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Original Article

Inhibitory effects of *Streptococcus salivarius* K12 on formation of cariogenic biofilm

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KEYWORDS
Streptococcus salivarius K12;
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Streptococcus mutans;
Glucosyltransferase

Abstract *Bacground/purpose: Streptococcus salivarius* (*S. salivarius*) K12 is known to be a probiotic bacterium. The purpose of this study was to investigate anti-cariogenic effects of *S. salivarius* K12 on cariogenic biofilm.
Materials and methods: S. salivarius K12 was cultured in M17 broth. The antimicrobial activity of spent culture medium (SCM) against *Streptococcus mutans* was investigated. *S. salivarius* K12 was co-cultivated with *S. mutans* using a membrane insert. When the biofilm was formed using salivary bacteria and *S. mutans*, the K12 was inoculated every day. The biomass of biofilm was investigated by a confocal laser scanning microscope. Also, bacterial DNA from the biofilm was extracted, and then bacteria proportion was analyzed by quantitative PCR using specific primers. The expression of *gtf* genes of *S. mutans* in the biofilm with or without *S. salivarius* K12 was analyzed by RT-PCR.
Results: The SCM of *S. salivarius* K12 inhibited the growth of *S. mutans*. Also, *S. salivarius* K12 reduced *S. mutans* growth in co-cultivation. The formation of cariogenic biofilm was reduced by adding *S. salivarius* K12, and the count of *S. mutans* in the biofilm was also decreased in the presence of *S. salivarius* K12. *gtf*B, *gtf*C, and *gtf*D expression of *S. mutans* in the biofilm was reduced in the presence of *S. salivarius* K12.
Conclusion: S. salivarius K12 may inhibit the formation of cariogenic biofilm by interrupting the growth and glucosyltransferase production of *S. mutans*.

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Introduction

Dental caries is a phenomenon of demineralization of tooth surface by acid and closely related with cariogenic bacteria such as *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus* (*S. sobrinus*), and *Lactobacillus* species which have the characteristics of vigorous acid production and aciduricity.[1] Especially, *S. mutans* is considered to be more related to dental caries because of their action in the microbial ecosystem in oral cavity rather than the characteristics of this bacterium alone. *S. mutans* produces water-soluble or -insoluble glucans from sucrose using glucosyltransferases (Gtf) and plays an important role in the development of biofilm on tooth surface through the production of glucans.[2,3] Furthermore, in the formed oral biofilm, *S. mutans* makes a strongly acidic environment inside the biofilm by its continuous acid production in sugar rich condition.[3,4] This low pH environment increases the growth of aciduric bacteria as cariogenic bacteria, and the proliferation of non-mutans streptococci were suppressed.[5] The increased duration of low pH leads to demineralization of tooth, eventually inducing caries.

Streptococcus salivarius (*S. salivarius*) K12 is early colonizer in microbial ecology of oral cavity and is a species isolated from tongue swabbing.[6] The K12 strain produces bacteriocins and safety characteristics in demonstrated in tests in human and animal models.[7,8] Therefore, this bacterium is considered with probiotic bacterium. Probiotics are known to be 'live microorganisms, which when administered in adequate amounts, confer a health benefit on the host,[9] and probiotics have been attempted to be used for prevention and treatment of human diseases. Among probiotics, *S. salivarius* K12, and secretes two bacteriocin such as salivaricin A2 and B.[10] Since *S. salivarius* K12 is isolated from the oral cavity and secretes antibacterial agents, it is better applied to oral bacterial diseases than other probiotics.

The aim of this study was to be investigated anti-cariogenic effects of *S. salivarius* K12 on cariogenic biofilm containing *S. mutans* and on effect of the K 12 strain on biofilm-forming related factors of *S. mutans*.

Materials and methods

Bacterial strain and culture condition

S. salivarius K12 was kindly donated from Green store Inc. (Bactoblis; Seongnam, Gyeonggi, Korea) and used in this study. The bacterium was cultivated with M17 broth (BD bioscience, Sparks, MD, USA) supplemented with 1% glucose to manage it and make bacterial stock. In order to investigate antimicrobial activity of *S. salivarius* K12, *S. salivarius* and *S. mutans* ATCC 25175 were cultivated with tryptic soy broth (BHI; BD bioscience).

Antimicrobial activity of *S. salivarius* K12 against *S. mutans*

The antibacterial activity of *S. salivarius* K12 against *S. mutans* was evaluated by a minimum inhibitory concentration using a microdilution methods according to methods recommended by Clinical and Laboratory Standards Institute (CLSI).[11] A milliliters of *S. salivarius* (1×10^7 bacteria/ml) was inoculated into 10 ml M17 broth, and the bacterial suspension was incubated for 24 h in an aerobic condition. The K12 suspension was centrifuged at 5000×*g* for 10 min, and the supernatant was transferred into a new 15 ml conical tube (SPL Life Sciences, Gyeonggi, Korea). The supernatant was filtered with a polyvinylidene fluoride filter (Millipore, Billerica, MA, USA). The filtered supernatant as a spent culture medium (SCM) was used to susceptibility assay. 180 μl of M17 broth was dispensed into 96-well plate (SPL Life Sciences). The SCM was added into 1st well containing the fresh medium and performed 2-fold serial dilution to the 11th column. *S. mutans* was counted with a bacterial counting chamber (Hausser Scientific, Horsham, PA, USA) and adjusted 2×10^6 bacteria/ml with fresh M17 broth. The prepared *S. mutans* suspension (20 μl) was inoculated into the well containing the mixed media. The plate was incubated at 37 °C in an aerobic incubator. The bacterial growth was measured using optical density at 660 nm of wavelength by a microplate reader (BioTek, Winooski, VT, USA). In another experiment, *S. mutans* (1×10^6 bacteria/ml) and *S. salivarius* K12 (1×10^7 bacteria/ml or 1×10^8 bacteria/ml) were co-cultivated using Transwell™ (pore size 0.4 μm; Corning Co., Corning, NY, USA) in 12-well plate. The bacterial suspension of *S. mutans* and *S. salivarius* were inoculated into inside and outside of Transwell™, respectively, and the plate was incubated at 37 °C for 24 h. The growth of *S. mutans* was measured optical density at 660 nm of wavelength, and the image of *S. mutans* was taken by CMOS camera of phase contrast microscope (Nikon Co., Tokyo, Japan).

Biofilm formation and observation

Biofilm was formed a method reported by Lee SH[3]). First, to form pellicle on plate, unstimulated saliva was collected from 10 healthy person, and pooled saliva was centrifuged at 7000×*g* for 10 min at 4 °C to remove debris and bacteria. The prepared saliva was dispensed into 12-well polystyrene plate (SPL Life Sciences) and 8-well culture slide (Corning Co.) and dried in dry oven at 40 °C. This step was repeated 5 times. The plates were carried out UV sterilization. In order to form saliva biofilm, the pooled saliva was mixed with BHI broth including 2% sucrose and 1% mannose and vortexed for 20 s. *S. mutans* (1×10^6 bacteria/ml) was added into the mixture with saliva and BHI broth to form cariogenic biofilm, and the prepared suspension was inoculated into saliva-coated 12-well plate and 8-well culture slide. The plates were incubated in an aerobic condition for 7 days, and the media were changed every day. To investigate effects of *S. salivarius* on the biofilm, when the cariogenic biofilm was formed, one hundred microliter of *S. salivarius* K12 suspension (1×10^8 and 1×10^9 bacteria/ml) was inoculated with fresh medium change every day. The biofilm was washed three times with phosphate buffered saline (PBS, pH7.2) and BHI broth (1 ml) was added in the biofilm formed 12-well plate. The biofilms were disrupted with a scraper (Corning Co.), and the biofilm suspensions were transferred into 1.5 ml tube. After vortexing for 30 s, the suspensions were performed 10-fold dilution to 10^6 with

Table 1 Primer sequences for real-time PCR.

Gene	Primer sequence	
Universal	Forward	5′-TGG AGC ATG TGG TTT AAT TCG A-3′
	Reverse	5′-TGC GGG ACT TAA CCC AAC A-3′
Streptococcus spp	Forward	5′-AAG CAA CGC GAA GAA CCT TA-3′
	Reverse	5′-GTC TCG CTA GAG TGC CCA AC-3′
S. mutans	Forward	5′-CTA CAC TTT CGG GTG GCT TG-3′
	Reverse	5′-GAA GCT TTT CAC CAT TAG AAG CTG-3′
S. salivarius	Forward	5′-TTG CCA CAT CTT CAC TCG CTT-3′
	Reverse	5′-GTT TAG TAG CAA CTT CTG GCT T-3′
*gtf*B	Forward	5′-AGC AAT GCA GCC AAT CTA CAA AT-3′
	Reverse	5′ -ACG AAC TTT GCC GTT ATT GTC A-3′
*gtf*C	Forward	5′ -CTC AAC CAA CCG CCA CTG TT-3′
	Reverse	5′ -GGT TAA CGT CAA AAT TAG CTG TAT TAG C-3′
*gft*D	Forward	5′-CAC AGG CAA AAG CTG AAT TAC A-3′
	Reverse	5′ -GAT GGC CGC TAA GTC AAC AG-3′
*rec*A	Forward	5′ -CTA ATT CAC CTG TAC GAG-3′
	Reverse	5′ -CCG AAT CTT CTG TAA G-3′

*gtf*B: glucosyltransferase B, *gtf*C: glucosyltransferase C, *gtf*D: glucosyltransferase D, and *rec*A: recombinase A.

BHI broth. The diluted suspensions were spread on Mitis-salivarius agar plate and Mitis-salivarius bacitracin agar plate (BD bioscience) to count oral streptococci and *S. mutans*, respectively. The plates were incubated at 37 °C for 48 h, and the colonies of oral streptococci and *S. mutans* were counted. In other experiment, the biofilm on 8-well culture slide was washed three times with PBS and stained with Live/dead bacterial viability kit (Invitrogen, Eugene, OR, USA) for 1 h at room temperature. After washing with PBS, the biofilm was observed by a confocal laser scanning microscope (CLSM) (LSM 700; Carl-Zeiss, Oberkochen, Germany). For 3D scanning of the biofilm, the z-stack scans were performed (0—30 μm), and the images of the biofilm were analyzed by ZEN program (Carl-Zeiss). To measure biofilm mass, after saving each slide of 3D image, dimension of fluorescence (bacteria) in each slide was measured by ImageJ software (National Institutes of Health, NY, USA). Percentage of bacteria biomass in each slide was calibrated by correlating the image dimension (160 μm × 160 μm) and each level of slides was sum it to calculate biofilm mass.

Investigation of bacterial proportion in the biofilm

To examine bacterial proportion in the biofilm, the biofilm was formed with or without *S. salivarius* K12 by method of 2.3. Biofilm formation and observation, and total bacterial DNA was extracted with bacterial genomic DNA extraction kit (iNtRON biotechnology, Gyeonggi, Korea). To generate standard curve, *S. mutans* and *S. salivarius* were counted by a bacterial counting chamber (Hausser Scientific), and 1×10^7 bacteria/ml of the bacteria was serially diluted to 10^5. Each diluted bacterial suspensions were harvested by a centrifugation at 7000×*g* for 10 min, and the supernatant was removed. Genomic DNA from the bacterial pellets were extracted with the kit. Extracted DNA (4 μl) was mixed with 25 μl of 2 × TB Green™ premix Ex Taq™ (Takara Co., Kyoto, Japan), 0.2 μM of each primer, ROX reference dye, and distilled water in 50 μl of final volume. The mixture was carried out PCR with 10 min template denaturation step at 94 °C and 40 cycles of amplification step (denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 33 s) with 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primers for real-time PCR are shown in Table 1. The bacteria level was calculated by critical threshold cycle (Ct) compared with generated standard curve from Ct values and standard bacterial count.

Change of virulent factor of *S. mutans* by *S. salivarius* K12

Total RNA from bacteria in the biofilm was extracted with a TRIzol® Max bacterial RNA isolation kit (Invitrogen Life

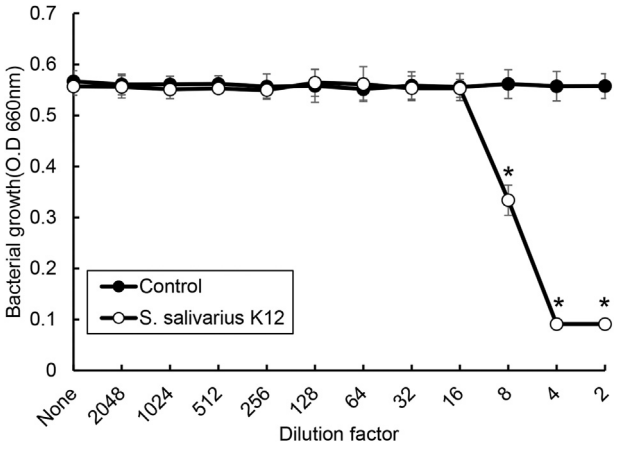

Figure 1 The Antimicrobial activity of *S. salivarius* K12 against *S. mutans*. After collecting the spent culture medium from *S. salivarius* K12, *S. mutans* was cultivated in various concentration of the SCM, and the bacterial growth was measured by optical density using a spectrophotometer. The experiment was performed three times in duplicate, and data are represented as mean ± standard deviation. * (asterisk) indicates statistically significant differences compared to control.

Tech, Carlsbad, CA) according to the manufacturer's recommended protocol. cDNA was synthesized by gene-specific primers as follows: 5′-CAT AAG GCG TTA ATT TCC CTT CA-3′ for *gtf*B, 5′-CCT GTG AAG TTA GCT TGC TAT TG-3′ for *gtf*C, and 5′-ATA GGC TGT CTT ATC GCT GTT GCT A-3′ for *gtf*D.[12] 1 μg of total RNA, 2 μM of gene-specific primer, 10 mM dNTA mix, and 200 U of SuperScript™ IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was mixed, and the mixture was incubated at 50 °C for 10 min and heated at 80 °C for 10 min to inactivate reaction. cDNAs were mixed with 10 μl of 2 × TB Green™ premix Ex Taq™ (Takara Co.), 0.4 μM of each primer, ROX reference dye, and distilled water in 20 μl of final volume. The mixture was carried out PCR with 5 min template denaturation step at 95 °C and 40 cycles of amplification step (denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 33 s) with 7500 real-time PCR system (Applied Biosystems). The PCR products were examined for each specific amplification product using a melting temperature. The primers for real-time PCR are shown in Table 1 recA gene was used as housekeeping gene.[12,13]

Statistical analysis

The data was investigated distribution using the Kolmogorov–Smirnov test. Significant differences between each group were analyzed by Kruskal Wallis test and Mann–Whitney U test using IBM SPSS statistics Ver. 23 (IBM, Armonk, NY, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Antibacterial activity of *S. salivarius* K12 against *S. mutans*

In antimicrobial experiment using SCM of *S. salivarius* K12, the growth of *S. mutans* was significantly inhibited at 8-fold diluted SCM and completely inhibited above 4-fold diluted SCM ($P < 0.05$) (Fig. 1). Next, in order to investigate the antibacterial activity when coexisting in the oral cavity, *S. mutans* was co-cultured with *S. salivarius* k12 of 10 and

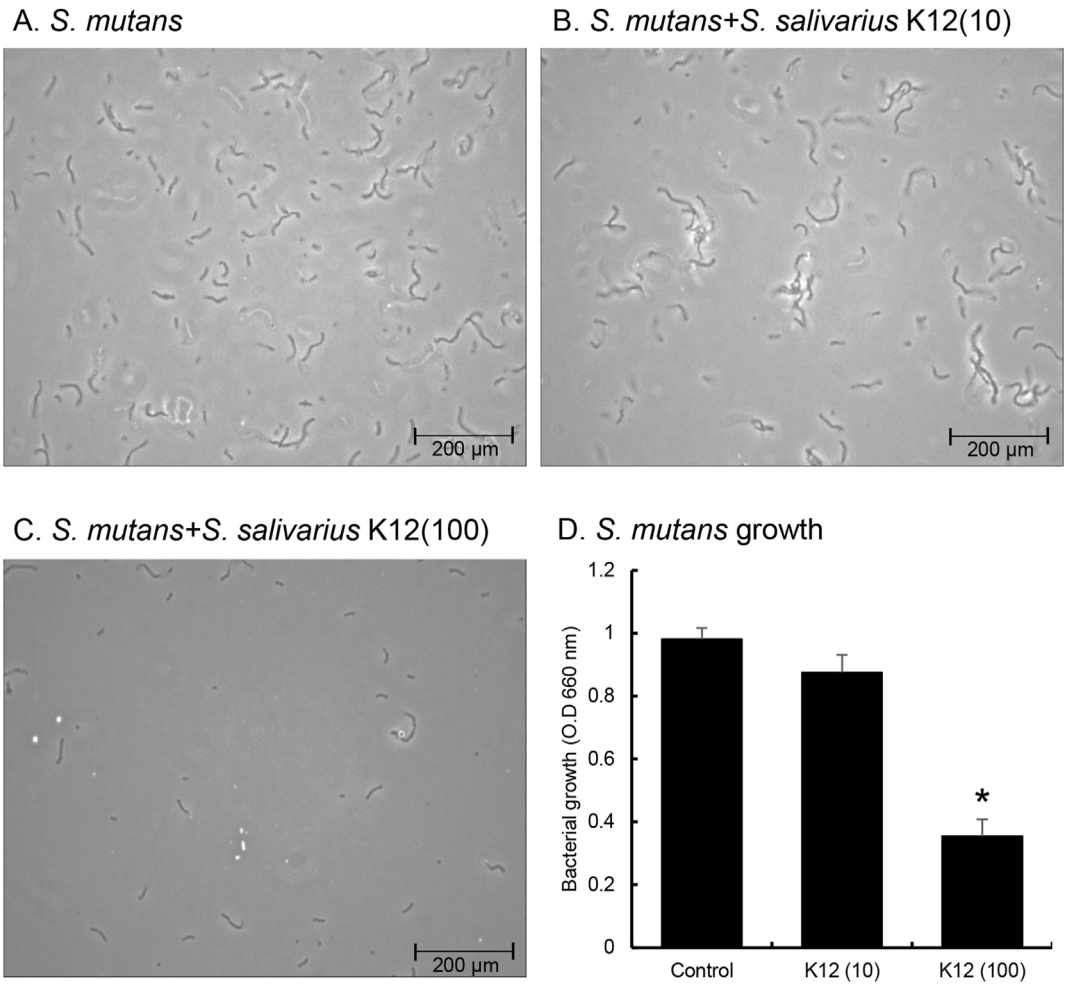

Figure 2 The growth of *S. mutans* in co-cultivation with *S. salivarius*. *S. mutans* was inoculated into inside of cell culture insert(A), and 10- or 100-fold of *S. salivarius* K12 was inoculated into outside of cell culture insert (B and C). The plate was incubated for 24 h, and the image of the suspension of *S. mutans* was obtained by a camera on the microscope (A, B, and C). The growth of *S. mutans* was measured by a spectrophotometer (D). The experiment was performed three times in duplicate, and data are represented as mean ± standard deviation. * (asterisk) indicates statistically significant differences compared to control.

100-fold concentration using Transwell. The growth of *S. mutans* was significantly inhibited by 100-fold concentration of *S. salivarius* K12 (Fig. 2).

Inhibition of cariogenic biofilm formation

When biofilm was formed using salivary bacteria added *S. mutans* for 7 days, cariogenic biofilm was confirmed by measuring pH of biofilm suspension using distilled water (data not shown). Next, when cariogenic biofilm was formed, *S. salivarius* K12 was treated at two concentrations. The biofilm formation was reduced when *S. salivarius* K12 was treated at a concentration of 10^7 cells (Fig. 3). Also, when biofilm biomass was calculated by ImageJ software, control sample showed biofilm biomass of $3.25 \times 10^5 \pm 8.86 \times 10^3$ μm,^{2} and *S. salivarius* K12-treated samples showed $3.19 \times 10^5 \pm 1.52 \times 10^3$ μm^2 and $2.00 \times 10^5 \pm 1.02 \times 10^3$ μm^2 in concentration of 10^7 and 10^8 cells, respectively (Fig. 3D). Comparing the control sample based on 100%, the levels of biofilm in *S. salivarius* K12-treated samples was 98.26 ± 2.44% (10^7 cells) and 63.31 ± 1.49% (10^8 cells).

Bacterial proportion in the biofilm

Next, to investigate whether *S. salivarius* K12 merely inhibits biofilm formation or alters the bacterial proportion in the biofilm, genomic DNA from total bacteria of the biofilm was extracted, and quantitative PCR was processed using the DNA. The average amount of total bacteria in the biofilm showed 2.05×10^8 cells/biofilm, and in the biofilm treated with 10^7 and 10^8 concentration of *S. salivarius* K12, the average amount of bacteria showed 1.90×10^8 and 1.47×10^8 cells/biofilm, respectively (Fig. 4A). When the number of oral streptococci in the biofilm were investigated, control and *S. salivarius* K12 (10^7) and (10^8)-treated sample showed the average level of 1.86×10^8, 1.56×10^8, and 1.32×10^8 cells/biofilm, respectively (Fig. 4B). Total bacteria and oral streptococci in the biofilm were significantly decreased by *S. salivarius* K12 at a concentration of 10^8 cells ($P < 0.05$). The amount of *S. mutans* in the biofilm was significantly reduced in both concentrations treated with *S. salivarius* K12 compared to control ($P < 0.05$) (Fig. 4C). On the other hand, the amount of *S. salivarius* in the biofilm was significantly increased in both concentrations treated

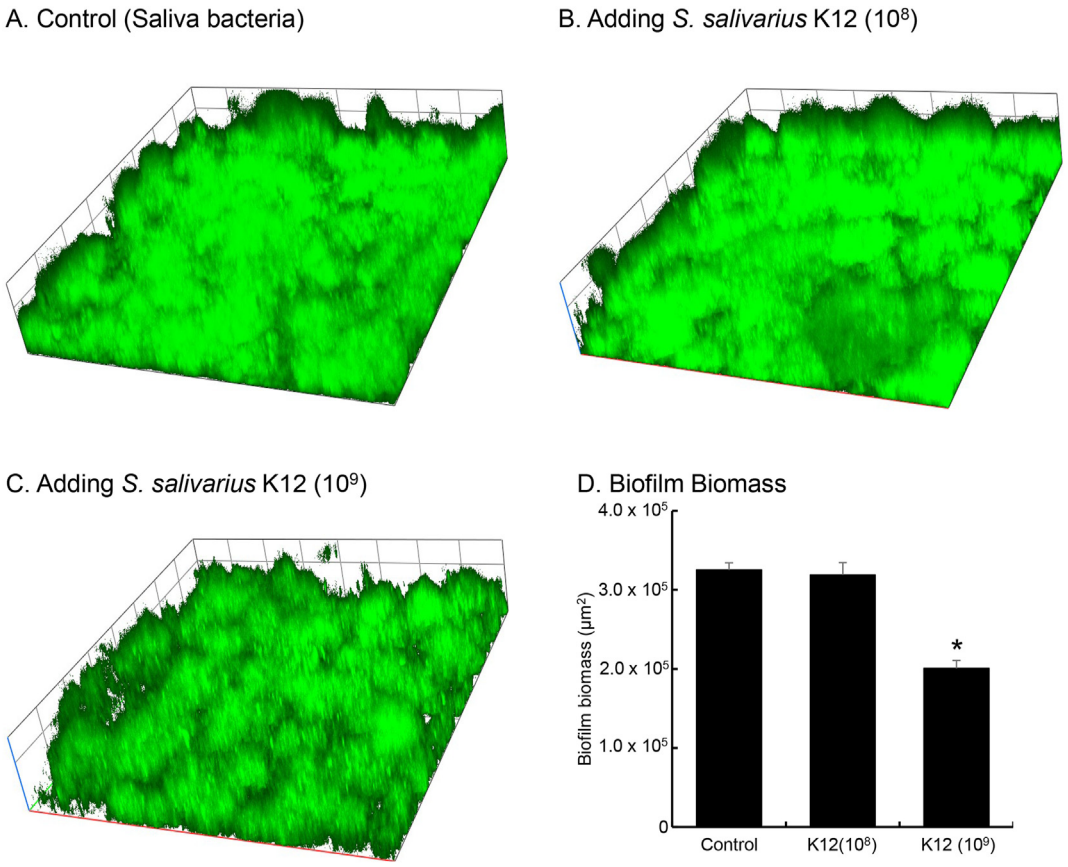

Figure 3 3D image of salivary biofilm. When the biofilm was formed with salivary bacteria and *S. mutans*, *S. salivarius* K12 was inoculated every day at a concentration of 10^8 (B) and 10^9 cells (C). The 3D image of biofilm was acquired by CLSM. The biomass of biofilm was measured by Image J software (D). The experiment was performed three times in duplicate, and data are represented as mean ± standard deviation. * (asterisk) indicates statistically significant differences compared to control.

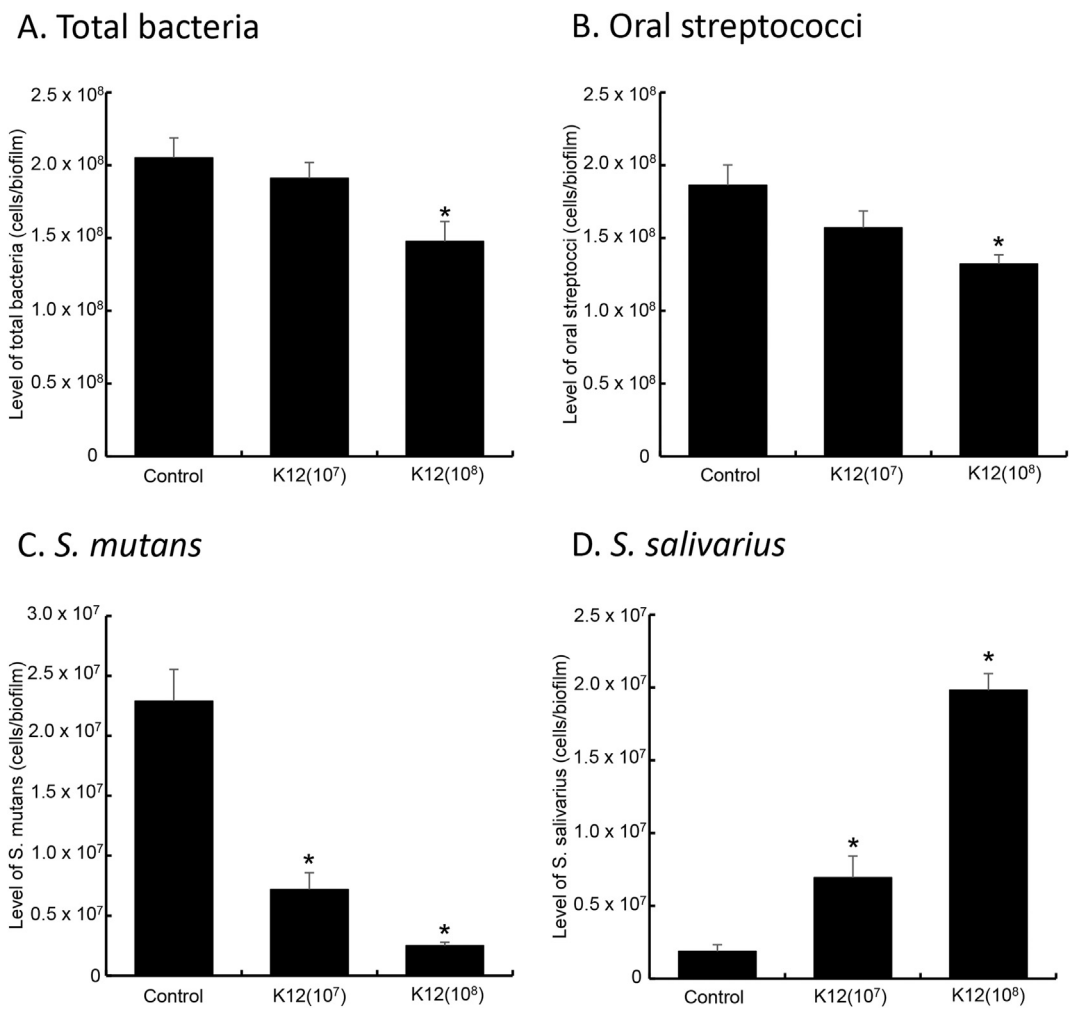

Figure 4 Bacterial proportion in the biofilm. When the biofilm was formed with salivary bacteria and *S. mutans*, *S. salivarius* K12 was inoculated every day at a concentration of 10^8 and 10^9 cells. After extracting total DNA, total bacteria (A), oral streptococci (B), *S. mutans* (C), and *S. salivarius* (C) in the biofilm was analyzed by qPCR using specific primers. The experiment was performed three times in triplicate, and data are represented as mean ± standard deviation. * (asterisk) indicates statistically significant differences compared to control.

with *S. salivarius* K12 ($P < 0.05$) (Fig. 4D). These results indicated that *S. salivarius* K12 colonizes in oral biofilm, thereby may inhibit the growth of *S. mutans*.

Reduction of virulent factor of *S. mutans* in the biofilm by *S. salivarius* K12

Finally, it was investigated whether the inhibition of biofilm formation by *S. salivarius* K12 is only caused by the reduction of the growth of *S. mutans*, or it also inhibits the expression of factors related with biofilm formation of *S. mutans*. Thus, it investigated the *gtf* expression of *S. mutans* in the biofilm was investigated. The expression of *gtf*B, *gtf*C, and *gtf*D was significantly reduced in *S. mutans* of *S. salivarius* K12 treated biofilm ($P < 0.05$) (Fig. 5).

Discussion

Recently, it is tried to treat and prevent diseases caused by pathogenic bacteria using beneficial bacteria.[14–16] These attempts have led to more research about probiotics. In dentistry, although many studies have been conducted to apply probiotics to bacteria-related diseases,[9] most probiotics have aciduricity and can induce dental caries.[17,18] *S. salivarius* is an early colonizer on epithelial surface of oral cavity in infant[5] and produces bacteriocin.[19] Among *S. salivarius*, *S. salivarius* K12 in known to be a probiotic bacterium and has strong antimicrobial activity against various bacteria and fungus.[20,21] The present study was to investigate effect of *S. salivarius* K12 on cariogenic biofilm and its mechanism for inhibiting formation of cariogenic biofilm.

First, *S. mutans* is considered to be a bacterium closely associated with cariogenic biofilm which creates localized low acidic environment.[3] Therefore, the susceptibility of *S. mutans* using spent culture medium (SCM) of *S. salivarius* K12 was investigated. The SCM of *S. salivarius* K12 inhibited the growth of *S. mutans* at or above the 8-fold dilution. Comparing SCM of other probiotics, the SCM of *S. salivarius* K12 showed weakly antimicrobial activity.[22] Therefore, it was evaluated using co-cultivation whether the growth of *S.*

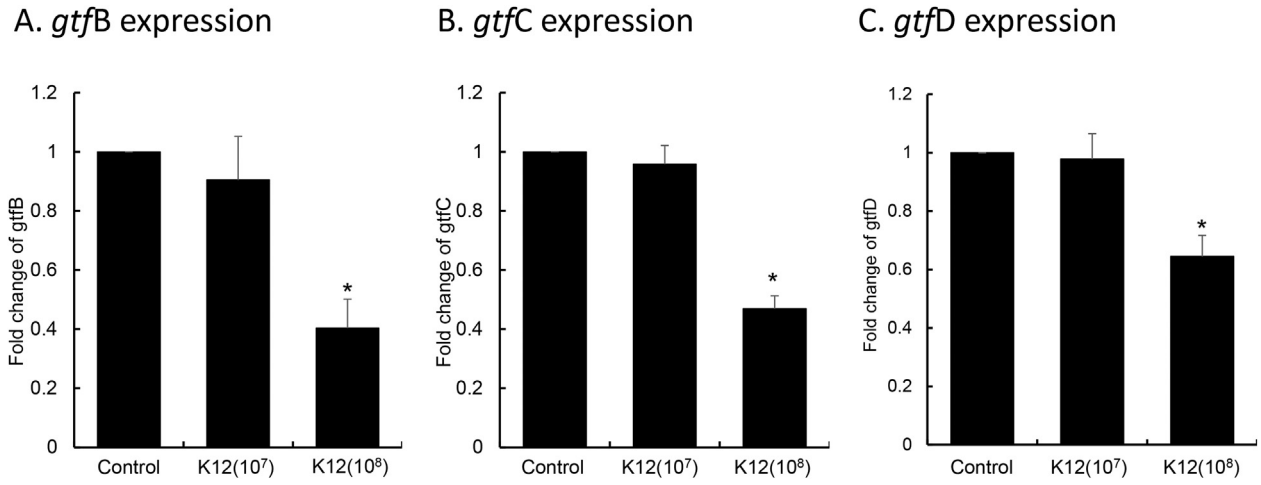

Figure 5 Effects of *S. salivarius* K12 on the expression of *gtf* genes of *S. mutans* in the biofilm. When the biofilm was formed with salivary bacteria and *S. mutans*, *S. salivarius* K12 was inoculated every day at a concentration of 10^8 and 10^9 cells. After extracting total RNA, the expression of gtfB (A), gtfC(B), and gtf D(C) was analyzed by RT-PCR. The experiment was performed three times in triplicate, and data are represented as mean ± standard deviation. * (asterisk) indicates statistically significant differences compared to control.

mutans was inhibited when *S. salivarius* K12 was present at which concentration. In co-cultivation of *S. mutans* and *S. salivarius* K12, *S. mutans* may inhibit the growth and metabolism of *S. salivarius* K12 due to production of abundant lactic acid,[23] and *S. salivarius* K12 may inhibit the growth of *S. mutans* by its salivaricin as a bacteriocin.[24] *S. salivarius* K12 inhibited *S. mutans* growth at 100-fold cells of initial inoculating concentration. These results showed the potential of *S. salivarius* K12 as a candidate probiotic for the prevention of dental caries induced by *S. mutans*. Therefore, effects of *S. salivarius* K12 on cariogenic biofilm using salivary bacteria and *S. mutans* was examined.

When salivary bacteria collected from healthy doner and *S. mutans* was formed biofilm for 7 days, the biofilm was considered to be a cariogenic biofilm because it showed a low pH (below pH 5.5) enough to induce dental caries,[25] and the effect of *S. salivarius* K12 on cariogenic biofilm was tested. To evaluated efficacy amount of *S. salivarius* K12 application for cariogenic biofilm, 10^7 and 10^8 cells of *S. salivarius* K12 were treated in forming biofilm every day (the concentrations showed antimicrobial effect in the co-cultivation). The formation of biofilm was reduced in treated with *S. salivarius* K12. When forming a biofilm, *S. salivarius* K12 inhibited the biofilm despite the sucrose-rich environment. These results indicate the possibility that dental caries can be prevented by continuous use of *S. salivarius* K12 products. Whether it is a healthy biofilm or cariogenic biofilm according to the microbial ecosystem was suggested by Marsh, P.D.[26] When the distribution of *S. mutans* in the biofilm was examined based on oral streptococci, the levels of *S. mutans* showed an average of 12.3% in the control group, and the ratio of *S. mutans* was 4.5% and 1.9% in the *S. salivarius* K12-treated group in a dose-dependent manner. Furthermore, the levels of *S. salivarius* was increased in the biofilm in the *S. salivarius* K12-treated group compared to control group. Finally, data showing that *S. salivarius* K12 may convert cariogenic biofilm to healthy biofilm.

The glucosyltransferases of *S. mutans* plays an important role in biofilm formation.[27] Therefore, the *gtf* gene expression of *S. mutans* in the biofilm was investigated using a real-time RT-PCR. When 10^7 and 10^8 cells of *S. salivarius* K12 were treated in forming biofilm every day, the expression of *gtf*B, C and D of *S. mutans* in the biofilm was significantly reduced. Eventually, *S. salivarius* K12 may inhibit the expression of *gtf* genes as well as *S. mutans* growth.

In conclusion, *S. salivarius* K12 inhibited the formation of cariogenic biofilm as well as the inhibition of *S. mutans* growth in the biofilm. These effects may appear by inhibition of the growth and glucosyltransferases production of *S. mutans* by antimicrobial activity of *S. salivarius* K12. Furthermore, *S. salivarius* K12 may colonize into oral biofilm. Therefore, among probiotics, *S. salivarius* K12 may be considered to be an effective candidate for the prevention of dental caries.

Declaration of competing interest

The authors claim to have no conflicts of interest related to this paper.

Acknowledgments

None.

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