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Soybean peptide inhibits the biofilm of periodontopathic bacteria via bactericidal activity

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ABSTRACT

Objective: This study aimed to clarify the antibacterial mechanism and antibiofilm effect of soybean-derived peptide BCBS-11 against periodontopathic bacteria.
Design: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of BCBS-11 against *Porphyromonas gingivalis* (*P. gingivalis*), *Fusobacterium nucleatum* (*F. nucleatum*), and *Streptococcus mitis* (*S. mitis*) were determined for the antibacterial mechanism. The effect of BCBS-11 on membrane permeability and depolarization activity were investigated using propidium iodide (PI) staining and 3, 3′-dipropylthiadicarbocyanine iodide ($DiSC_3$-(5)) analysis. Monospecies and multispecies biofilms were cultured on 96-well plates. The amount of biofilm was determined using crystal violet staining to determine the inhibition of biofilm formation and the eradication of established biofilm using BCBS-11. The cytotoxicity of BCBS-11 was evaluated using 3-(4, 5-Dimethylthiazol-2-yl)− 2, 5-diphenyltetrazolium bromide (MTT) assay.
Results: The MIC and MBC indicated the bactericidal activity of BCBS-11 against *P. gingivalis* and *F. nucleatum.* The PI staining revealed that BCBS-11 disrupted the bacterial membrane integrity. The $DiSC_3$-(5) analysis indicated that BCBS-11 depolarized the bacterial cytoplasmic membrane. These results indicate the antimicrobial action of BCBS-11 through membrane disruption and the collapse of membrane electrochemical gradient. BCBS-11 significantly inhibited the monospecies biofilm formation of *P. gingivalis* and *F. nucleatum* and also inhibited dual-species biofilm. BCBS-11 was not cytotoxic toward human oral epithelial cells.
Conclusions: BCBS-11 inhibits the monospecies and multispecies biofilm formation of *P. gingivalis* and *F. nucleatum*, and their bactericidal activity results from membrane disruption.

1. Introduction

Periodontitis is a biofilm infection characterized by gingival inflammation, alveolar bone resorption, and tooth loss (Caton et al., 2018). In addition to mechanical biofilm removal, antimicrobial agents may be used as an adjunctive therapy for preventing and treating periodontitis. However, bacteria in biofilms are highly resistant to antibiotics, in contrast to planktonic bacteria, because the matrix enclosing biofilms prohibits the infiltration of pharmacological substances (Hoiby et al., 2011; Olson et al., 2002). Furthermore, antibiotic-resistant genes spread via horizontal migration between biofilms (Abe et al., 2020; Olsen et al., 2013; Roberts & Kreth, 2014). Oral bacteria also demonstrate drug resistance, such as the resistant strains of *Fusobacterium nucleatum*, to amoxicillin, clindamycin, and

Abbreviations: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; CHX, chlorhexidine; CFU, colony-forming unit; PI, propidium iodide; $DiSC_3$-5, 3, 3′-Dipropylthiadicarbocyanine iodide; HEPES, 4-(2-hydroxyethyl)− 1-piperazineethanesulfonic acid; MTT, 3-(4, 5-Dimethylthiazol-2-yl)− 2, 5-diphenyltetrazolium Bromide.

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metronidazole (Ardila et al., 2010; Mosca et al., 2007; van Winkelhoff et al., 2005).

The number of bacteria that are resistant to existing antibiotics has increased yearly and deaths resulting from multidrug-resistant bacteria are expected to reach 10 million worldwide by 2050 (Willyard, 2017). In general, antimicrobial agents demonstrating a broad spectrum of antibacterial activity are effective against various strains of bacteria; however, they transfer the risk of inducing drug resistance across a wide range of bacteria. In contrast, narrow-spectrum antimicrobial agents are effective against only certain species, but the range acquiring drug resistance is limited (Alm & Lahiri, 2020). Therefore, the following two issues are important to achieve effective periodontal treatment in the context of drug-resistant bacteria. First, limiting the usage of existing antibiotics, and second, developing alternative methods to control biofilm that are specific to periodontopathogenic bacteria.

Antimicrobial peptides represent an alternative to existing antibiotics (Chen & Lu, 2020). They exhibit membrane-damaging antimicrobial activity, such as membrane pore formation and lysis, which decreases the potential of bacteria acquiring drug resistance (Rodriguez-Rojas et al., 2002). Plants are one source of antimicrobial peptides, and these peptides exhibit significant antimicrobial activity against human and plant pathogens (Li et al., 2021). Since the first report of plant antimicrobial peptide thionine in 1972 (Fernandez de Caleya et al., 1972), studies on plant antimicrobial peptides have increased considerably. Generally, the activity of antimicrobial peptides has been reported to be influenced by temperature and pH conditions (Mackay & Chilkoti, 2008; Walkenhorst et al., 2013). Peptide toxicity, immunogenicity, drug resistance, and hemolytic effects have also been reported (Moravej et al., 2018). Applying antimicrobial peptides for treating various human diseases has attracted considerable attention recently, but their clinical application is very limited (Lei et al., 2019).

Previously, we demonstrated that the rice peptide inhibits biofilm formation in *Porphyromonas gingivalis* and *F. nucleatum* (Matsugishi et al., 2021). Although its antimicrobial activity is weak, its ability was enhanced by increasing its cationic property through amino acid substitution (Matsugishi et al., 2021). Therefore, soybean peptide, a natural peptide with high cationic property, was utilized in this study. The soybean-derived peptide, BCBS-11, which is the focus of the present study, exhibits antibacterial activity against planktonic *P. gingivalis* (Taniguchi et al., 2017); however, its antibiofilm properties have not been developed. This study aimed to clarify the antibacterial mechanism and antibiofilm effect of BCBS-11 against periodontopathic bacteria. Three species of bacteria were used for monospecies and multispecies biofilm experiments: two representative periodontal bacteria, i.e., *P. gingivalis* and *F. nucleatum*, and a harmless oral commensal, i.e., *Streptococcus mitis*.

2. Materials and methods

2.1. Peptides and reagents

The properties, amino acid sequence and the secondary structures of soybean peptides, BCBS-11 and a control peptide Glycinin-17 used in this study are summarized in Supplementary Tables 1 and 2A. These peptides were chemically synthesized and purified to > 95% using high-performance liquid chromatography (Eurofins Genomics, Tokyo, Japan). Chlorhexidine, CHX (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), was used as the reference medium.

2.2. Bacterial strains and culture conditions

The *P. gingivalis* strain, FDC 381, and the *F. nucleatum* strain, ATCC 25586, were cultured in modified Gifu anaerobic medium broth (Nissui, Tokyo, Japan) in an anaerobic jar (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) in the presence of AnaeroPack™ at 37 °C. *S. mitis* strain ATCC 903 was cultured in brain heart infusion broth (Thermo Fisher Scientific, San Diego, CA, USA) at 37 °C under aerobic conditions. We determined the number of colony-forming units (CFUs) using our established growth curves. The bacterial concentrations of the mid-log phase were $1 \times 10^9 - 1 \times 10^{10}$ CFU/mL (*P. gingivalis*), $1 \times 10^{10} - 1 \times 10^{11}$ CFU/mL (*F. nucleatum*), and $1 \times 10^8 - 1 \times 10^9$ CFU/mL (*S. mitis*). The concentration of the bacterial suspension was obtained by optical density at 600 nm using a UV–visible Spectronic Genesys 10 Bio spectrophotometer (Thermo Fisher Scientific, San Diego, CA, USA). The growth curves were prepared for each species of bacteria, and the following experimental methods were established based on these curves.

2.3. Determination of the MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for BCBS-11 were determined using a microplate dilution assay as described previously (Wang et al., 2015). Bacterial cultures of *P. gingivalis*, *F. nucleatum,* and *S. mitis* were measured by a spectrophotometer and adjusted to 1×10^7 CFU/mL using growth curves (Maezono et al., 2011). Bacteria and BCBS-11 were mixed and aliquoted into 96-well round-bottomed plates (Techno Plastic Products AG, Trasadingen, Switzerland). The plates were incubated at 37 °C for 5 days (*P. gingivalis*) and 2 days (*F. nucleatum* and *S. mitis*) under anaerobic (*P. gingivalis* and *F. nucleatum*) or aerobic (*S. mitis*) conditions.

2.4. Membrane permeability assay

P. gingivalis, *F. nucleatum,* and *S. mitis* biofilms were formed in a Lab-Tek™ Chamber Slide (Nunc, Rochester, NY, USA) at a density of 2×10^8 CFU/mL to check the membrane permeability assay as previous study (Matsugishi et al., 2021). *P. gingivalis* was incubated with BCBS-11 or CHX at 25% MBC with each reagent (BCBS-11: 25 μM, CHX: 4.4 μM) for 48 h under anaerobic conditions. *F. nucleatum* was incubated at 12.5% MBC for 72 h under anaerobic conditions. For *S. mitis*, 100 μM of BCBS-11 or 70 μM of CHX was added to the bacterial suspension and incubated for 48 h under aerobic conditions. Biofilm samples were stained using the LIVE/DEAD BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, San Diego, CA, USA) according to the manufacturer's instructions. Samples were examined using a BZ-X710 microscope (Keyence, Osaka, Japan), and the images were analyzed using the imaging software ImageJ 1.52k (National Institute of Health, Bethesda, MD, USA).

2.5. Membrane depolarization assay

The membrane depolarization activity of BCBS-11 was determined using intact *F. nucleatum* or *S. mitis* cells and a membrane potential-sensitive fluorescent probe, 3, 3′-dipropylthiadicarbocyanine iodide ($DiSC_3$-(5)) (Sigma-Aldrich, Burlington, USA). Bacterial cells were centrifuged at 3000 x g for 20 min and then suspended in 4-(2-hydroxyethyl)− 1-piperazineethanesulfonic acid (HEPES) buffer (5 mM HEPES (Thermo Fisher Scientific, San Diego, CA, USA), 0.5 M EDTA (Sigma-Aldrich, Burlington, USA), and 20 mM Glucose, pH 7.2 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) with 4 μM $DiSC_3$-(5). Bacterial cell suspensions were incubated in a shaking water bath at 37 °C for 45 min under dark conditions to introduce the dye into the bacterial membrane. Next, stained cells were rinsed with HEPES buffer and the cell concentrations were modified to 2×10^8 CFU/mL as previous study (Matsugishi et al., 2021). BCBS-11 or CHX was introduced to the 96-well flat-bottomed black microplate (Thermo Fisher Scientific, San Diego, CA, USA) containing the bacterial cell suspension and incubated for 5 min. The release of $DiSC_3$-(5) from the bacterial cells was monitored by calculating the fluorescence intensity at an excitation wavelength of 622 nm and an emission wavelength of 670 nm utilizing a GloMax® Discover multimode microplate reader (Promega Japan KK, Chiba, Japan).

2.6. Biofilm quantification

We examined three types of biofilm assays in this study:

2.6.1. Monospecies biofilm formation assay

To assess the effects of BCBS-11 on the monospecies biofilm formation of *P. gingivalis*, *F. nucleatum,* or *S. mitis*, bacterial cultures were diluted to 2×10^8 CFU/mL to produce standardized biofilm of each bacterium (Matsugishi et al., 2021; Wang et al., 2015). A total of 100 μL of bacterial suspension was transferred into 96-well flat-bottomed plates (Techno Plastic Products AG, Trasadingen, Switzerland) with BCBS-11 at the final concentrations of 0–400 μM and incubated for 48 h (*P. gingivalis*), 72 h (*F. nucleatum*), or 48 h (*S. mitis*), and the biofilm was quantified. The biofilm quantification was assessed using a crystal violet (Chroma-Gesellschaft Co. Ltd., Münster, Germany) staining approach as described previously (Onozawa et al., 2015; Zhang et al., 2017). The same procedures included CHX at final concentrations of 0–280 μM, which served as a positive control. Phosphate-buffered saline (PBS) served as the control medium.

2.6.2. Monospecies biofilm eradication assay

First, the developed biofilm was generated; 2×10^8 bacterial CFU/mL were incubated at 37 °C for 24 h (*P. gingivalis*) or 48 h (*F. nucleatum*) anaerobically or 24 h (*S. mitis*) aerobically. Then, BCBS-11 was introduced to the bacterial suspensions; this was followed by additional 24 h incubation to measure the amount of remaining biofilm removed using the peptide.

2.6.3. Multispecies biofilm formation assay

The inhibition effect of BCBS-11 on biofilm formation was also evaluated using the multispecies biofilm model (Kuboniwa et al., 2009; Matsugishi et al., 2021). The dual-species biofilm comprised *P. gingivalis* and *F. nucleatum*. The triple-species biofilm was a complex of *P. gingivalis*, *F. nucleatum*, and *S. mitis*. A total of 2×10^8 bacterial CFU/mL culture suspensions of bacteria were combined in equal proportions and incubated for 48 h to develop biofilms under anaerobic conditions. Bacteria were cultivated in brain heart infusion broth supplemented with 5 μg/mL of hemin (Sigma-Aldrich, Burlington, USA) and 1 μg/mL of menadione (Sigma-Aldrich, Burlington, USA).

2.7. Cell viability assay

The effect of BCBS-11 on cell viability was evaluated using the 3-(4, 5-Dimethylthiazol-2-yl)− 2, 5-diphenyltetrazolium bromide (MTT) assay (Sigma, St. Louis, MO, USA). Briefly, the human oral epithelial cell line, Ca9–22 cells, were plated into 96-well plates at a concentration of 1×10^5 cells/well and the plate was incubated overnight. Then, the plate was stimulated by introducing 100 μL/well of BCBS-11 (100 μM, 200 μM, and 400 μM) or CHX (35–280 μM) and the plate was further incubated for 24 h. Absorbance was measured using a Molecular Devices SpectraMAX ABS Plus (MOLECULAR DEVICES, San Jose, CA, USA) at a wavelength of 570 nm.

2.8. Statistical analysis

The data are expressed as the means ± standard errors of the mean (SEMs). Statistical analyses were conducted with GraphPad Prism 7.0 graphing and statistical software (GraphPad Software, Inc., San Diego, CA, USA). A one-way analysis of variance was conducted. A *P* value less than 0.05 was statistically considered significant. All experiments were independently repeated at least twice on separate days.

3. Results

3.1. BCBS-11 shows bactericidal activity against P. gingivalis and F. nucleatum

Major antibiofilm reagents exhibit bactericidal or bacteriostatic properties (Gorr & Abdolhosseini, 2011). To clarify the mechanism of the antibacterial effect of BCBS-11 (Maezono et al., 2011), MIC and MBC values were determined (Table 1). The MIC and MBC for BCBS-11 were similar against *P. gingivalis* and *F. nucleatum,* indicating bactericidal activity of BCBS-11. CHX also demonstrated bactericidal activity against these two bacteria. The growth curves for *P. gingivalis* and *F. nucleatum* and the growth inhibition effect of BCBS-11 are shown in Supplementary Figure 1. The results identify that BCBS-11, at ½ MIC or 1x MIC, significantly inhibited the increase in each bacterium. Moreover, CHX exhibited bactericidal action against *S. mitis*; however, the MIC and MBC for BCBS-11 against *S. mitis* were higher than 400 μM, considering that BCBS-11 does not demonstrate significant antimicrobial activity against *S. mitis.*

3.2. BCBS-11 shows membrane disintegration in both P. gingivalis and F. nucleatum

Major antimicrobial peptides primarily disrupt the integrity of bacterial membranes (Chung & Khanum, 2017; Leontiadou et al., 2006). To determine the extent of membrane permeability, we utilized the propidium iodide (PI) stain, which crosses damaged bacterial membranes and stains the nucleus (Zhang et al., 2017). As a PBS-incubated control, PI-positive cells were not observed (Fig. 1A, E, and I). BCBS-11 induced PI influx into *P. gingivalis* (Fig. 1B) and the CHX effect was greater compared with BCBS-11 (Fig. 1C and D). PI-positive bacteria were observed in BCBS-11- and CHX-treated *F. nucleatum* (Fig. 1F, G, and H). In contrast, BCBS-11 treatment did not result in PI influx in *S. mitis,* even at 100 μM (Fig. 1J), although CHX demonstrated significant activity at 70 μM (Fig. 1K and L). These results indicate that BCBS-11 disrupts the integrity of bacterial membranes in both *P. gingivalis* and *F. nucleatum*.

3.3. BCBS-11 induces the disruption of bacterial membranes and causes depolarization

Membrane disintegration by positively charged peptides causes the collapse of transmembrane electrochemical gradients, resulting in cell swelling and osmolysis (Shai, 2002). $DiSC_3$-(5) may be used to monitor changes in bacterial membrane potential. Here, we compared *F. nucleatum*, for which BCBS-11 exhibited high membrane permeability, and *S. mitis*, for which no membrane permeability was observed, based on PI staining results. BCBS-11 demonstrated significant $DiSC_3$-(5) release in *F. nucleatum*, which was higher compared with CHX (Fig. 2A). BCBS-11 caused minimal $DiSC_3$-(5) release of *S. mitis*, although 70 μM CHX exhibited significant release, indicating membrane depolarization (Fig. 2B). These results suggest that the antimicrobial action of BCBS-11 results from membrane disruption and collapse of the membrane electrochemical gradient.

Table 1
MIC and MBC values of soy peptide BCBS-11 with regard to *P. gingivalis*, *F. nucleatum* and *S. mitis*.

(μM)	*Porphyromonas gingivalis*		*Fusobacterium nucleatum*		*Streptococcus mitis*	
	MIC	MBC	MIC	MBC	MIC	MBC
BCBS-11	100	100	50	100	> 400	> 400
Chlorhexidine	17.5	17.5	35	35	70	70

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

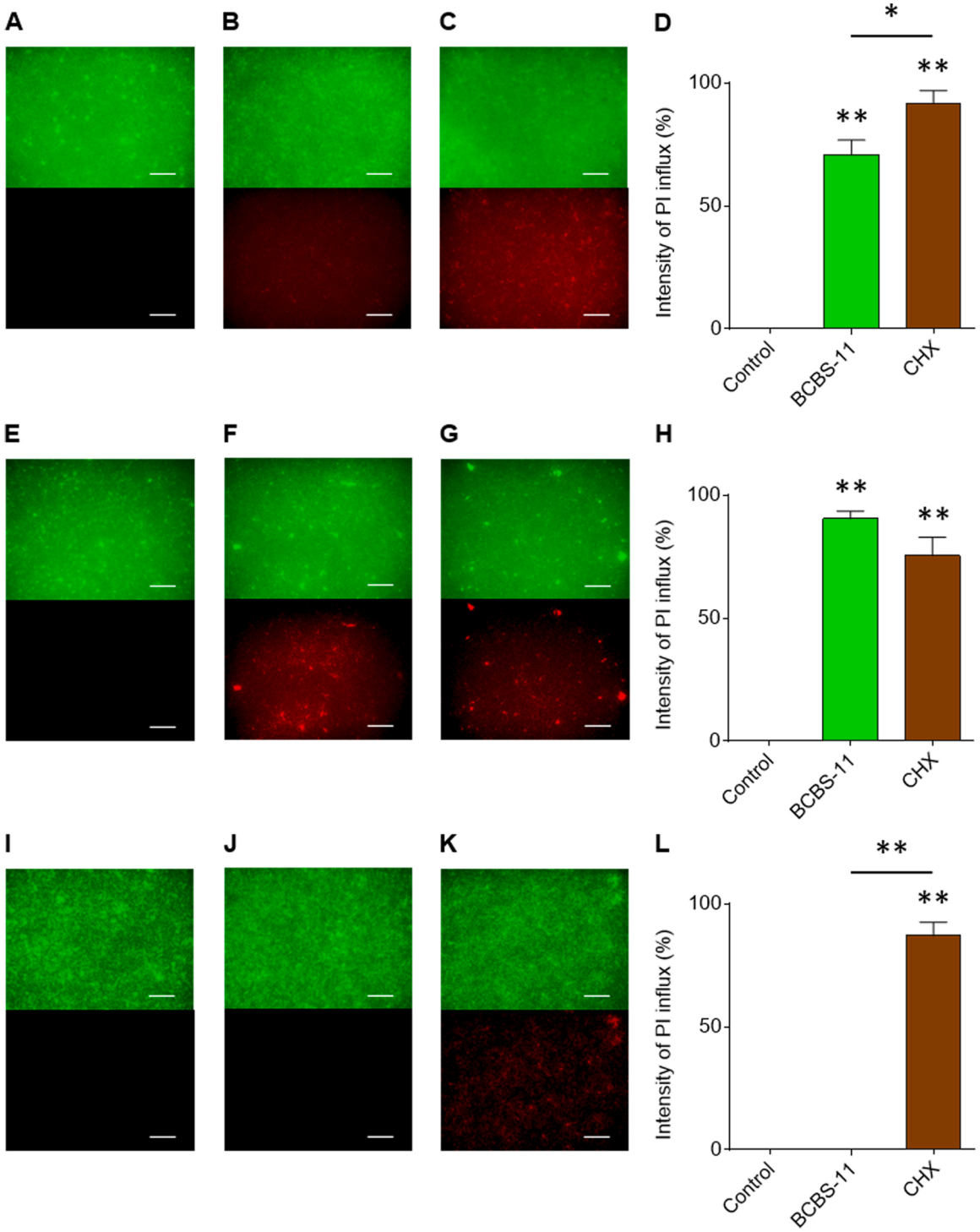

Fig. 1. BCBS-11 shows membrane disintegration in both *P. gingivalis* and *F. nucleatum.* SYTO 9-positive staining (green) shows live bacteria and PI-positive staining (red) shows membrane damage in *P. gingivalis* (A: control, B: BCBS-11, and C: CHX), *F. nucleatum* (E: control, F: BCBS-11, and G: CHX), and *S. mitis* (I: control, J: BCBS-11, and K: CHX). Scale bars: 100 μm. The intensity of PI influx was quantified and normalized to SYTO 9-positive intensity (D: *P. gingivalis*, H: *F. nucleatum* and L: *S. mitis*; n = 3). The results are presented as the means ± standard errors of the mean (SEMs). * $P < 0.05$ or * * $P < 0.01$ versus control or as indicated according to analysis of variance.

3.4. The soy bean peptide BCBS-11 inhibits the monospecies biofilm formation of P. gingivalis or F. nucleatum

To determine the antibiofilm effects of the soy bean peptide against periodontopathic bacteria, we evaluated the effect of BCBS-11 on monospecies biofilm formation. This peptide significantly inhibited the monospecies biofilm formation of *P. gingivalis* (Fig. 3A) and *F. nucleatum* (Fig. 3B). The minimum biofilm inhibitory concentration of BCBS-11 against *F. nucleatum* (100 μM) was lower compared with *P. gingivalis* (400 μM), considering that BCBS-11 exhibits a stronger antibiofilm effect against *F. nucleatum* than *P. gingivalis*. Furthermore, BCBS-11 was almost as effective as CHX, a positive control, against *F. nucleatum* (Fig. 3B). BCBS-11 did not inhibit the biofilm formation of *S. mitis*, a Gram-positive bacterium, despite CHX having a significant effect

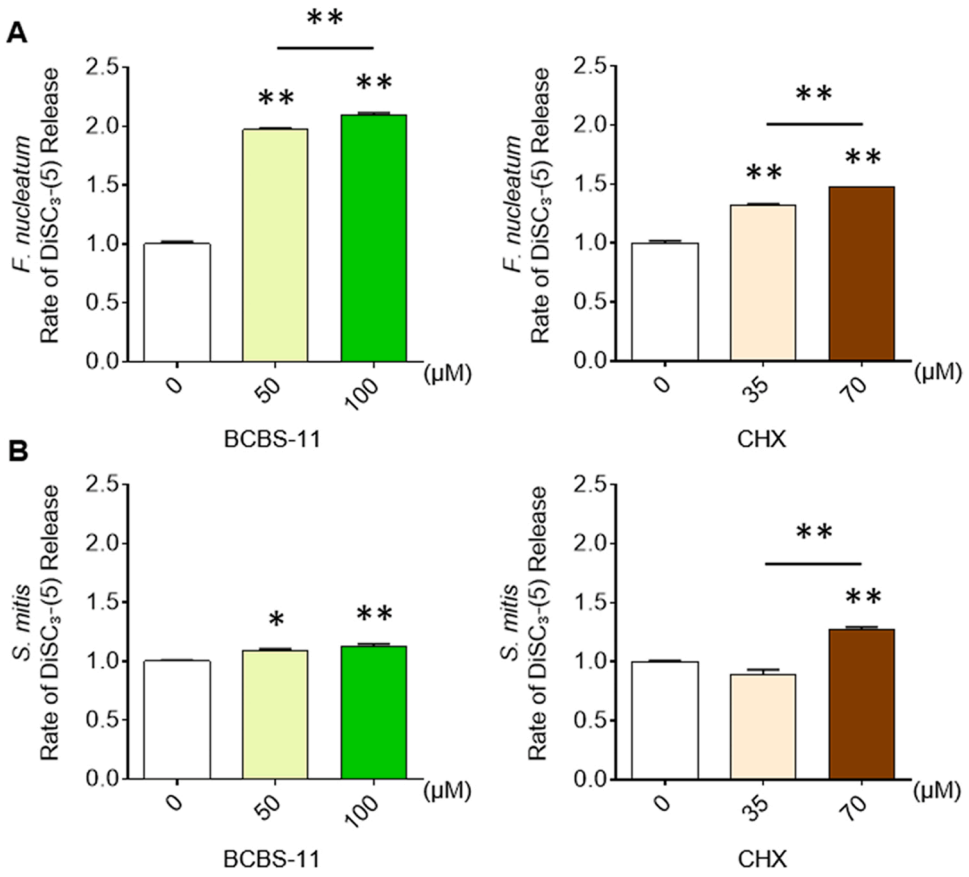

Fig. 2. BCBS-11 induces disruption of the bacterial membrane and depolarization. The effects of BCBS-11 or CHX on membrane depolarization of *F. nucleatum* (A) or *S. mitis* (B). The rate of fluorescence intensity was quantified and 0 μM is shown as 1.0 (n = 4). The results are represented as the means ± standard errors of the mean (SEMs). * $P < 0.05$ or ** $P < 0.01$ versus 0 μM or as indicated according to analysis of variance.

(Fig. 3C).

3.5. BCBS-11 reduces the amount of the monospecies established biofilm of F. nucleatum

Next, we investigated the eradicating effect toward established biofilms. BCBS-11 did not eradicate the established biofilm of *P. gingivalis* (Fig. 4A). The amount of *F. nucleatum* biofilm was reduced significantly using BCBS-11 at 400 μM, although neither BCBS-11 nor CHX completely eradicated the mature biofilm (Fig. 4B). BCBS-11 did not reduce the mature biofilm of *S. mitis* (Fig. 4C).

3.6. BCBS-11 inhibits the dual-species biofilm formation of P. gingivalis and F. nucleatum

We examined the effect of BCBS-11 on dual- and triple-species biofilm formation. BCBS-11 peptide also inhibited the dual-species biofilm formation of *P. gingivalis* and *F. nucleatum* (Fig. 5A), whereas it does not inhibit the triple-species biofilm formation (*P. gingivalis*, *F. nucleatum*, and *S. mitis*) at 400 μM (Fig. 5B). CHX demonstrated effective inhibitory effects on dual- (Fig. 5A) and triple-species (Fig. 5B) biofilm formation.

3.7. BCBS-11 do not show any cytotoxic effect on human oral epithelial cells

Low off-target toxicity is important for clinical applications. Therefore, the cytotoxicity of the BCBS-11 peptide was measured using an MTT assay. BCBS-11 did not demonstrate any cytotoxicity at the maximum concentration examined (i.e., 400 μM), although CHX exhibited toxicity toward Ca9–22 at 70 μM (Fig. 6). Thus, BCBS-11 demonstrated lower cytotoxicity compared with CHX.

4. Discussion

In the present study, we demonstrated that soybean-derived BCBS-11 peptide exhibits the growth inhibition (Supplementary Figure 1) and bactericidal antibacterial activity against *P. gingivalis* and *F. nucleatum* (Table 1). Moreover, the peptide inhibits monospecies and dual-species biofilm formation (Figs. 3 and 5). *P. gingivalis* is the most common periodontopathogenic bacterium, which occurs in deep periodontal pockets (Noiri et al., 2004). *F. nucleatum* plays an important role in biofilm formation by bridging bacteria during biofilm maturation (Kolenbrander & London, 1993). In a previous report, we demonstrated that the antimicrobial control of *F. nucleatum* was extremely crucial in inhibiting the formation of complex biofilms for multiple bacteria (Matsugishi et al., 2021). Therefore, BCBS-11 may be an effective agent for the prevention and control of periodontal disease.

Several peptides have strong physiological influences and are relatively easy to synthesize, but few have been successfully utilized as pharmaceuticals. One factor is that peptides are chemically unstable and quickly broken down by digestive enzymes in the gastrointestinal tract (Yao et al., 2018). The heat-treated BCBS-11 retained their antimicrobial activity (Supplementary Table 3), but further studies are needed on the method of administration because the peptides may be degraded or denatured by various factors, such as pH and enzymes in saliva.

The results shown in Figs. 1 and 2 indicate that the bactericidal effect of BCBS-11 results from membrane disruption of *P. gingivalis* and *F. nucleatum*, resulting in increased membrane permeability and depolarization. BCBS-11 causes the release of $DiSC_3$-(5) in *P. gingivalis* in a concentration-dependent manner (Taniguchi et al., 2017), which is

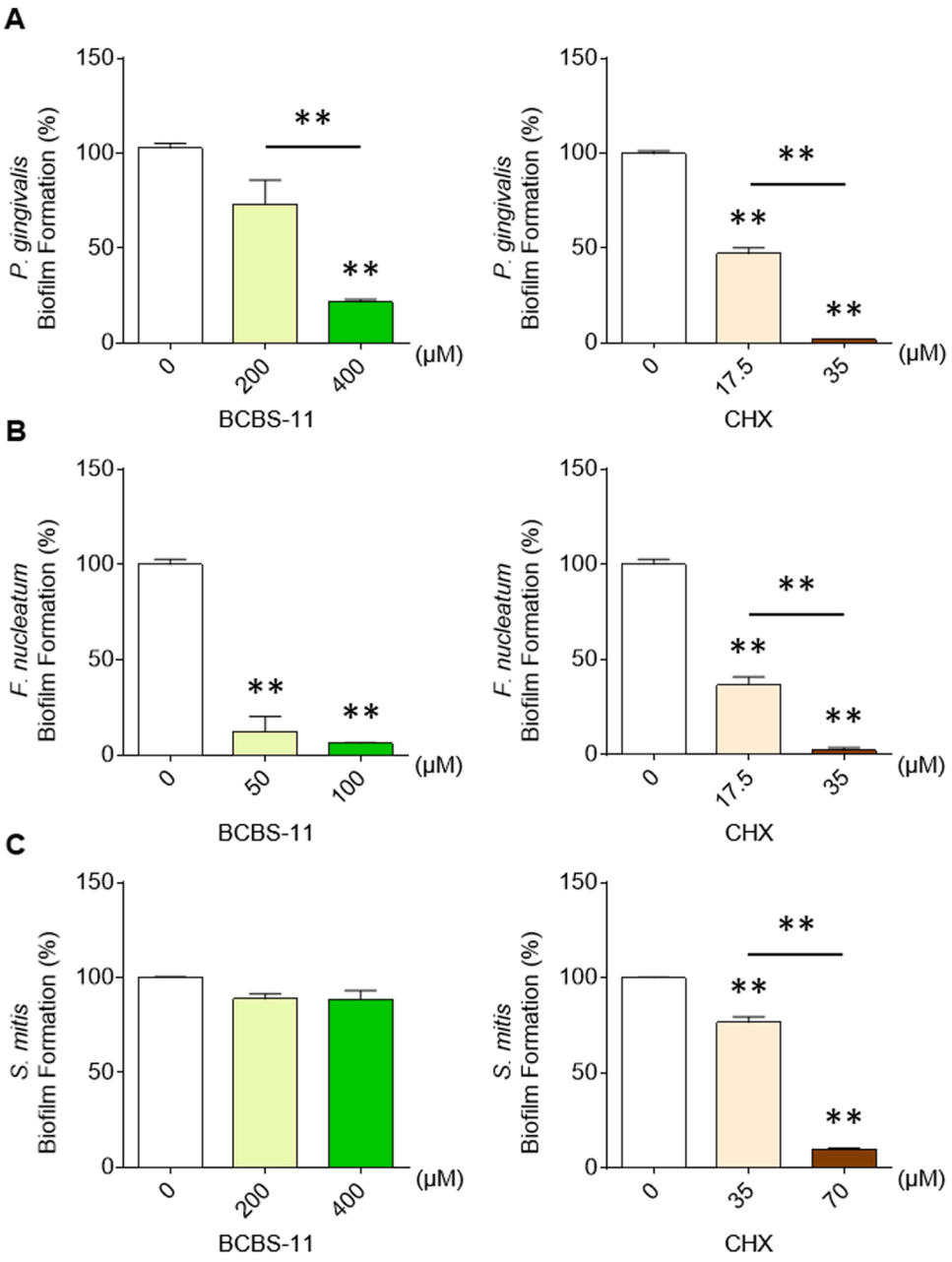

Fig. 3. The soy bean peptide BCBS-11 inhibits monospecies biofilm formation of *P. gingivalis* or *F. nucleatum.* The effects of BCBS-11 on monospecies biofilm formation of *P. gingivalis* (A), *F. nucleatum* (B), or *S. mitis* (C). After incubation with each reagent, the amount of remaining biofilm was evaluated with crystal violet stain (n = 4). The results are presented as the means ± standard errors of the mean (SEMs). ** $P < 0.01$, versus 0 μM or as indicated according to an analysis of variance.

consistent with the PI staining, MIC, and MBC results of this present study. Since BCBS-11 contains several hydrophobic amino acids and is amphiphilic, the "carpet" mechanism is one potential mechanism of membrane disruption (Jarva et al., 2018; Shai, 2002). This term is used describing the mechanism of other antimicrobial peptides, such as dermaseptin natural analogs (La Rocca et al., 1999; Strahilevitz et al., 1994), the human antimicrobial peptide, LL-37 (Oren et al., 1999), and melittin in anionic lipids (Ladokhin & White, 2001). These peptides bind to the surface of the membrane with their hydrophobic surfaces facing the membrane and their hydrophilic surfaces facing the solvent. A threshold concentration of peptide leads to transient pores and membrane disintegration.

The antimicrobial mechanism of BCBS-11 requires further study. In general, net charge, hydrophobicity, amphipathicity, and structural features are crucial for peptides to demonstrate antimicrobial activity (Bhattacharjya & Ramamoorthy, 2009). In particular, the positive charge of the peptide is crucial for its interaction with negatively-charged membrane phospholipids. Glycinin-17 (Taniguchi et al., 2017) as a control peptide, is also a charged cationic, more so than BCBS-11 (Supplementary Table 2A), but it did not show any antimicrobial effects against *P. gingivalis* and *F. nucleatum* (Supplementary Table 2B). An increase in the total amount of positive charge often increases the affinity for microbial membranes and enhances antimicrobial activity; however, there may be a threshold for this effect (Dong et al., 2012). Furthermore, the most antimicrobial activity of peptides have α-helix or β-sheet contents (Nguyen et al., 2011; Zasloff, 2002). BCBS-11 contains α-helix and β-sheet, whereas Glycinin-17 does not contain these structures (Supplementary Tables 1 and 2A), suggesting that the difference in the secondary structure of these peptides influences the intensity of their antibacterial activity. Moreover, the

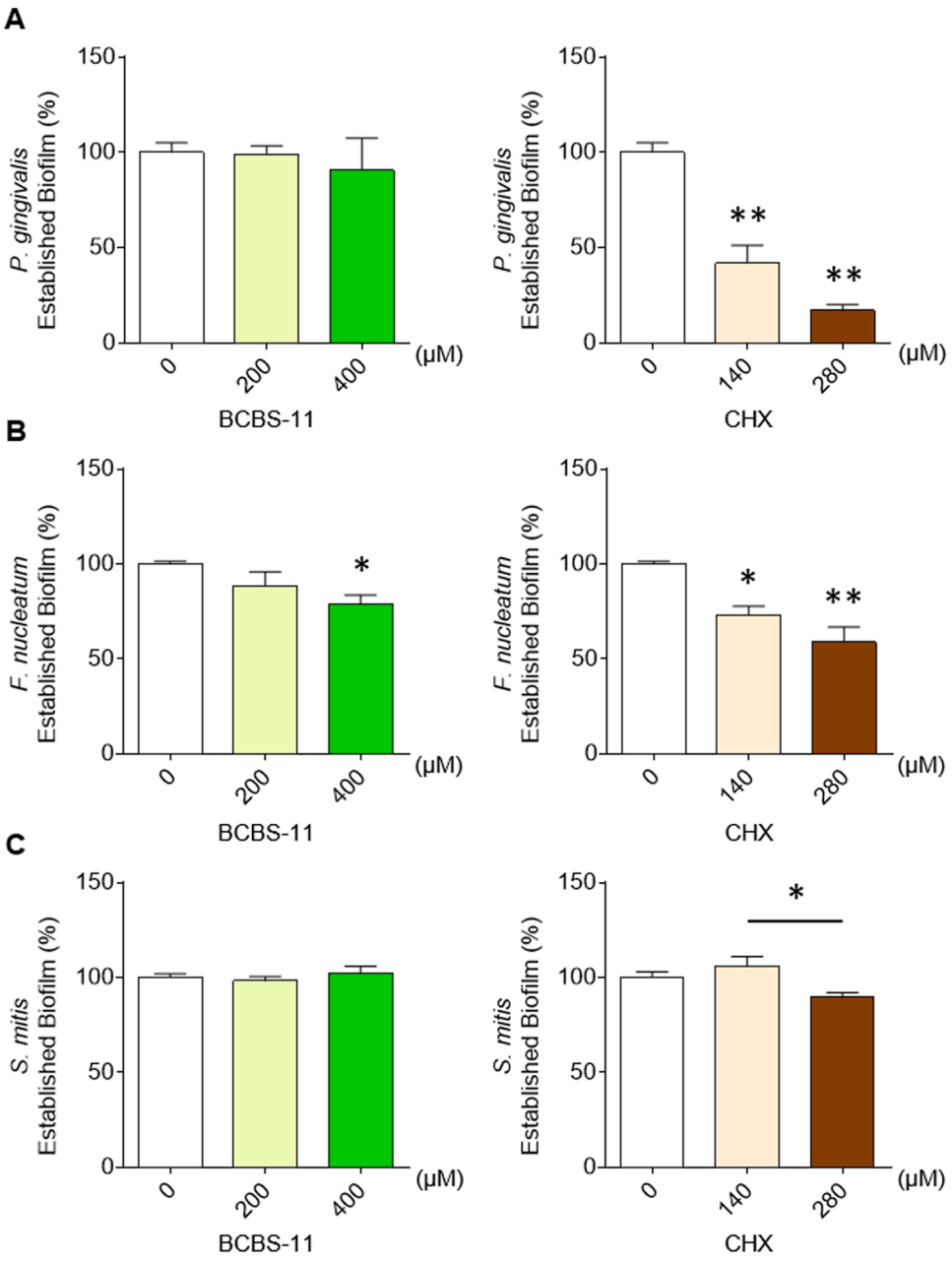

Fig. 4. BCBS-11 reduces the amount of established biofilm of *F. nucleatum.* The effects of BCBS-11 on the established biofilm of *P. gingivalis* (A), *F. nucleatum* (B), or *S. mitis* (C). The reagents were added after the biofilms were established. The remaining amount of biofilm was evaluated with crystal violet stain (n = 4). The results are presented as the means ± standard errors of the mean (SEMs). *$P < 0.05$ or ** $P < 0.01$ versus 0 μM or as indicated according to analysis of variance.

combination of these factors may function specifically for bacteria, but more comparisons of bacteria and peptides are required.

Bacteria-specific antimicrobial activity is desirable, especially to enhance antimicrobial resistance, which is currently prevalent worldwide. In this present study, we utilized the Gram-positive bacterium, *S. mitis*, a harmless oral commensal, as a contrast to periodontopathogenic bacteria. BCBS-11 demonstrated strong antibacterial activity against the Gram-negative bacteria, *P. gingivalis* and *F. nucleatum*; however, BCBS-11 did not show any antibacterial activity against *S. mitis* or any inhibition of biofilm formation (Table 1, Fig. 3C). The putative mechanism of action of the peptide against the bacteria is presented in Fig. 7. In general, the antimicrobial activity of membrane-targeted antimicrobial peptides is influenced by the structure and lipid composition of the bacterial membrane (Deleu et al., 2013). The outer membrane of Gram-negative bacteria comprises a barrier that maintains cell structure and contains lipopolysaccharides, which are targets for antimicrobial peptides because of their electrostatic interactions with hydrophobic moments (Schmidtchen et al., 2014; Torres et al., 2018; Ulmschneider et al., 2014; Yin et al., 2012). Conversely, Gram-positive bacteria have a cell wall that contains a thick peptidoglycan layer and a strong negatively-charged teichoic acid on the cell wall surface. In this study, compared with CHX, which also showed strong antibacterial activity against *S. mitis*, the antibacterial activity of BCBS-11 was limited. Its low impact on indigenous bacteria may be an advantage of BCBS-11; however, further studies are needed to elucidate the bacterial specificity of this peptide.

We conducted an experiment using multispecies biofilms to investigate a bacterial flora potentially close to the diversity of the oral cavity. The comparison of the results for dual- and triple-species biofilms demonstrated that BCBS-11 failed to inhibit the formation of triple-

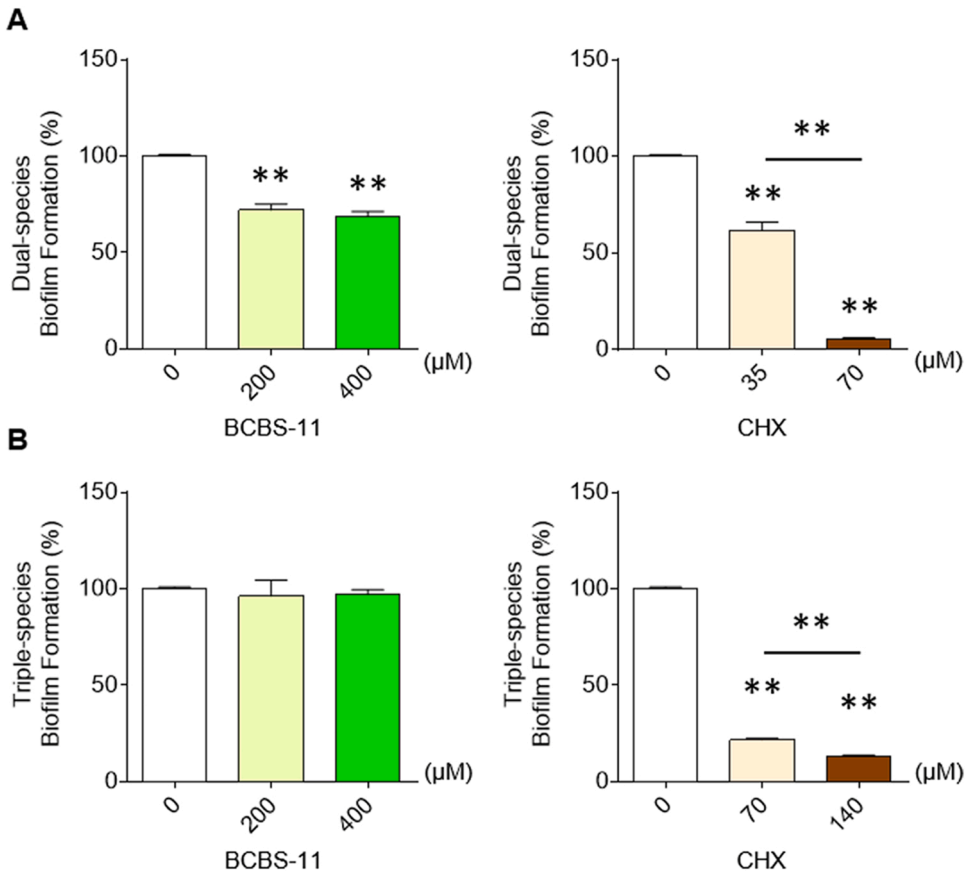

Fig. 5. BCBS-11 inhibits dual-species biofilm formation of *P. gingivalis* and *F. nucleatum.* The effects on dual-species (A) and triple-species (B) biofilm formation. The amount of biofilm was calculated with crystal violet stain (n = 4) after incubation with BCBS-11 or CHX. The results are presented as the means ± standard errors of the mean (SEMs). ** $P < 0.01$, versus 0 μM or as indicated according to an analysis of variance.

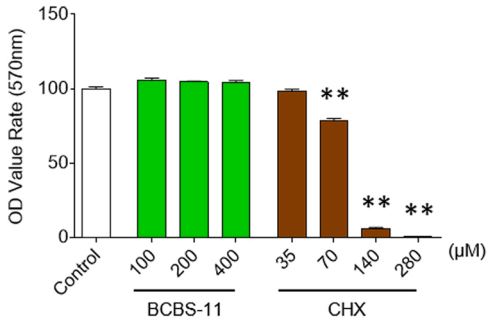

Fig. 6. BCBS-11 does not exhibit any cytotoxicity on human oral epithelial cells. The effect of BCBS-11 on cell viability was evaluated using an MTT assay. BCBS-11 or CHX was added to each well and incubated for 24 h. Absorbance was measured at a wavelength of 570 nm (n = 4). The results are presented as the means ± standard errors of the mean (SEMs). * * $P < 0.01$ for control according to analysis of variance.

species biofilms because of its weak antimicrobial activity against *S. mitis* (Fig. 5B). Therefore, it is insufficient to inhibit only Gram-negative bacteria such as *P. gingivalis* and *F. nucleatum* to inhibit biofilm formation, and it is essential to show antibacterial activity against Gram-positive bacteria, which are considered to be the initial colonizers of biofilms.

Although BCBS-11 did not entirely remove the established biofilm for any bacteria (Fig. 4), it significantly reduced the amount of biofilm for *F. nucleatum*. In general, antimicrobial peptides with strong cationic properties adhere to the biofilm surface with electrostatic force (Rzhepishevska et al., 2013). BCBS-11 also exhibits strong cationic properties (Supplementary Table 1), so it may adhere to the biofilm surface and exhibit antimicrobial activity against surface bacteria. To eradicate the established biofilm, it is ideal that the peptides are able to penetrate into the biofilm.

Comparing the effects of BCBS-11 and CHX, CHX was demonstrated to have more effective antibacterial activity against Gram-positive and Gram-negative bacteria (Table 1). Moreover, CHX demonstrated strong activity in terms of the inhibition of biofilm formation and removal of established biofilm (Figs. 3, 4 and 5). It may be difficult for BCBS-11 to surpass CHX in terms of efficacy in the biofilm control. However, the clinical use of selective antibacterial agents for treating periodontal diseases may be advantageous to prevent the increase in antimicrobial-resistant bacteria because membrane-acting antimicrobial peptides are generally less likely to induce resistance in bacteria compared with other existing antibiotics that target bacterial metabolism (Rodriguez-Rojas et al., 2018; Zasloff, 2002).

Another advantage of BCBS-11 may be that it is less cytotoxic than CHX (Fig. 6). The less pronounced effect of this antimicrobial peptide on host cells may be due to a difference of the electrostatic charge of the bacterial and host cell membranes. The cationic peptide BCBS-11 binds strongly to the negatively-charged membrane of bacteria with acidic phospholipids by electrostatic interaction. In contrast, the host mammalian cell membrane is generally rich in neutral phospholipids and charge-neutral (Op den Kamp, 1979), thus considering that the binding of the peptide is weak.

The primary limitations of this study is that it was conducted using a biofilm model in vitro, thus the efficacy of the peptide as an orally administered drug must be valid by additional experiments including in vivo experiments before future clinical studies. Second, the stability of

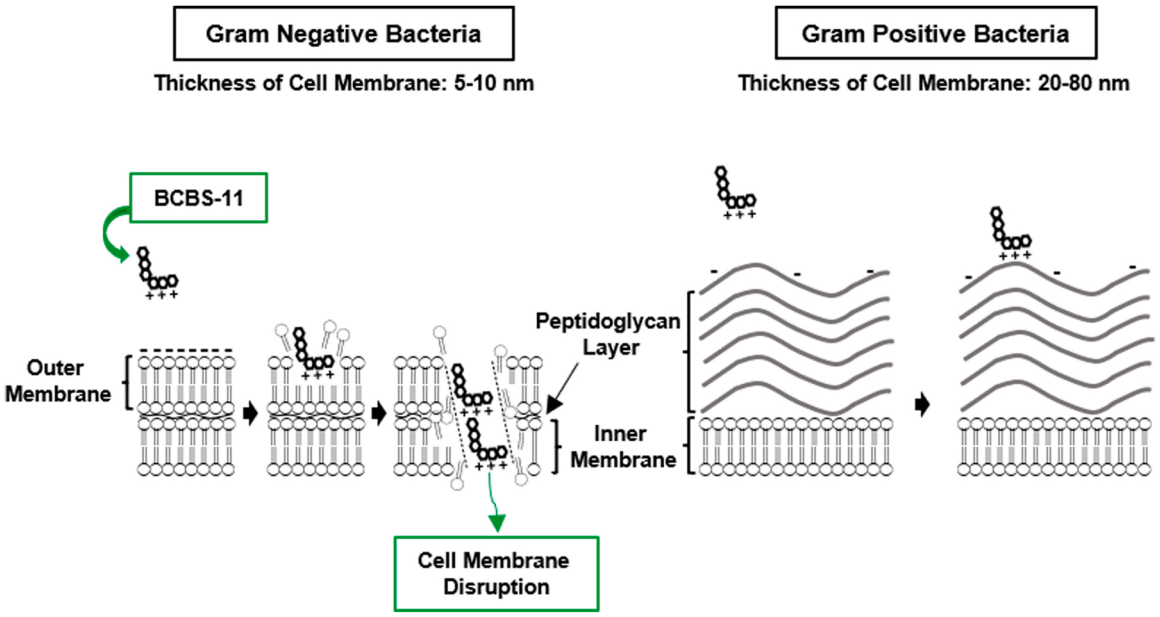

Fig. 7. The mechanism of peptide activity against the bacteria used in this study. Gram-negative bacteria generally have a thin membrane structure. Their surfaces are negatively-charged and BCBS-11 interacts with them electrostatically. Gram-positive bacteria have a cell wall that contains a thick peptidoglycan layer, indicating that the peptides do not have a membrane-disrupting effect.

the peptides in saliva and gingival sulcus fluid should be valid, and adiministration methods and dosage forms should be considered. Third, the effects of BCBS-11 peptide on EPS and quorum sensing other than its antimicrobial activity against biofilms are unknown and are the subject of future research. Finally, it has not yet been evaluated the risk that this peptide may influence the organism and its pathology by disrupting the diversity of natural biofilms and their ecological balance in oral cavity. The identification of peptides with antimicrobial activity against periodontal pathogens may permit personalized treatment of biofilm-related diseases and provide alternatives to existing antibiotics.

5. Conclusion

BCBS-11 inhibited biofilm formation of *P. gingivalis* and *F. nucleatum,* and bactericidal activity resulted from membrane disruption.

Ethics

No animal and human samples were used in this study, thus there is no IRB statement to show.

CRediT authorship contribution statement

Hnin Yu Lwin: Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Yukari Aoki-Nonaka:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Aoi Matsugishi:** Writing – review & editing, Validation, Investigation. **Naoki Takahashi:** Writing – review & editing, Formal analysis, Visualization. **Takumi Hiyoshi:** Writing – review & editing, Formal analysis, Visualization. **Koichi Tabeta:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.archoralbio.2022.105497.

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Supplementary Table 1. Properties of peptide BCBS-11.

Peptide	Sequence	Molecular Weight (g/mol)		pI (-)	MH[a]	Net Charge[b]	α-helix Content[c] (%)	β-sheet Content[c] (%)	β-turn Content[c] (%)
		Measured	Calculated						
BCBS-11	RIRLLQRFNKR	1499.81	1499.85	13	−1.27	+5	27.27	36.36	36.36

[a]The mean hydrophobicity (MH) value of the peptide was calculated using the Kyte and Doolittle scale.

[b]The net charge of the peptide was calculated using the Peptide Property Calculator (https://pepcalc.com).

[c]The peptide secondary structure prediction, α-helix, β-sheet, and β-turn contents, were estimated using the GOR1 method, available at the Network Protein Sequence @nalysis (NPS@) (https://npsa-prabi.ibcp.fr).

Supplementary Table 2.

(A) Properties of peptide Glycinin-17.

Peptide	Sequence	Molecular Weight (g/mol)		pI (-)	MH[a]	Net Charge[b]	α-helix Content[c] (%)	β-sheet Content[c] (%)	β-turn Content[c] (%)
		Measured	Calculated						
Glycinin-17	RKSREWRSKKTQPRRPR	2252.59	2253.07	12.8	−3.06	+8	0	0	58.82

[a]The mean hydrophobicity (MH) value of the peptide was calculated using the Kyte and Doolittle scale.

[b]The net charge of the peptide was calculated using the Peptide Property Calculator (https://pepcalc.com).

[c]The peptide secondary structure prediction, α-helix, β-sheet, and β-turn contents, were estimated using the GOR1 method, available at the Network Protein Sequence @nalysis (NPS@) (https://npsa-prabi.ibcp.fr).

(B) MIC and MBC values of soybean peptide Glycinin-17 with regard to *P. gingivalis* and *F. nucleatum*.

(μM)	*P. gingivalis*		*F. nucleatum*	
	MIC	MBC	MIC	MBC
Glycinin-17	>400	>400	>400	>400

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

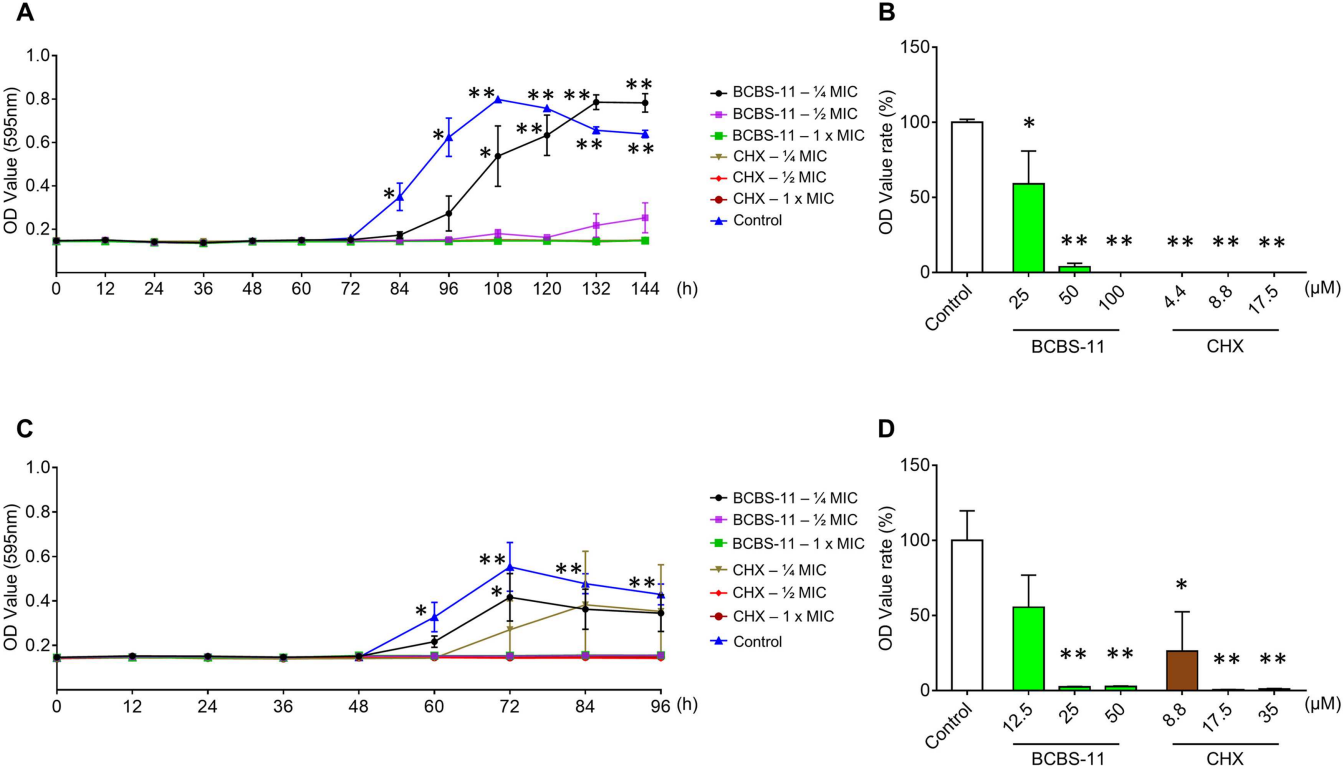
Supplementary Table 3. MIC and MBC values of soybean peptide BCBS-11 (heat-treated) with regard to *F. nucleatum*.

(μM)	Heat-treated BCBS-11		Untreated BCBS-11	
	MIC	MBC	MIC	MBC
F. nucleatum	50	100	50	100

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

Supplementary Table 3. The thermostability was measured by the determination of the antimicrobial activity of the peptide after incubation for 10 minutes at 100°C. Compared with untreated BCBS-11, the results showed that BCBS-11 retained bactericidal activity even after heating at high temperature. It was suggested that soybean peptide BCBS-11 has high thermal stability.

Materials and method. Soybean peptide BCBS-11 was heated at 100°C for 10 minutes, and as a control, an untreated BCBS-11 that was kept at room temperature was used. After BCBS-11 was heated, MIC was determined using 96-well round-bottomed plates (Techno Plastic Products AG, Trasadingen, Switzerland). *F. nucleatum* was used as a test strain. Bacterial cultures of *F. nucleatum,* was diluted to 1×10^7 CFU/mL. Equal volumes of the diluted bacterial cultures, and BCBS-11 (both heated and untreated types) were mixed and aliquoted into plates. The plates were incubated at 37°C for 2 days under anaerobic conditions.



Supplementary Figure 1.

Inhibition of bacterial growth by soybean peptide BCBS-11

The growth curves of *P. gingivalis* and *F. nucleatum* are shown in Supplementary Figure 1 (A: *P. gingivalis*, C: *F. nucleatum*). The bacterial growth rate was plotted as a percentage of the OD value of *P. gingivalis* at 108 h (B) and *F. nucleatum* at *72* h (D), respectively. BCBS-11 and CHX, at ½ MIC or 1 x MIC, significantly inhibited bacterial growth. The 1 × MIC of BCBS-11 concentrations were 100 μM for *P. gingivalis* and 50 μM for *F. nucleatum*.

The growth curves (A and C) are expressed as the mean ± SEM (**$p < 0.01$ vs. 0 h, by paired t-test , n = 4). The growth rates (B and D) are expressed as the mean ± SEM (**$p < 0.01$ vs. Control, by ANOVA, n = 4).

Materials and Methods

The growth curve measurement

The inhibitory effect of BCBS-11 against *P. gingivalis* and *F. nucleatum* growth was determined. Briefly, bacterial cultures were diluted to 1 × 10^7 CFU/mL and added to 96-well plates. The diluted bacterial cultures were treated with ¼ MIC, ½ MIC and 1 × MIC concentrations of BCBS-11 and CHX. The bacterial culture without BCBS-11 or CHX was used as a bacterial growth control. The bacterial plates were incubated at 37°C under anaerobic conditions. The absorbance at 600 nm was recorded with a microplate reader (MOLECULAR DEVICES, San Jose, CA, USA). The growth curves were constructed by plotting the OD value versus time.