S. mutans* was analyzed by qPCR after treatment with D-galactose. *S. mutans* was maintained in Trypitase soy broth containing 1 % sucrose and grown to biofilm in the absence or presence of D-galactose at 2-200 mM under aerobic conditions at 37 ˚C. After 24 h, total bacterial RNA of the biofilm was isolated by using Easy-RED™ Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacture's protocol. Isolated RNA was treated with DNaseI(Amplification grade, Invitrogen, Carlsbad, CA, USA) for eliminating DNA during RNA purification procedures prior to RNA-PCR amplification. cDNA was synthesized in a 30-μl reaction volume using 1 μg RNA, random primers (Promega, Madison, WI, USA), and an M-MLV Reverse Transcriptase kit according to the manufacturer's instructions (Promega, Madison, WI, USA). cDNA (2 μl) was mixed with primer pairs (10 pmol each) and 10 μl of Power SYBR® Green Master mix (Applied Biosystems, Warrington, UK) in a 20-μl reaction volume. After an initial denaturation at 95℃ for 5 min, cDNA was amplified for 42 cycles of denaturation (95℃, 15 s), annealing (60℃, 15 s), and extension (72℃, 33 s) with a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The 16S rRNA gene was used as an internal control. Gene expression levels determined by qPCR were normalized against the 16S rRNA gene (16S rDNA) and calculated by the $2^{-\triangle\triangle ct}$ method. Primer sequences for qPCR 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 *gtfB*; 5′-CTC AAC CAA CCG CCA CTG TT-3′ and 5′-GGT TAA CGT CAA AAT TAG CTG TAT TAG C-3′ for *gtfC*; 5′-CAC AGG CAA AAG CTG AAT TAA CA-3′ and 5′-AAT GGC CGC TAA GTC AAC AG-3′ for *gtfD*; 5′-GAA AGT CTG GAG TAA AAG GCT A-3′ and 5′-GTT AGC TCC GGC ACT AAG CC-3′ for the 16S rRNA gene.

2.6. Statistical analysis

Statistical analyses were performed using Student's *t*-test. Statistically significant differences between the control and galactose groups were analyzed. A *p*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of D-galactose on biofilm formation of oral streptococci

Biofilm formation of a cariogenic bacteria *S. mutans* and an oral commensal *S. oralis* was assessed using D-galactose at concentrations of 0.002 − 200 mM. D-Galactose significantly inhibited biofilm formation of *S. mutans* at all concentrations used compared to a control without D-galactose (Fig. 1A and B). In contrast, D-galactose significantly increased *S. oralis* biofilm formation at the concentration of 2 − 200 mM (Fig. 1A and B) and had no effect at concentrations below 2 mM. For further experiments, we chose D-galactose concentrations of 2 − 200 mM, which inhibit *S. mutans* biofilm formation and increase *S. oralis* biofilm formation.

Next, we compared the effect of D-galactose on biofilm formation of two bacteria with that of L-galactose at concentrations of 2 − 200 mM. L-Galactose also significantly inhibited biofilm formation of *S. mutans* at concentrations of 20 mM and 200 mM but not at 2 mM (Fig. 2). However, the inhibitory effect of L-galactose was much less than that of D-galactose at the concentrations (20 mM and 200 mM), which inhibit biofilm formation. The application of 200 mM L-galactose showed a 70 % inhibitory effect on biofilm formation relative to 200 mM D-galactose, whereas only a 15 % inhibitory activity of 20 mM L-galactose was observed relative to 20 mM D-galactose. In contrast to D-galactose, L-galactose did not increase *S. oralis* biofilm formation. Rather, this treatment inhibited *S. oralis* biofilm formation by 52 % at 200 mM compared to the control, and no significant change was observed at the concentrations of 2 mM or 20 mM (Fig. 2). We also tested the effect of D-galactose on the biofilm formation of two strains of *S. mitis*. As shown in Fig. 3, D-galactose significantly enhanced biofilm formation of the *S. mitis* strains similar to that of *S. oralis*.

Because the effect of D-galactose on the biofilm formation of oral streptococci could be attributed to the effect on bacterial growth, we tested whether D-galactose inhibits or increases bacterial growth in a planktonic state. Interestingly, D-galactose increased the growth of *S. mutans* at 20 − 200 mM, while D-galactose at 2 mM did not affect the growth (Fig. 4). The growth of *S. oralis* and *S. mitis* was not affected by D-galactose (Fig. 4). These results indicate that the effect of D-galactose on biofilm formation is independent of bacterial growth.

3.2. Effect of D-galactose on biofilm formation and lactic acid production of oral streptococci in coculture

We also tested the effect of D-galactose on *S. mutans* biofilm formation in the presence of *S. oralis* and *S. mitis*. *S. mutans* labelled with pHrodo and then cocultured with *S. oralis* and *S. mitis* labelled with SYTO9. The fluorescence intensity of *S. mutans* in the biofilm was significantly reduced by D-galactose, whereas that of *S. oralis* and *S. mitis* was significantly increased (Fig. 5A and B). These results suggest that D-galactose exerts the inhibitory effect on biofilm formation of *S. mutans* present alone or with other streptococci. Furthermore, D-galactose significantly reduced lactic acid production in the biofilm of coculture composed of *S. mutans* and two commensal streptococci (Fig. 5C).

3.3. Effect of D-galactose on glucosyltransferases of S. mutans

Glucosyltransferases of *S. mutans* are involved in the synthesis of glucans, which play a critical role in colonization of the bacterium on the tooth surface to develop dental caries. We tested whether D-galactose is able to inhibit the gene expression of three types of glycosyltransferases of *S. mutans* in a biofilm state by qPCR. D-Galactose treatment of *S. mutans* for 24 h resulted in a significant decrease in the mRNA expression of glucosyltransferase B (*gtfB*), C (*gtfC*), and D (*gtfD*) (Fig. 6).

3.4. Effect of D-galactose in a solution and a paste form on biofilm growth of S. mutans on tooth surface

For the application of D-galactose, we evaluated its effect on biofilm formation as a solution and paste that imitate a mouthwash and toothpaste, respectively. Saliva-coated bovine teeth were pretreated with a solution or a paste form containing 100 mM galactose for 3 min followed by incubation with *S. mutans* culture for 24 h. The biofilm formation of *S. mutans* on the bovine teeth was significantly inhibited by pretreatment with 100 mM D-galactose in both forms compared to that on the untreated bovine teeth (Fig. 7). D-Galactose inhibited approximately 57 % biofilm formation in a solution form compared to the control without D-galactose. D-Galactose inhibited approximately 80 % biofilm formation in a paste form compared to the control only with CMC. In addition, after washing the bovine teeth exposed to D-galactose in a paste form with a saline solution, the biofilm formation of *S. mutans* was slightly increased compared to that without washing but was still less than biofilm on the bovine bone teeth exposed to D-galactose solution. These results suggest that D-galactose can be used as a

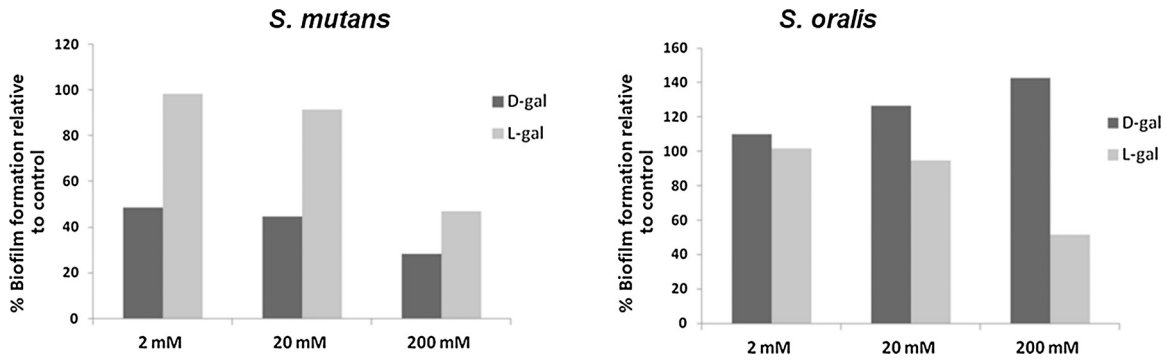

Fig. 2. Comparison of biofilm growth of *S. mutans* and *S. oralis* exposed to D- and L-galactose. *S. mutans* and *S. oralis* were grown in the absence or presence of D- or L-galactose on saliva-coated glass slips. The biofilms formed were stained with 1 % crystal violet for 10 min. The staining was eluted with 1 ml of acetone-alcohol solution. The optical density at 590 nm of the destaining solution containing crystal violet was measured using a microplate reader. The data are presented in % relative to the control value (100 %). The data represent the mean ± SD of three independent experiments.

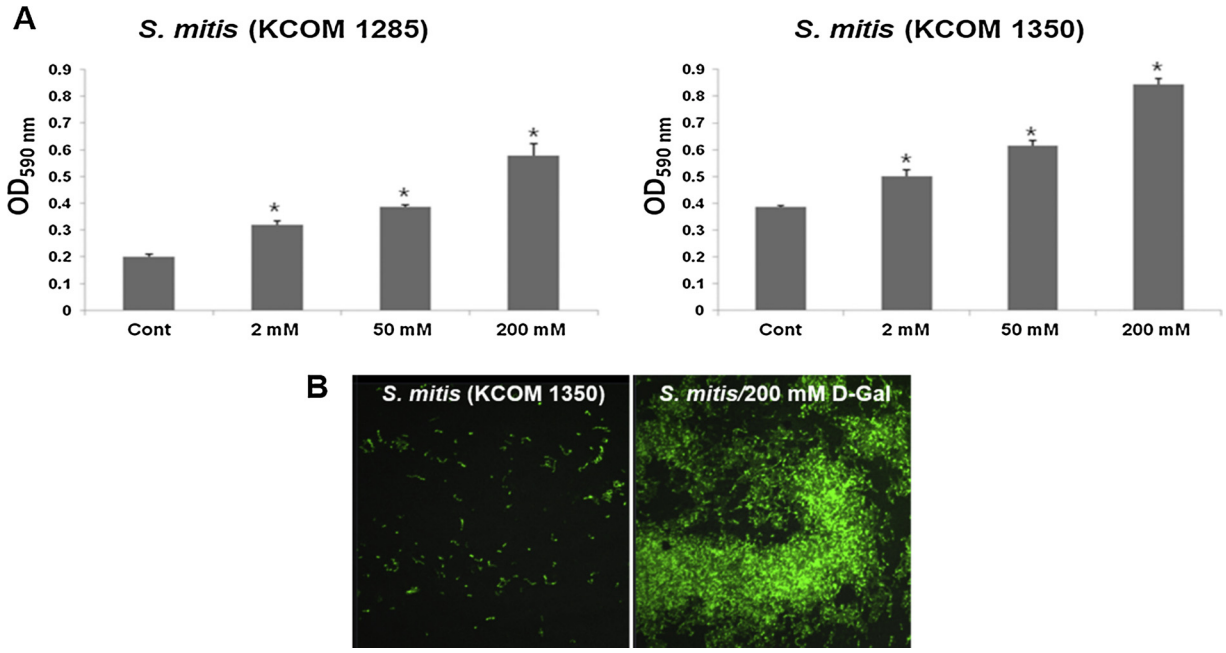

Fig. 3. Biofilm growth of *S. mitis* exposed to D-galactose. (A) *S. mitis* was grown in the presence of D-galactose on saliva-coated glass slips. The biofilms formed were stained with 1 % crystal violet for 10 min. The staining was eluted with 1 ml of acetone-alcohol solution. The optical density at 590 nm of the destaining solution containing crystal violet was measured using a microplate reader. The data represent the mean ± SD of three independent experiments. $*p < 0.05$ compared to the control group. (B) *S. mitis* biofilms formed on glass slips were stained using a Live/Dead-BacLight bacterial viability kit and observed using a confocal laser scanning microscope at a magnification of 1,000×.

mouthwash or a toothpaste.

4. Discussion

In this study, we demonstrated that D-galactose was able to inhibit biofilm growth of a cariogenic bacterium *S. mutans* and, at the same time, enhance biofilm growth of oral commensals *S. oralis* and *S. mitis*. The effect of D-galactose showed the same pattern in both monospecies culture and a coculture by reducing *S. mutans* biofilm growth and increasing *S. oralis and S. mitis* biofilm growth. Interestingly, even in the absence of D-galactose, the attachment of *S. mutans* to glass slips was hampered in a coculture, indicating inhibition of *S. mutans* attachment by *S. oralis* and *S. mitis*. Crystal violet staining is a widespread method for biofilm evaluation and can detect the whole area of biofilm formed on glass slips for quantification. The limitation of this method is that it does not distinguish between different bacteria. Confocal laser scanning microscopy (CLSM) is a sensitive and specific tool to study biofilms, but, sometimes quantitative measurement is difficult because of the matrix thickness of images. In the present study, we used crystal violet staining to evaluate monospecies biofilm formation and used CLSM to distinguish bacteria which were labeled with different fluorescent dyes in a coculture biofilm. Strategies to inhibit biofilm formation of cariogenic bacteria are important to prevent caries, and many studies have attempted to identify substances to inhibit the interaction between cariogenic bacteria and tooth surface. As lectin-like adhesins of bacterial surfaces have an important role in the initial attachment by binding carbohydrate chains of salivary components on the tooth surface, competitive inhibition of lectin-mediated adhesion of cariogenic bacteria can be a valuable means of inhibiting dental caries. Soluble lectins interacting with a carbohydrate chain Galβ1-3GalNAc of salivary glycoprotein inhibited *S. mutans* binding and biofilm formation (Ito et al., 2018).

Glucosyltransferases are crucial virulence factors of *S. mutans* (Bowen & Koo, 2011). These enzymes are engaged in the synthesis of extracellular polysaccharide glucans, which play an essential role in *S. mutans* biofilm formation by crosslinking bacterial cells. *gftB*

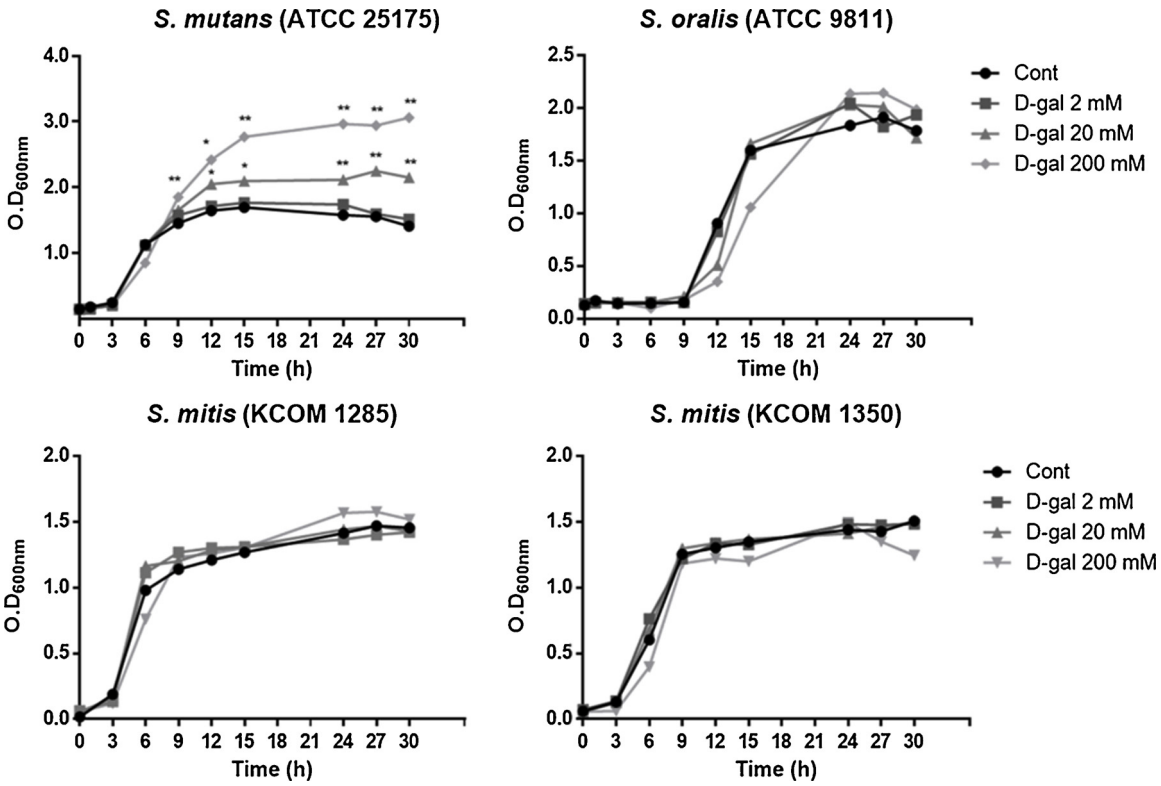

Fig. 4. Growth curves of streptococci exposed to D-galactose. *S. mutans*, *S. oralis, and S. mitis* were grown in the absence or presence of D-galactose. The optical density at 600 nm of the culture was monitored for 48 h using a spectrophotometer. Experiments were repeated three times, and the results of three independent experiments were presented. $*p < 0.05$ and $**p < 0.001$ compared to the control group.

synthesizes insoluble glucans rich in α-1,3 glycosidic linkages (Aoki, Shiroza, Hayakawa, Sato, & Kuramitsu, 1986), *gtfD* synthesizes soluble glucans rich in α-1,6 glycosidic linkages (Hanada & Kuramitsu, 1989), and *gtfC* synthesizes a mixture of soluble and insoluble glucans (Hanada & Kuramitsu, 1988). D-Galactose inhibited the gene expression of three glucosyltransferases. These results suggest that decreased glucosyltransferases by D-galactose contributed to the reduction of *S. mutans* biofilm growth.

Cariogenic activity is associated with the production of lactic acid which causes the demineralization of tooth surface. D-Galactose significantly inhibited lactic acid production in coculture, indicating reduction of lactic acid production may be mainly attributed to decreased

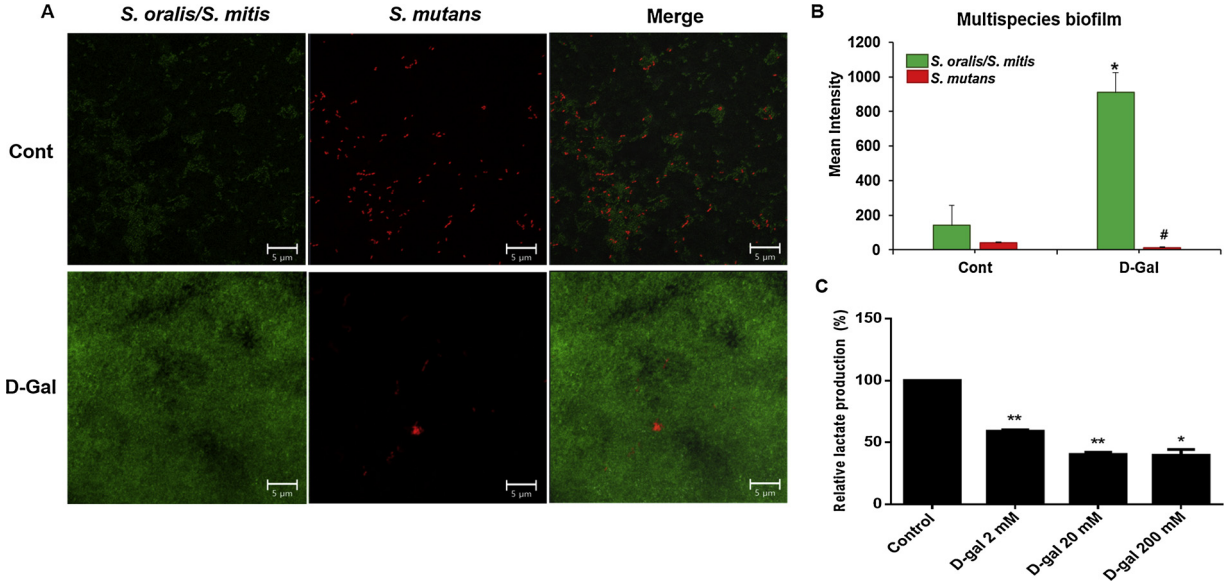

Fig. 5. Biofilm growth and lactic acid production of streptococci coculture. *S. mutans* labelled with pHrodo (red fluorescence) was cocultured on saliva-coated glass slips with *S. oralis* and *S. mitis* which were labelled with SYTO9 (green fluorescence) in the absence or presence of D-galactose (200 mM). (A) Biofilms formed on glass slips were observed using a confocal laser scanning microscope at a magnification of 1,000× and (B) Mean fluorescence intensity was calculated from five selected microscopic images. $*p$ and $^{\#}p < 0.05$ compared to the control group (C) Culture supernatants of the biofilm were subjected to measuring lactic acid produced from the bacteria. $*p < 0.05$ and $**p < 0.001$ compared to the control group.

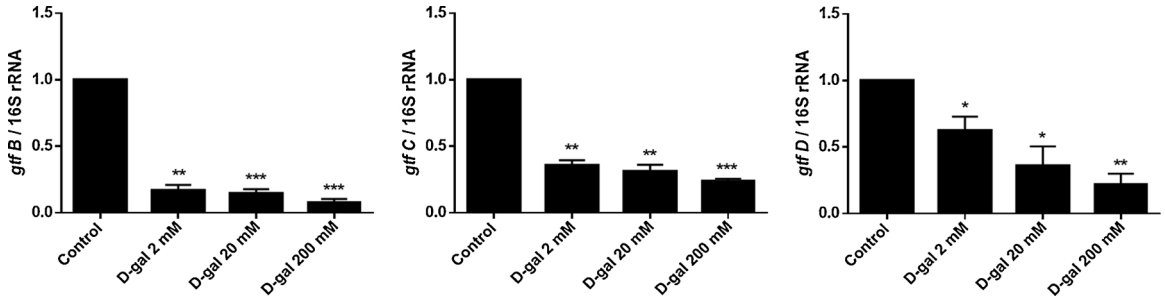

Fig. 6. mRNA expression of *S. mutans* glucosyltransferases by qPCR. *S. mutans* was grown in a biofilm state in the absence or presence of D-galactose for 24 h, and total bacterial RNA was isolated. The mRNA expression of three glucosyltransferases (GtfB, GtfC, and GtfD) was analyzed by qPCR. The data represent the mean ± SD of three independent experiments. $*p < 0.05$ and $**p < 0.001$ compared to the control group.

number of *S. mutans*.

Recently, we reported that D-galactose reduced AI-2 production of *V. harveyi* BB152 and *Fusobacterium nucleatum* and inhibited AI-2-induced biofilm formation of major periodontal pathogens, including *Porphyromonas gingivalis* and *Tannerella forsythia* (Ryu et al., 2016). D-Galactose may compete with AI-2 for binding to the D-galactose binding protein, a possible AI-2 receptor. *S. mutans* is known to produce QS molecules, such as competence inducing peptide (CSP), sigX inducing peptide (XIP), and AI-2 (Reck, Tomasch, & Wagner-Döbler, 2015; Yoshida, Ansai, Takehara, & Kuramitsu, 2005). AI-2 has been shown to play an important role in *S. mutans* biofilm formation and virulence expression. Biofilm formation of the *luxS* mutant strain of *S. mutans* GS-5 was significantly reduced compared to the wild type (Merritt, Qi, Goodman, Anderson, & Shi, 2003; Yoshida et al., 2005). LuxS is engaged in AI-2 production. The synthetic (Z)-5-bromomethylene-2(5 H)-furanone that inhibited AI-2 activity of *V. harveyi* BB152 and *S. mutans* inhibited *S. mutans* biofilm growth (Lönn-Stensrud, Petersen, Benneche, & Scheie, 2007), suggesting that AI-2 is engaged in biofilm formation. Reduced biofilm formation of *S. mutans* by D-galactose may be attributed to both inhibiting AI-2 activity and competitive inhibition of lectin-mediated adhesion. The fact that D-galactose increased biofilm formation of *S. oralis* and *S. mitis* suggests the involvement of another mechanism. It is well known that there is an antagonistic relationship between *S. mutans* and *Streptococcus sanguinis*. A recent report evaluated the effect of D-cysteine on biofilm formation by *S. mutans* and *S. sanguinis* (Guo et al., 2009). D-Cysteine inhibited biofilm formation of *S. mutans*, whereas it hardly affected *S. sanguinis* biofilm formation. In dual-species biofilm, D-cysteine increased the proportion of *S. sanguinis* and reduced extracellular polysaccharides and lactic acid production, suggesting a benefial role of D-cysteine in caries prevention and control.

In contrast to the biofilm growth, D-galactose enhanced the growth of *S. mutans* in the planktonic state at concentrations of 20 and 200 mM. *S. mutans* is known to metabolize galactose by the tagatose 6-phosphate and Leloir pathways (Abranches, Chen, & Burne, 2004). These results indicate that D-galactose differentially regulates the growth of oral streptococci in the biofilm state and planktonic state and support the hypothesis that the inhibition of *S. mutans* biofilm growth is partly attributable to AI-2 inhibition by D-galactose. There may be concerns about the enhanced growth rate of *S. mutans* by D-galactose. However, biofilm formation on teeth is a prerequisite for developing dental caries. D-galactose at concentrations ranging between 0.002 − 2 mM, which did not affect the growth of *S. mutans,* was able to significantly inhibit the biofilm formation of the bacterium.

S. oralis is known to produce hydrogen peroxide, which inhibits the growth of *S. mutans* and periodontal pathogens (Abranches et al., 2018). In human subjects, a probiotic mouthwash containing *S. oralis*, *S. uberis*, and lactic acid-deficient *S. rattus* significantly reduced the number of *S. mutans* and *Porphyromonas gingivalis* in saliva and subgingival plaque, respectively (Zahradnik et al., 2009). Moreover, *S. oralis* strain KJ3-induced hydrogen peroxide was demonstrated to exert a whitening effect on stained ceramic discs (Hillman, McDonell, Hillman, & Handfield, 2016).

In summary, D-galactose inhibited biofilm formation of *S. mutans* alone or in a coculture with *S. oralis* and *S. mitis* while it increased biofilm formation of oral commensals *S. oralis* and *S. mitis* at concentrations ranging from 2 to 200 mM. Although L-galactose also inhibited biofilm formation by *S. mutans* at a concentration of 200 mM or 20 mM, the inhibitory effect was considerably lower than that of D-galactose. The biofilm formation of *S. mutans* on the bovine teeth was reduced after application of D-galactose in a solution or paste form. Accordingly, D-galactose can be an excellent substance for the development of oral hygiene products to inhibit the biofilm growth of *S. mutans* and, at the same time, increase the biofilm growth of oral commensals, such as *S. oralis* and *S. mitis*.

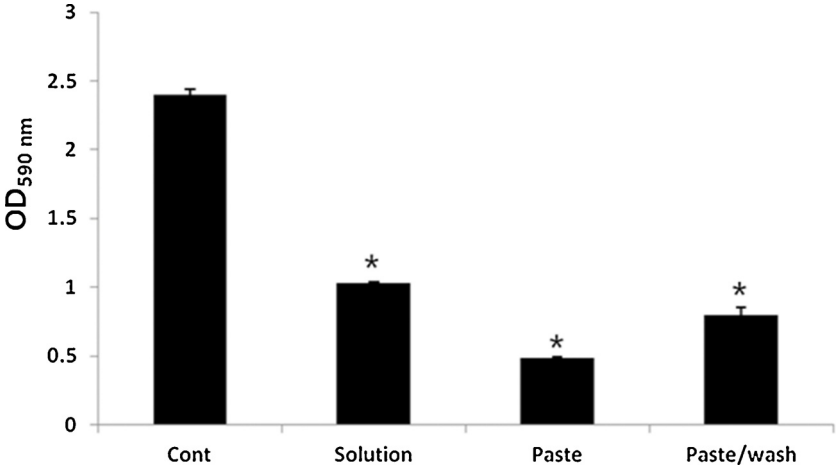

Fig. 7. Biofilm formation of *S. mutans* on bovine teeth pretreated with D-galactose. Bovine teeth with a circular opening with a diameter of 7 mm were treated with 100 mM D-galactose in a solution and a paste form prior to incubation with *S. mutans*. Biofilms formed on the teeth were stained with 1 % crystal violet, and the optical density at 590 nm of the destaining solution containing crystal violet was measured using a microplate reader. Experiments were repeated three times, and the results of a representative experiment were presented. $*p < 0.05$ and $**p < 0.001$ compared to the control group. Cont: CMC-coated bovine teeth were incubated with *S. mutans*.

Disclosure statement

This work covers the contents of patents (PC-KR 10-1870239; US 10,292,993) and patent applications (PC-CN 201580050473.5).

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Author contributions

Eun-Ju Ryu: acquisition of data, analysis of data, drafting the article.

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Jaehyun Sim: conception of the study.

Jun Sim: interpretation of data.

Julian Lee: conception of the study.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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