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Effect of Bacteriocin-Like Inhibitory Substance (BLIS) from *Enterococcus faecium* DB1 on Cariogenic *Streptococcus mutans* Biofilm Formation

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Abstract The aim of the study was to investigate the effect of bacteriocin-like inhibitory substance (BLIS) from *Enterococcus faecium* DB1 on cariogenic *Streptococcus mutans* biofilm. Crystal violet staining, fluorescence, and scanning electron microscopy analyses demonstrated that the BLIS from *Enterococcus faecium* DB1 (DB1 BLIS) inhibited *S. mutans* biofilm. When DB1 BLIS was co-incubated with *S. mutans*, biofilm formation by *S. mutans* was significantly reduced ($p < 0.05$). DB1 BLIS also destroyed the preformed biofilm of *S. mutans*. In addition, DB1 BLIS decreased the viability of *S. mutans* biofilm cells during the development of biofilm formation and in the preformed biofilm. DB1 BLIS significantly decreased the growth of *S. mutans* planktonic cells. Furthermore, *S. mutans* biofilm on the surface of saliva-coated hydroxyapatite discs was reduced by DB1 BLIS. Taken together, DB1 BLIS might be useful as a preventive and therapeutic agent against dental caries caused by *S. mutans*.

Keywords *Enterococcus faecium*, bacteriocin-like inhibitory substance, *Streptococcus mutans*, biofilm, dental caries

Introduction

It is currently recognized that the bacterial viability of probiotics is not an essential requirement for physiological benefits to the host (Cuevas-González et al., 2020). Accumulating evidence shows that postbiotics, that is, cell components or metabolites derived from probiotics, exert probiotic effects (Teame et al., 2020). Postbiotics are soluble bioactive compounds, such as organic acids, short-chain fatty acids, antimicrobial peptides including bacteriocins, enzymes, and vitamins (Moradi et al., 2019; Nataraj et al., 2020). Among the many beneficial effects of postbiotic compounds on the host health are antagonistic activities against pathogenic bacteria and antitumor and immunomodulatory effects (Teame et al., 2020). Several advantages of postbiotics over probiotics have been suggested, such as no risk of a blood infection, suitable

absorption, directly interacting with the host and stimulating a range of biological responses, higher stability, and easier to standardize and incorporate into functional foods and pharmaceutical products (Aguilar-Toalá et al., 2018; Homayoni Rad et al., 2012).

Biofilm formed by *Streptococcus mutans* on tooth and dentine surfaces is a major cause of dental caries (Tong et al., 2014). During the transition from planktonic to biofilm cells, *S. mutans* changes its physiological characteristics (Ahn et al., 2018). For example, glucans synthesized by *S. mutans* from fermentable sugars, such as sucrose, via glucosyltransferases enhance bacterial adhesion to tooth surfaces and bacterial cell-to-cell adhesion, contributing to cariogenic biofilm formation (Jeong et al., 2013). It is known that bacteria in the biofilm are 10 to 1,000 times more resistant to antibiotics than planktonic bacteria (Penesyant et al., 2015). Hence, inhibiting or removing *S. mutans* biofilm to prevent dental caries remains a challenge in oral health care. Although a variety of cariostatic agents have been developed and clinically used to control *S. mutans*, their use is controversial because of some adverse side effects, including host cytotoxicity (Arabaci et al., 2013). Thus, alternative agents with minimal adverse effects are needed to control *S. mutans*.

Bacteriocins are small, cationic, membrane-active, antimicrobial peptides ribosomally synthesized by lactic acid bacteria with bacteriostatic or bactericidal functions (Perez et al., 2014). A most attractive feature of bacteriocins as clinical antimicrobial candidates is their GRAS (generally regarded as safe) status (Imran, 2016). A recent review has already described the role of bacteriocins in preventing biofilm formation and eradicating existing biofilms (Mathur et al., 2018). Certain antibiotic-resistant, infectious strains among enterococci, including *Enterococcus faecium*, have been reported, but some members of enterococci are used as probiotics (Hanchi et al., 2018). Although bacteriocin-like inhibitory substance (BLIS) produced by *E. faecium* DB1 exerted antimicrobial activity against *Listeria monocytogenes* (Choi et al., 2011), it has not been well documented whether BLIS from *E. faecium* DB1 (DB1 BLIS) inhibits *S. mutans* biofilm. Therefore, in this study, we examined whether DB1 BLIS exerts an antibiofilm effect against *S. mutans*.

Materials and Methods

Bacterial strains and culture conditions

E. faecium DB1 was kindly provided by Prof. Wang June Kim (Dongguk University, Goyang, Korea). Cariogenic *S. mutans* KCTC 3065 was purchased from the Korean Collection for Type Cultures (KCTC, Jeongeup, Korea). *E. faecium* DB1 was grown in de Man, Rogosa, and Sharpe (MRS) medium (Neogen, Lansing, MI, USA) at 37°C, and *S. mutans* KCTC 3065 was maintained in brain heart infusion (BHI) medium (Becton Dickinson, Sparks, MD, USA) at 37°C.

DB1 bacteriocin-like inhibitory substance (BLIS) extraction

DB1 BLIS was isolated as described previously (Kim et al., 2019). In brief, *E. faecium* DB1 was grown in MRS broth at 37°C for 24 h. Afterward, cell-free supernatants were obtained by filtering the bacterial suspension through a 0.2- μ m membrane filter. DB1 BLIS in the cell-free supernatants was precipitated by adding 70% saturation of ammonium sulfate (Samchum Chemical, Seoul, Korea) at 4°C overnight and collected by centrifugation at 17,000 \times g for 15 min. Precipitated DB1 BLIS was resuspended in distilled water, then placed in dialysis tubing (molecular weight cutoff 1,200; Sigma-Aldrich, St. Louis, MO, USA) and dialyzed against distilled water at 4°C for 24 h. DB1 BLIS was diluted in phosphate-buffered saline (PBS) for further experiments. The concentration of DB1 BLIS was determined by the bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL, USA).

Measurement of biofilm using crystal violet assay

Overnight culture of *S. mutans* was diluted to 5×10^7 CFU/mL. The diluted bacterial suspension in BHI broth containing sucrose (0.05%) were transferred to wells of a 96-well microtiter plate with or without DB1 BLIS (5 mg/mL) and incubated at 37°C for the indicated times. To investigate whether the preformed biofilm of *S. mutans* was disrupted by DB1 BLIS, *S. mutans* biofilm was formed at 37°C for 24 h without DB1 BLIS. Afterward, the preformed biofilm was treated with DB1 BLIS (5 mg/mL) for 1, 3, 6, 12, and 24 h. Unattached bacteria were removed by gently rinsing with PBS and *S. mutans* biofilm was stained with 0.1% crystal violet for 30 min. The stained *S. mutans* biofilm was washed again with PBS to remove non-specific staining and dissolved in a mixture of 0.1% acetic acid and 95% ethanol. The biofilm formation by *S. mutans* was determined by measuring the absorbance at 595 nm using a microtiter plate reader (AMR-100, Allsheng, Hangzhou, China).

Viability of *Streptococcus mutans* biofilm cells

A tetrazolium salt, sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT), is reduced by metabolically active cells to water-soluble formazan (Wadhawan et al., 2010). Since the XTT reduction assay has commonly used for the viability of *S. mutans* within biofilm (Liu et al., 2017), we examined the viability of *S. mutans* biofilm. After biofilm formation by *S. mutans* in the presence or absence of DB1 BLIS (5.0 mg/mL) at 37°C for 1, 3, 6, 12, and 24 h, the biofilm was washed with PBS, followed by the addition of 100 μ L of XTT (Biotium, Fermtom, CA, USA) in 200 μ L PBS and incubation at 37°C for 5 h. To examine the viability of preformed *S. mutans* biofilm cells treated with DB1 BLIS, *S. mutans* was incubated for 24 h, followed by DB1 BLIS (5.0 mg/mL) treatment for 1, 3, 6, 12, and 24 h. The treated *S. mutans* biofilm was washed with PBS, followed by the addition of 100 μ L XTT in 200 μ L PBS and incubation at 37°C for 5 h. The viability of *S. mutans* biofilm cells was determined by measuring the absorbance at 492 nm. The optical density of the background at 630 nm was subtracted from the measured absorbance at 492 nm for normalization.

Confocal laser scanning microscopy

S. mutans was grown in BHI broth containing 0.05% sucrose on cover glass-bottom dishes (SPL Life Sciences, Pocheon, Korea) with or without DB1 BLIS (5.0 mg/mL) at 37°C for 24 h. After washing with PBS, *S. mutans* biofilm cells were stained for 10 min using the LIVE/DEAD Bacterial Viability Kit containing SYTO9 and propidium iodide (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. *S. mutans* biofilm was observed under a confocal laser scanning microscope (Eclipse Ti-E, Nikon, Tokyo, Japan).

Scanning electron microscopy

S. mutans was cultured in BHI broth containing 0.05% sucrose on a coverslip in the wells of a 24-well culture plate at 37°C for 24 h with or without DB1 BLIS (5.0 mg/mL). Non-adherent *S. mutans* was removed by washing with PBS. *S. mutans* biofilm was fixed with PBS containing 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C overnight and dehydrated by replacing the buffer with increasing grades of ethanol (70%, 80%, 90%, 95%, and 99% for 15 min each). *S. mutans* biofilm was dried with hexamethyldisilazane for 15 min, sputter-coated using an ion sputter coater (E-1010, Hitachi, Tokyo, Japan), and examined under a scanning electron microscope (S-3000N, Hitachi) at $\times 1,000$ and $\times 5,000$ magnifications.

Growth of *Streptococcus mutans* planktonic cells

S. mutans was cultured with or without DB1 BLIS (5.0 mg/mL) in BHI broth containing 0.05% sucrose at 37°C for 1, 3, 6,

12, and 24 h. After incubation for the indicated times, the growth of *S. mutans* planktonic cells was determined by measuring the absorbance at 595 nm. Furthermore, the culture of *S. mutans* planktonic cells was serially diluted and enumerated by plating on BHI agar plates.

Saliva-coated hydroxyapatite (HA) disc assay

The saliva-coated HA assay was performed as previously described (Ansari et al., 2017). Sterile saliva-coated HA discs of 9 mm in diameter (3D Biotek, Bridgewater, NJ, USA) were placed in the wells of a 48-well microtiter plate and coated with human saliva at 37°C for 2 h. Bacterial suspension of *S. mutans* diluted in BHI broth containing 0.05% sucrose was added to the wells and incubated with or without DB1 BLIS at 37°C for 48 h. After incubation, saliva-coated HA discs were rinsed with PBS to remove planktonic bacteria and transferred to conical tubes containing PBS. After vigorous vortex mixing and sonication, the supernatants were collected and serially diluted for enumeration (CFU per disc) on BHI agar.

Statistical analysis

All data are expressed as mean±SD of triplicate samples. Statistical comparisons between the control and treated groups were determined at $p < 0.05$ by unpaired two tailed t-test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data are representative of two or three independent experiments.

Results and Discussion

Different concentrations of DB1 BLIS (0.6, 1.2, 2.5, and 5.0 mg/mL) were tested antibiofilm activity against *S. mutans*. Particularly, 5.0 mg/mL of DB1 BLIS inhibited *S. mutans* biofilm by approximately 50% (data not shown). Therefore, this concentration of DB1 BLIS (5.0 mg/mL) was used for further experiments. When *S. mutans* was co-incubated with DB1 BLIS for 1, 3, 6, 12, and 24 h, DB1 BLIS significantly inhibited *S. mutans* biofilm at 3, 6, 12, and 24 h ($p < 0.05$; Fig. 1A). The decreased viability of *S. mutans* biofilm cells in the presence of DB1 BLIS was confirmed by the XTT assay. As shown in Fig. 1B, the viability was significantly reduced at 3, 6, 12, and 24 h in the presence of DB1 BLIS ($p < 0.05$). To examine whether DB1 BLIS eradicates established *S. mutans* biofilm, preformed 24-h biofilm was treated with DB1 BLIS and incubated for 1, 3, 6, 12, and 24 h. The preformed *S. mutans* biofilm was significantly reduced by DB1 BLIS treatment for 6, 12, and 24 h ($p < 0.05$) but not by the shorter 1- and 3-h treatments (Fig. 1C). There was also reduced viability of preformed *S. mutans* biofilm cells for all treatments (3, 6, 12, and 24 h), except for the 1 h-treatment (Fig. 1D). Furthermore, microscopic analyses indicated that DB1 BLIS inhibited biofilm formation and aggregation of *S. mutans*. The biofilm was comprised of dense, thick layers with heterogeneously aggregated cells. However, a relatively less dense and sparsely populated biofilm was observed in the presence of DB1 BLIS (Figs. 2A and B). Studies have already shown the inhibition of *S. mutans* biofilm by probiotics, such as *Lactobacillus plantarum* (Zhang et al., 2020) and *Lactobacillus salivarius* (Wu et al., 2015). However, acidogenic lactobacilli are closely associated with the process of dental caries (Chhour et al., 2005; Yang et al., 2010), posing a potential dental health risk. By contrast, the bacteriocin activity of postbiotics does not have inherent cariogenic activities, such as acid production. It is widely demonstrated that bacteriocins derived from lactic acid bacteria inhibit the biofilm formation of several pathogens. Nisin of *Lactococcus lactis* decreased the biofilm formation of methicillin-resistant *Staphylococcus aureus* (Okuda et al., 2013). In our previous studies of inhibition of foodborne pathogens, we showed that *Lactobacillus brevis* bacteriocin effectively inhibited the biofilm formation of *Escherichia coli* and *Salmonella* Typhimurium

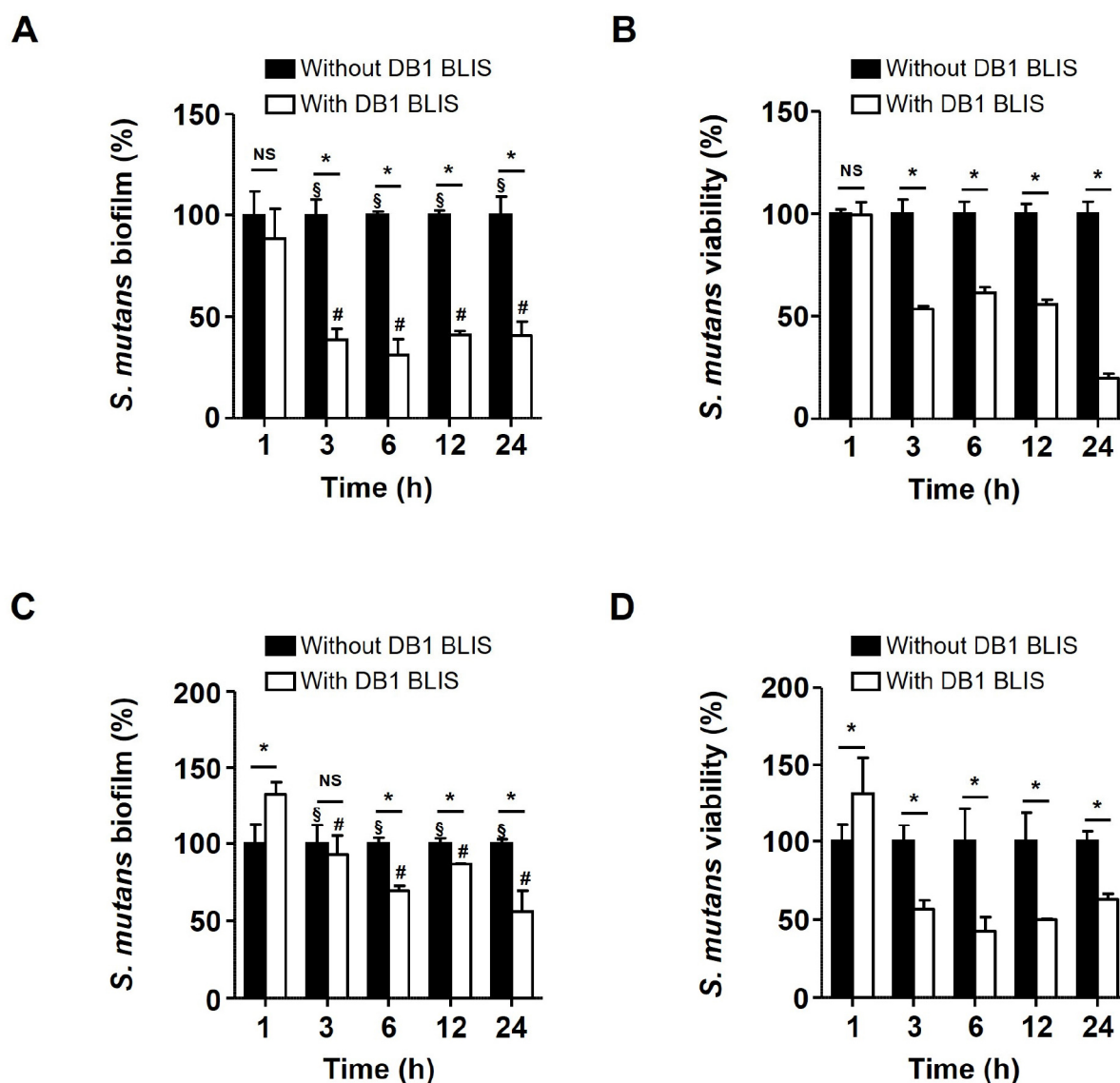


Fig. 1. Inhibitory effect of DB1 BLIS against biofilm formation by *Streptococcus mutans*. (A) To examine whether DB1 BLIS inhibits the biofilm formation, *S. mutans* was co-incubated with DB1 BLIS (5.0 mg/mL) for 1, 3, 6, 12, and 24 h at 37°C. *S. mutans* biofilm was assessed by staining with crystal violet. (B) After co-incubation of *S. mutans* with DB1 BLIS for the indicated times, biofilm cells of *S. mutans* were subjected to the XTT assay. (C) To examine whether DB1 bacteriocin disrupts the established biofilm, *S. mutans* was pre-incubated at 37°C for 24 h and treated with DB1 BLIS (5.0 mg/mL) for 1, 3, 6, 12, and 24 h. *S. mutans* biofilm was assessed by staining with crystal violet. (D) After treating the preformed *S. mutans* biofilm with DB1 BLIS for the indicated times, *S. mutans* biofilm cells were subjected to the XTT assay. * Indicates statistical significance ($p < 0.05$) compared to each control without DB1 BLIS. # Indicates statistical difference from the group treated with DB1 BLIS for 1 h and § indicates statistical difference from the group treated without DB1 BLIS for 1 h ($p < 0.05$). The results are representative of three independent experiments. NS, not significant; BLIS, bacteriocin-like inhibitory substance; XTT, 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate.

(Kim et al., 2019), and *Pediococcus acidilactici* bacteriocin displayed antibiofilm activities against *S. Typhimurium*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (Lee et al., 2020; Seo and Kang, 2020; Yoon and Kang, 2020). Recent studies have also shown that bacteriocins of lactic acid bacteria possess antibiofilm activity against *S. mutans* (Conrads et al., 2019; Dufour et al., 2020; Molham et al., 2021). In accordance with the previous reports, the current study demonstrated that DB1 BLIS could be an effective antibiofilm agent to control *S. mutans*.

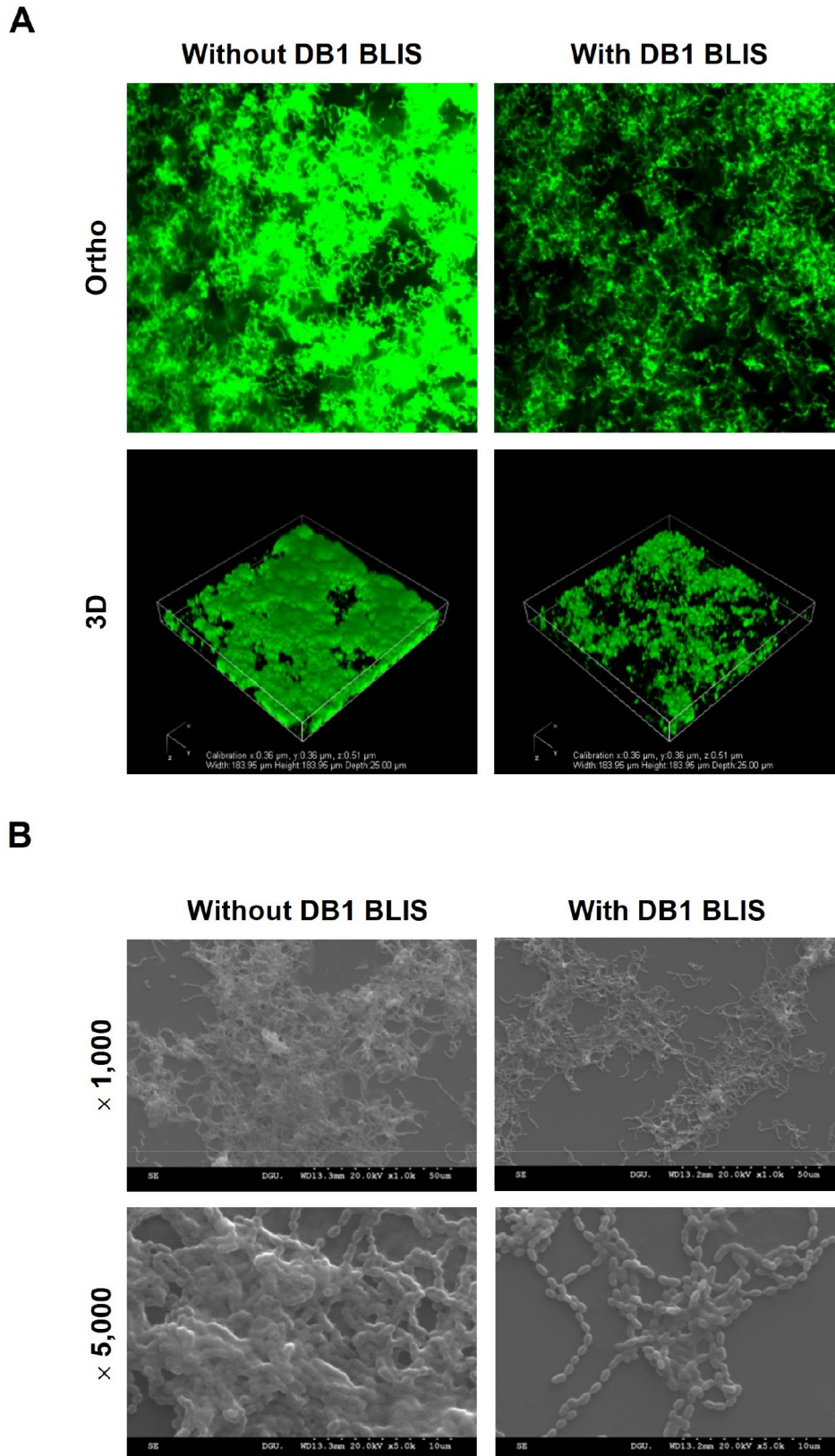


Fig. 2. Microscopic analyses of *Streptococcus mutans* biofilm with and without DB1 BLIS. *S. mutans* was incubated with or without DB1 BLIS (5.0 mg/mL) at 37°C for 24 h and biofilm formation was assessed by confocal laser scanning microscopy (A) and scanning electron microscopy (B). Images taken from one of three similar results are shown. BLIS, bacteriocin-like inhibitory substance.

We next examined the effect of DB1 BLIS on the viability of *S. mutans* planktonic cells. Although short treatments with DB1 BLIS (1 and 3 h) had no effect, treatment for 6 h significantly decreased *S. mutans* planktonic cells viability and a decrease in viability was observed up to 24 h ($p < 0.05$; Fig. 3A). This result was confirmed by enumeration of *S. mutans* planktonic cells plated on BHI agar plates. As shown in Fig. 3B, *S. mutans* planktonic cell count significantly decreased from approximately 1.8×10^8 CFU/mL without DB1 BLIS to approximately 1.3×10^8 CFU/mL with DB1 BLIS at 12 h (approximately 30% reduction; $p < 0.05$), albeit that no considerable reduction in the viability of *S. mutans* planktonic cells was observed following shorter treatments (1, 3, and 6 h). Furthermore, DB1 BLIS more significantly reduced the viability of *S. mutans* planktonic cells by approximately 65%, from 1.7×10^8 CFU/mL without DB1 BLIS to 6×10^7 CFU/mL with DB1 BLIS at 24 h ($p < 0.05$). Therefore, DB1 BLIS reduces the viability of *S. mutans* planktonic cells at 12 and 24 h. Since lactic acid bacteria produce different types of BLIS or bacteriocins, the antimicrobial potential of BLIS or bacteriocins may be different. In addition, bacteriocins exhibit antimicrobial potential with various spectrum depending on their types of structure, against target pathogens (Simons et al., 2020).

Bacterial attachment to the surface is the first step in biofilm. Our observations demonstrated that a decrease in the biofilm formation by *S. mutans* was not attributed to competitive inhibition of bacteria to the surface by DB1 BLIS. When *S. Typhimurium* was co-cultured with the bacteriocin of *P. acidilactici* HW01, both biofilm formation and viability of *S. Typhimurium* planktonic cells were reduced (Seo and Kang, 2020). The C-terminal 15 amino acids of synthetic human beta-defensin-3 also suppressed *S. mutans* growth and biofilm formation (Ahn et al., 2017). Our findings supported the previous reports that the reduction of biofilm formation by *S. mutans* could be associated with the inhibition of bacterial growth. Conversely, lipoteichoic acid of *L. plantarum* did not affect *S. mutans* growth but displayed exceptional ability to inhibit

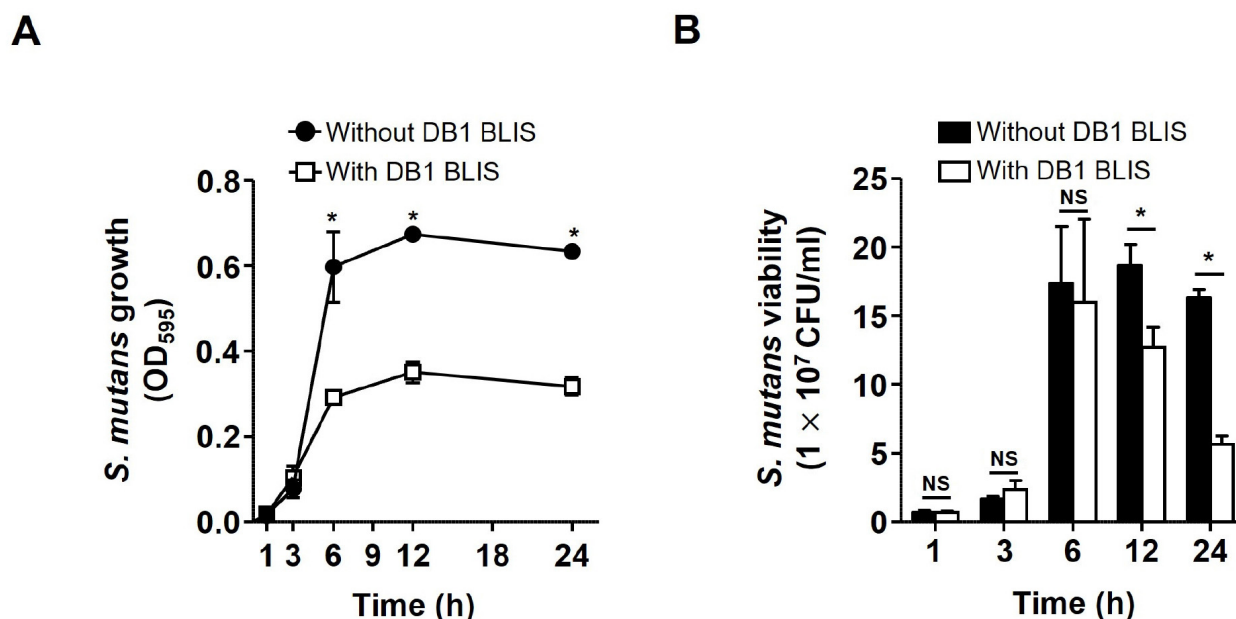


Fig. 3. Growth kinetics of *Streptococcus mutans* planktonic cells with and without DB1 BLIS. *S. mutans* was cultured with or without DB1 BLIS (5.0 mg/mL) for 1, 3, 6, 12, and 24 h, and the absorbance was measured at 595 nm (A). After incubation of *S. mutans* with or without DB1 BLIS, the viability of *S. mutans* planktonic cells was determined by plating on BHI agar (B). * Indicates statistical significance ($p < 0.05$) compared to each control without DB1 BLIS. The results are representative of three independent experiments. NS, not significant; BLIS, bacteriocin-like inhibitory substance; BHI, brain heart infusion.

biofilm formation of the bacteria (Ahn et al., 2018). As mentioned above, the bacteriocin of *P. acidilactici* HW01 inhibited both bacterial growth and biofilm of *S. Typhimurium* (Seo and Kang, 2020) but markedly inhibited *P. aeruginosa* biofilm without suppressing bacterial growth (Lee et al., 2020). Although the inhibitory mechanism of bacteriocin or BLIS against biofilm formation remains unclear, it appears to be dependent on the target bacteria and inhibitory molecules. Most of the characterized bacteriocins of enterococci including *E. faecium* belong to class II bacteriocins (Nes et al., 2007). Their mechanism of antimicrobial action is based on the ability to induce membrane permeabilization, subsequently resulting in the leakage of molecules from target bacteria (Cotter et al., 2005). Our findings showed that the viability of *S. mutans* in biofilm was reduced by DB1 BLIS. Thus, it can be elucidated that DB1 BLIS could induce permeabilization of the cell membrane that leads to the reduction of *S. mutans* biofilm.

As alluded to above, inhibiting the biofilm formation of *S. mutans* might inhibit the occurrence of dental caries (Ahn et al., 2018). In order to examine the inhibitory effect of DB1 BLIS against *S. mutans* biofilm in a dental biofilm model representing dental caries, *S. mutans* was inoculated and allowed to form biofilm on the surface of saliva-coated HA discs with and without DB1 bacteriocin. The treatment with DB1 bacteriocin resulted in an almost 50% reduction of *S. mutans* biofilm (Fig. 4), suggesting the possible clinical use of DB1 bacteriocin to prevent or treat dental caries.

Besides *S. mutans* biofilm, multispecies bacteria in the oral cavity form biofilm, aggravating dental caries. These multispecies biofilms protect the embedded bacteria from the external environment, leading to the maintenance of biofilm architecture. Thus, it would be worth investigating the inhibitory activity of DB1 BLIS on multispecies biofilms in the oral cavity. Although further studies are needed to confirm certain molecule(s) of DB1 BLIS that are involved in the inhibition of *S. mutans* biofilm, our preliminary study showed that DB1 BLIS, as a postbiotic compound produced by *E. faecium* DB1, effectively inhibited the biofilm formation of *S. mutans* by decreasing the viability of *S. mutans*. Thus, DB1 BLIS could be a promising agent to prevent and treat dental caries caused by *S. mutans* biofilm.

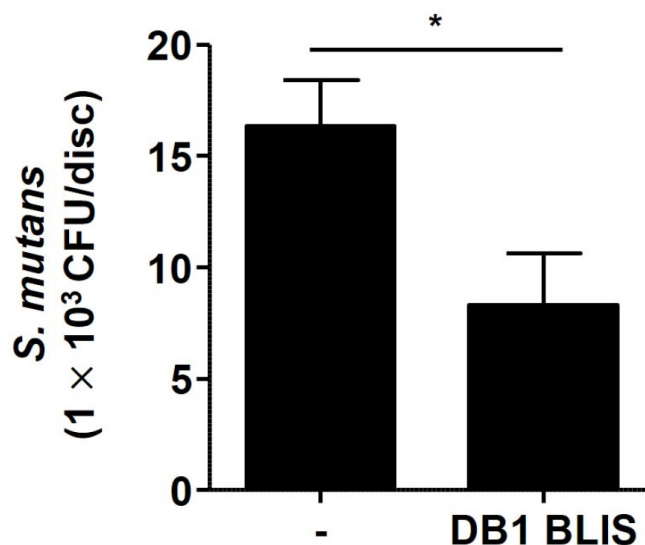


Fig. 4. Antibiofilm effect of DB1 BLIS against *Streptococcus mutans* on the surface of saliva-coated HA discs. *S. mutans* was incubated on the surface of saliva-coated HA discs at 37°C for 24 h with or without DB1 BLIS (5.0 mg/mL). After incubation, adherent *S. mutans* was detached and enumerated by plating on BHI agar. * Indicates statistical significance ($p < 0.05$) compared to the control without DB1 BLIS. The results are representative of three independent experiments. BLIS, bacteriocin-like inhibitory substance; HA, hydroxyapatite; BHI, brain heart infusion.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Author Contributions

Conceptualization: Kim NN, Kang SS. Data curation: Kim NN, Kim BS. Formal analysis: Kim NN, Kim BS, Lee HB, An S. Methodology: Kim NN, Kim BS, Lee HB, An S, Kim D. Software: Kim NN, Kim BS. Validation: Kim NN, Lee HB. Investigation: Kim NN, Kim BS. Writing - original draft: Kim NN, Kang SS. Writing - review & editing: Kim NN, Kim BS, Lee HB, An S, Kim D, Kang SS.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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