TITLE PAGE
- Food Science of Animal Resources -
Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title	Effect of bacteriocin-like inhibitory substance (BLIS) from *Enterococcus faecium* DB1 on cariogenic *Streptococcus mutans* biofilm formation
Running Title (within 10 words)	BLIS of *E. faecium* DB1 inhibits *S. mutans* biofilm formation
Author	Ni-Na Kim, Bong Sun Kim, Han Bin Lee, Sunghyun An, Donghan Kim, Seok-Seong Kang
Affiliation	Department of Food Science and Biotechnology, College of Life Science and Biotechnology, Dongguk University, Goyang, 10326, Korea
Special remarks – if authors have additional information to inform the editorial office	None
ORCID (All authors must have ORCID) https://orcid.org	Ni-Na Kim (https://orcid.org/0000-0001-9992-7559) Bong Sun Kim (https://orcid.org/0000-0001-7676-6238) Han Bin Lee (https://orcid.org/0000-0002-1885-6128) Sunghyun An (https://orcid.org/0000-0003-2930-1168) Donghan Kim (https://orcid.org/0000-0003-0013-2807) Seok-Seong Kang (https://orcid.org/0000-0001-7029-9122)
Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (NRF-2020R1A2C1010010).
Author contributions (This field may be published.)	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 (IRB/IACUC) (This field may be published.)	This article does not require IRB/IACUC approval because there are no human and animal participants.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Seok-Seong Kang
Email address – this is where your proofs will be sent	sskang@dgu.ac.kr
Secondary Email address	Kangelo72@gmail.com
Postal address	Department of Food Science and Biotechnology, Dongguk University, 32 Dongguk-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do, 10326, Korea

Cell phone number	+82-10-3669-6409
Office phone number	
Fax number	

7
8

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 (Penesyan 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 *Enterococcus 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 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-μm membrane filter. DB1 BLIS in the cell-free supernatants was precipitated by adding 70% saturation of ammonium sulfate (Samchum Chemical Co., Ltd., Seoul, Korea) at 4°C overnight and collected by centrifugation at 17,000 × *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 (BCA) 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 *S. 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 μL of XTT (Biotium, Fermont, CA, USA) in 200 μL 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 μL XTT in 200 μL 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 ×1,000 and ×5,000 magnifications.

Growth of *S. 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 disc assay

The saliva-coated hydroxyapatite (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,

149 USA) were placed in the wells of a 48-well microtiter plate and coated with human saliva at
150 37°C for 2 h. Bacterial suspension of *S. mutans* diluted in BHI broth containing 0.05% sucrose
151 was added to the wells and incubated with or without DB1 BLIS at 37°C for 48 h. After
152 incubation, saliva-coated HA discs were rinsed with PBS to remove planktonic bacteria and
153 transferred to conical tubes containing PBS. After vigorous vortex mixing and sonication, the
154 supernatants were collected and serially diluted for enumeration (CFU per disc) on BHI agar.
155
156 ***Statistical analysis***
157 All data are expressed as mean ± standard deviation of triplicate samples. Statistical
158 comparisons between the control and treated groups were determined at $P < 0.05$ by unpaired
159 two tailed *t*-test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data
160 are representative of two or three independent experiments.
161
162 **Results and discussion**
163 Different concentrations of DB1 BLIS (0.6, 1.2, 2.5, and 5.0 mg/mL) were tested antibiofilm
164 activity against *S. mutans*. Particularly, 5.0 mg/mL of DB1 BLIS inhibited *S. mutans* biofilm
165 by approximately 50% (data not shown). Therefore, this concentration of DB1 BLIS (5.0
166 mg/mL) was used for further experiments. When *S. mutans* was co-incubated with DB1 BLIS
167 for 1, 3, 6, 12, and 24 h, DB1 BLIS significantly inhibited *S. mutans* biofilm at 3, 6, 12, and 24
168 h ($P < 0.05$) (Fig. 1A). The decreased viability of *S. mutans* biofilm cells in the presence of
169 DB1 BLIS was confirmed by the XTT assay. As shown in Fig. 1B, the viability was
170 significantly reduced at 3, 6, 12, and 24 h in the presence of DB1 BLIS ($P < 0.05$). To examine
171 whether DB1 BLIS eradicates established *S. mutans* biofilm, preformed 24-h biofilm was
172 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 (Fig. 2A and 2B). Studies have already shown the inhibition of *S. mutans* biofilm by probiotics, such as *Lactobacillus plantarum* (Zhang et al., 2020) and *L. 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 *S. aureus* (Okuda et al., 2013). In our previous studies of inhibition of foodborne pathogens, we showed that *L. brevis* bacteriocin effectively inhibited the biofilm formation of *Escherichia coli* and *Salmonella* Typhimurium (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*.

196 We next examined the effect of DB1 BLIS on the viability of *S. mutans* planktonic cells.
197 Although short treatments with DB1 BLIS (1 and 3 h) had no effect, treatment for 6 h
198 significantly decreased *S. mutans* planktonic cells viability and a decrease in viability was
199 observed up to 24 h ($P < 0.05$) (Fig. 3A). This result was confirmed by enumeration of *S.*
200 *mutans* planktonic cells plated on BHI agar plates. As shown in Fig. 3B, *S. mutans* planktonic
201 cell count significantly decreased from approximately 1.8×10^8 CFU/mL without DB1 BLIS
202 to approximately 1.3×10^8 CFU/mL with DB1 BLIS at 12 h (approximately 30% reduction)
203 ($P < 0.05$), albeit that no considerable reduction in the viability of *S. mutans* planktonic cells
204 was observed following shorter treatments (1, 3, and 6 h). Furthermore, DB1 BLIS more
205 significantly reduced the viability of *S. mutans* planktonic cells by approximately 65%, from
206 1.7×10^8 CFU/mL without DB1 BLIS to 6×10^7 CFU/mL with DB1 BLIS at 24 h ($P < 0.05$).
207 Therefore, DB1 BLIS reduces the viability of *S. mutans* planktonic cells at 12 and 24 h. Since
208 lactic acid bacteria produce different types of BLIS or bacteriocins, the antimicrobial potential
209 of BLIS or bacteriocins may be different. In addition, bacteriocins exhibit antimicrobial
210 potential with various spectrum depending on their types of structure, against target pathogens
211 (Simons et al., 2020).
212 Bacterial attachment to the surface is the first step in biofilm. Our observations demonstrated
213 that a decrease in the biofilm formation by *S. mutans* was not attributed to competitive
214 inhibition of bacteria to the surface by DB1 BLIS. When *S.* Typhimurium was co-cultured with
215 the bacteriocin of *P. acidilactici* HW01, both biofilm formation and viability of *S.* Typhimurium
216 planktonic cells were reduced (Seo and Kang, 2020). The C-terminal 15 amino acids of
217 synthetic human beta-defensin-3 also suppressed *S. mutans* growth and biofilm formation (Ahn
218 et al., 2017). Our findings supported the previous reports that the reduction of biofilm formation
219 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 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.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (NRF-2020R1A2C1010010).

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.

References

Aguilar-Toalá J, Garcia-Varela R, Garcia H, Mata-Haro V, González-Córdova A, Vallejo-Cordoba B, Hernández-Mendoza A. 2018. Postbiotics: An evolving term within the functional foods field. Trends Food Sci Technol 75:105-114.

Ahn KB, Baik JE, Park OJ, Yun CH, Han SH. 2018. *Lactobacillus plantarum* lipoteichoic acid inhibits biofilm formation of *Streptococcus mutans*. PLoS One 13:e0192694.

Ahn KB, Kim AR, Kum KY, Yun CH, Han SH. 2017. The synthetic human beta-defensin-3 c15 peptide exhibits antimicrobial activity against *Streptococcus mutans*, both alone and in combination with dental disinfectants. J Microbiol 55:830-836.

Ansari JM, Abraham NM, Massaro J, Murphy K, Smith-Carpenter J, Fikrig E. 2017. Anti-biofilm activity of a self-aggregating peptide against *Streptococcus mutans*. Front Microbiol 8:488.

Arabaci T, Turkez H, Canakci CF, Ozgoz M. 2013. Assessment of cytogenetic and cytotoxic effects of chlorhexidine digluconate on cultured human lymphocytes. Acta Odontol Scand 71:1255-1260.

Chhour KL, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N. 2005. Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 43:843-849.

Choi H-Y, Kim J-S, Kim W-J. 2011. Optimization of conditions for the maximum bacteriocin production of *Enterococcus faecium* DB1 using response surface methodology. Korean J Food Sci Ani Resour 31:176-182.

Conrads G, Westenberger J, Lurkens M, Abdelbary MMH. 2019. Isolation and bacteriocin-related typing of *Streptococcus dentisani*. Front Cell Infect Microbiol 9:110.

Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol 3:777-788.

291 Cuevas-González P, Liceaga A, Aguilar-Toalá J. 2020. Postbiotics and paraprobiotics: From
292 concepts to applications. Food Res Int 136:109502.
293 Dufour D, Barbour A, Chan Y, Cheng M, Rahman T, Thorburn M, Stewart C, Finer Y, Gong
294 SG, Levesque CM. 2020. Genetic analysis of mutacin b-ny266, a lantibiotic active
295 against caries pathogens. J Bacteriol 202:e00762-19.
296 Hanchi H, Mottawea W, Sebei K, Hammami R. 2018. The genus *Enterococcus*: Between
297 probiotic potential and safety concerns-an update. Front Microbiol 9:1791.
298 Homayoni Rad A, Akbarzadeh F, Mehrabany EV. 2012. Which are more important: Prebiotics
299 or probiotics? Nutrition 28:1196-1197.
300 Imran S. 2016. Bacteriocin: An alternative to antibiotics. World J Pharm Res 5:467-477.
301 Jeong SI, Kim BS, Keum KS, Lee KH, Kang SY, Park BI, Lee YR, You YO. 2013. Kaurenoic
302 acid from aralia continentalis inhibits biofilm formation of *Streptococcus mutans*. Evid
303 Based Complement Alternat Med 2013:160592.
304 Kim N-N, Kim WJ, Kang S-S. 2019. Anti-biofilm effect of crude bacteriocin derived from
305 *Lactobacillus brevis* DF01 on escherichia coli and salmonella typhimurium. Food
306 Control 98:274-280.
307 Lee DH, Kim BS, Kang SS. 2020. Bacteriocin of *Pediococcus acidilactici* HW01 inhibits
308 biofilm formation and virulence factor production by *Pseudomonas aeruginosa*.
309 Probiotics Antimicrob Proteins 12:73-81.
310 Liu Y, Xu Y, Song Q, Wang F, Sun L, Liu L, Yang X, Yi J, Bao Y, Ma H, Huang H, Yu C, Huang
311 Y, Wu Y, Li Y. 2017. Anti-biofilm activities from bergenia crassifolia leaves against
312 *Streptococcus mutans*. Front Microbiol 8:1738.
313 Mathur H, Field D, Rea MC, Cotter PD, Hill C, Ross RP. 2018. Fighting biofilms with
314 lantibiotics and other groups of bacteriocins. NPJ Biofilms Microbiomes 4:9.

315 Molham F, Khairalla AS, Azmy AF, El-Gebaly E, El-Gendy AO, Abdelghani S. 2021. Anti-
316 proliferative and anti-biofilm potentials of bacteriocins produced by non-pathogenic
317 *Enterococcus* sp. Probiotics Antimicrob Proteins 13:571-585.
318 Moradi M, Mardani K, Tajik H. 2019. Characterization and application of postbiotics of
319 *Lactobacillus* spp. on *Listeria monocytogenes in vitro* and in food models. LWT-Food
320 Sci Technol 111:457-464.
321 Nataraj BH, Ali SA, Behare PV, Yadav H. 2020. Postbiotics-parabiotics: The new horizons in
322 microbial biotherapy and functional foods. Microb Cell Fact 19:168.
323 Nes IF, Diep DB, Holo H. 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. J
324 Bacteriol 189:1189-1198.
325 Okuda K, Zendo T, Sugimoto S, Iwase T, Tajima A, Yamada S, Sonomoto K, Mizunoe Y. 2013.
326 Effects of bacteriocins on methicillin-resistant *Staphylococcus aureus* biofilm.
327 Antimicrob Agents Chemother 57:5572-5579.
328 Penesyan A, Gillings M, Paulsen IT. 2015. Antibiotic discovery: Combatting bacterial
329 resistance in cells and in biofilm communities. Molecules 20:5286-5298.
330 Perez RH, Zendo T, Sonomoto K. 2014. Novel bacteriocins from lactic acid bacteria (LAB):
331 Various structures and applications. Microb Cell Fact 13 Suppl 1:S3.
332 Seo H-J, Kang S-S. 2020. Inhibitory effect of bacteriocin produced by *Pediococcus acidilactici*
333 on the biofilm formation of *Salmonella* typhimurium. Food Control 117:107361.
334 Simons A, Alhanout K, Duval RE. 2020. Bacteriocins, antimicrobial peptides from bacterial
335 origin: Overview of their biology and their impact against multidrug-resistant bacteria.
336 Microorganisms 8:639.

337 Teame T, Wang A, Xie M, Zhang Z, Yang Y, Ding Q, Gao C, Olsen RE, Ran C, Zhou Z. 2020.
338 Paraprobiotics and postbiotics of probiotic lactobacilli, their positive effects on the host
339 and action mechanisms: A review. Front Nutr 7:570344.
340 Tong Z, Zhang L, Ling J, Jian Y, Huang L, Deng D. 2014. An *in vitro* study on the effect of
341 free amino acids alone or in combination with nisin on biofilms as well as on planktonic
342 bacteria of *Streptococcus mutans*. PLoS One 9:e99513.
343 Wadhawan T, Mcevoy J, Prüβ BM, Khan E. 2010. Assessing tetrazolium and ATP assays for
344 rapid *in situ* viability quantification of bacterial cells entrapped in hydrogel beads.
345 Enzyme Microb Technol 47:166-173.
346 Wu CC, Lin CT, Wu CY, Peng WS, Lee MJ, Tsai YC. 2015. Inhibitory effect of *Lactobacillus*
347 *salivarius* on *Streptococcus mutans* biofilm formation. Mol Oral Microbiol 30:16-26.
348 Yang R, Argimon S, Li Y, Gu H, Zhou X, Caufield PW. 2010. Determining the genetic diversity
349 of lactobacilli from the oral cavity. J Microbiol Methods 82:163-169.
350 Yoon JW, Kang SS. 2020. *In vitro* antibiofilm and anti-inflammatory properties of bacteriocins
351 produced by *Pediococcus acidilactici* against enterococcus faecalis. Foodborne Pathog
352 Dis 17:764-771.
353 Zhang G, Lu M, Liu R, Tian Y, Vu VH, Li Y, Liu B, Kushmaro A, Li Y, Sun Q. 2020. Inhibition
354 of *Streptococcus mutans* biofilm formation and virulence by *Lactobacillus plantarum*
355 k41 isolated from traditional sichuan pickles. Front Microbiol 11:774.

356
357
358
359
360

Figure legends

Fig. 1. Inhibitory effect of DB1 BLIS against biofilm formation by *S. 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. The asterisk (*) 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 1h ($P < 0.05$). N.S. denotes not significant. The results are representative of three independent experiments.

Fig. 2. Microscopic analyses of *S. 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.

Fig. 3. Growth kinetics of *S. 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).

385 The asterisk (*) indicates statistical significance ($P < 0.05$) compared to each control without
386 DB1 BLIS. N.S. denotes not significant. The results are representative of three independent
387 experiments.
388
389 **Fig. 4.** Antibiofilm effect of DB1 BLIS against *S. mutans* on the surface of saliva-coated HA
390 discs. *S. mutans* was incubated on the surface of saliva-coated HA discs at 37°C for 24 h with
391 or without DB1 BLIS (5.0 mg/mL). After incubation, adherent *S. mutans* was detached and
392 enumerated by plating on BHI agar. The asterisk (*) indicates statistical significance ($P < 0.05$)
393 compared to the control without DB1 BLIS. The results are representative of three independent
394 experiments.
395
396
397
398
399

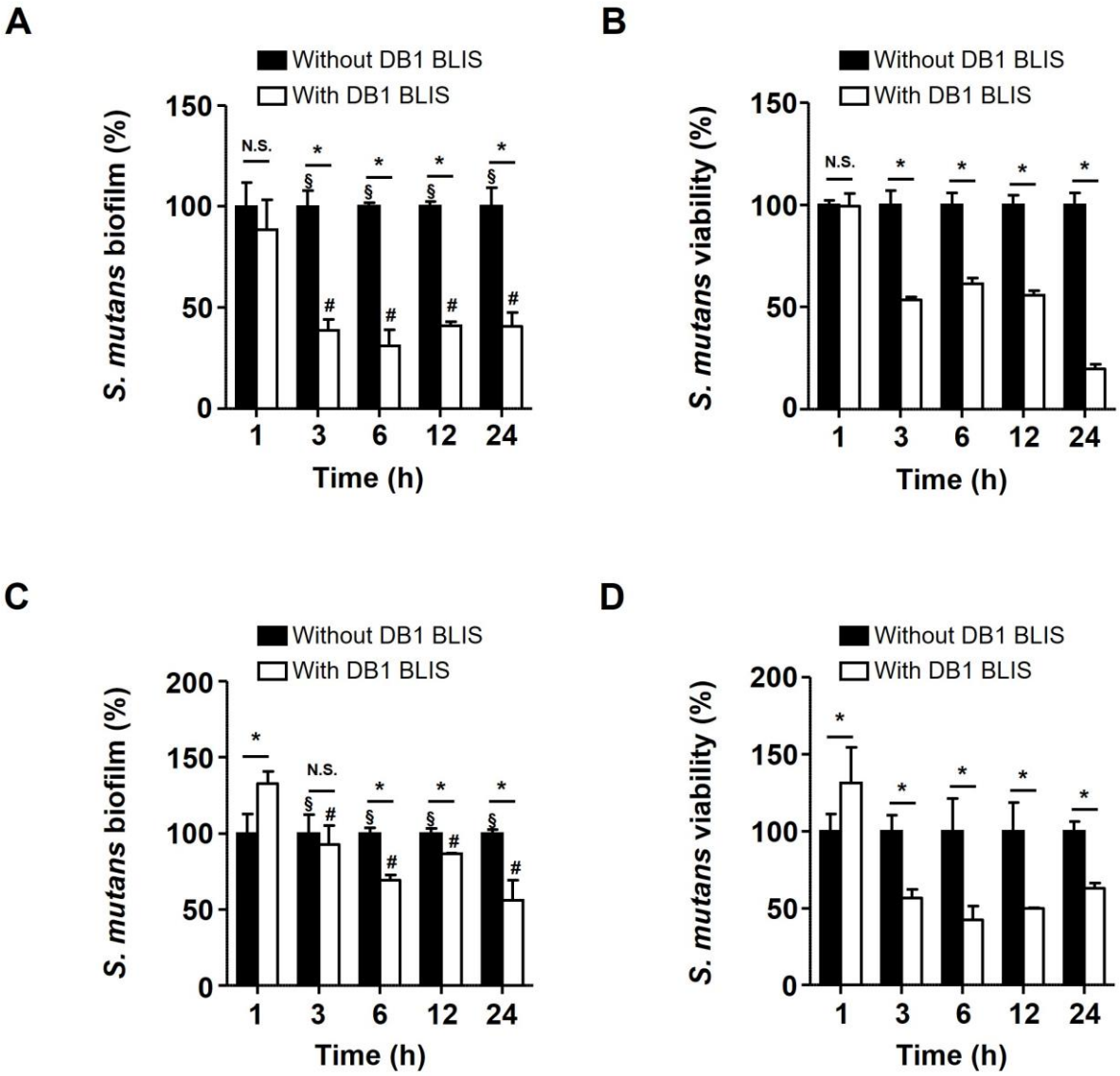
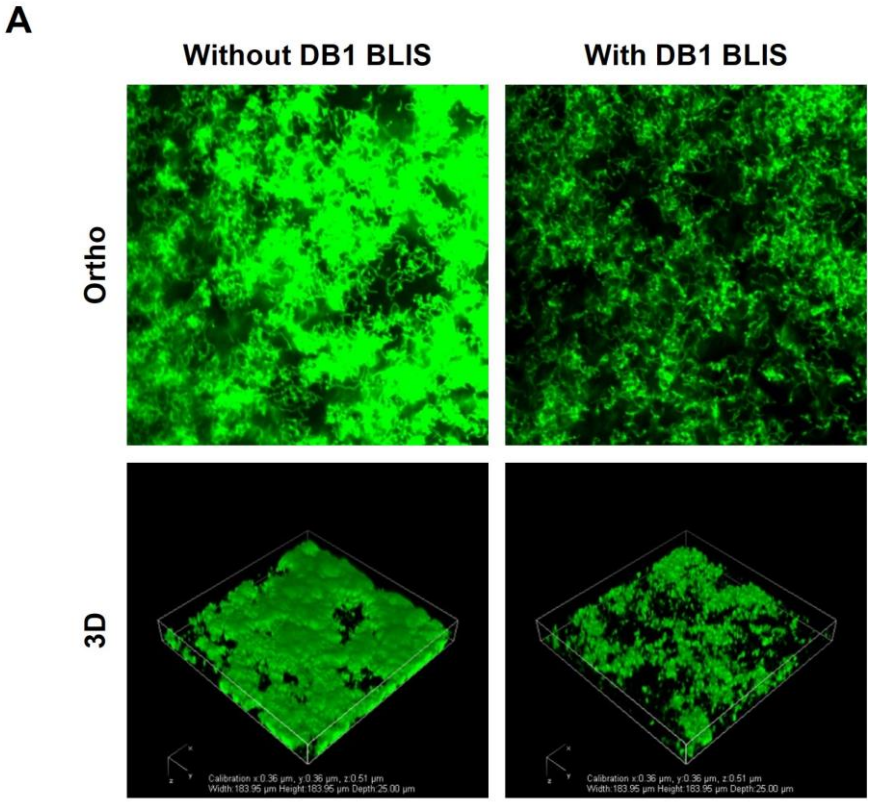

A
Without DB1 BLIS
With DB1 BLIS
S. mutans biofilm (%)
150
100
50
0
N.S.
*
§
#
1
3
6
12
24
Time (h)
B
Without DB1 BLIS
With DB1 BLIS
S. mutans viability (%)
150
100
50
0
N.S.
*
1
3
6
12
24
Time (h)
C
Without DB1 BLIS
With DB1 BLIS
S. mutans biofilm (%)
200
150
100
50
0
*
N.S.
§
#
1
3
6
12
24
Time (h)
D
Without DB1 BLIS
With DB1 BLIS
S. mutans viability (%)
200
150
100
50
0
*
1
3
6
12
24
Time (h)

Figure 1


A
Without DB1 BLIS
With DB1 BLIS
Ortho
3D
Calibration x:0.36 µm, y:0.36 µm, z:0.51 µm
Width:183.95 µm Height:183.95 µm Depth:25.00 µm
Calibration x:0.36 µm, y:0.36 µm, z:0.51 µm
Width:183.95 µm Height:183.95 µm Depth:25.00 µm

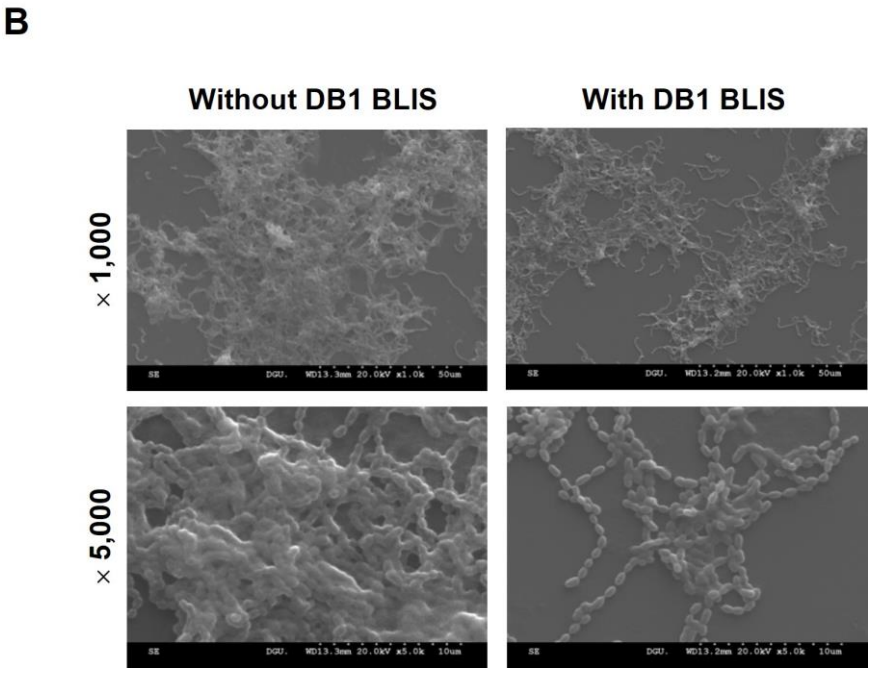

B
Without DB1 BLIS
With DB1 BLIS
× 1,000
× 5,000

Figure 2

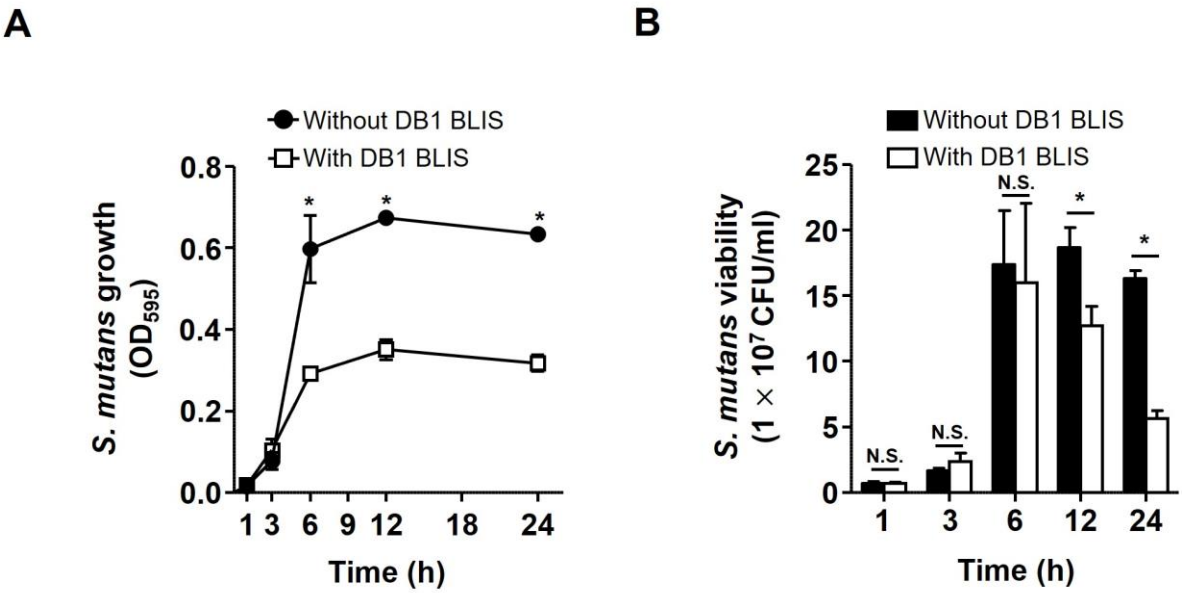
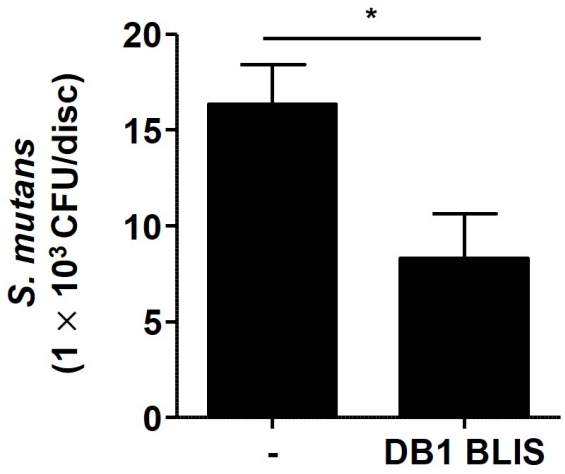


Figure 3



425

426 Figure 4