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Rice peptide with amino acid substitution inhibits biofilm formation by *Porphyromonas gingivalis* and *Fusobacterium nucleatum*

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ABSTRACT

Objective: Rice peptide has antibacterial properties that have been tested in planktonic bacterial culture. However, bacteria form biofilm at disease sites and are resistant to antibacterial agents. The aim of this study was to clarify the mechanisms of action of rice peptide and its amino acid substitution against periodontopathic bacteria and their antibiofilm effects.

Design: Porphyromonas gingivalis and *Fusobacterium nucleatum* were treated with AmyI-1-18 rice peptide or its arginine-substituted analog, G12R, under anaerobic conditions. The amount of biofilm was evaluated by crystal violet staining. The integrity of the bacteria cytoplasmic membrane was studied in a propidium iodide (PI) stain assay and transmission electron microscopy (TEM).

Results: Both AmyI-1-18 and G12R inhibited biofilm formation of *P. gingivalis* and *F. nucleatum;* in particular, G12R inhibited *F. nucleatum* at lower concentrations. However, neither peptide eradicated established biofilms significantly. According to the minimum inhibitory concentration and minimum bactericidal concentration against *P. gingivalis,* AmyI-1-18 has bacteriostatic properties and G12R has bactericidal activity, and both peptides showed bactericidal activity against *F. nucleatum.* PI staining and TEM analysis indicated that membrane disruption by G12R was enhanced, which suggests that the replacement amino acid reinforced the electostatic interaction between the peptide and bacteria by increase of cationic charge and α-helix content.

Conclusions: Rice peptide inhibited biofilm formation of *P. gingivalis* and *F. nucleatum,* and bactericidal activity via membrane destruction was enhanced by amino acid substitution.

1. Introduction

Periodontitis is a biofilm-mediated disease that is characterized by inflammation of gingival tissue and subsequent tooth loss (Van Dyke & Serhan, 2003). Regulating the levels of dental plaque biofilm is essential to address the primary cause of this disease. However, matrix-enclosed biofilms prohibit the infiltration and subsequent action by pharmacological substances, an essential prerequisite to tackle this issue. Also, in contrast to planktonic bacteria, those present in formed biofilms are highly resistant to antibiotics (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Hoiby et al., 2011; Olson, Ceri, Morck, Buret, & Read, 2002). It is interesting to note that *Porphyromonas gingivalis* biofilms are highly resistant to antibiotics such as minocycline and metronidazole, despite these being quite effective against planktonic *P. gingivalis* (Noiri et al., 2003).

The influence of antimicrobial resistance to existing antibiotics has

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become a major concern worldwide in the past several decades.(Willyard, 2017) Existing biofilms in the oral cavity can enable the horizontal gene transfer of antibiotic-resistance genes (Olsen, Tribble, Fiehn, & Wang, 2013; Roberts & Kreth, 2014), which promotes the migration of periodontopathic bacteria from the oral cavity to other organs (Naylor et al., 2018). For instance, resistance of *Fusobacterium nucleatum* strains to amoxicillin, clindamycin, and metronidazole has been reported (Ardila, Granada, & Guzman, 2010; Mosca, Miragliotta, Iodice, Abbinante, & Miragliotta, 2007; van Winkelhoff, Herrera, Oteo, & Sanz, 2005). Therefore, the use of antibiotics must be restricted, and alternative medicine to control plaque biofilm must be developed to achieve effective periodontal treatment and prepare a counterplan for microbial resistance.

Antimicrobial peptides have been studied extensively as potential alternatives to existing antibiotics (Chen & Lu, 2020). One of the advantages of using antimicrobial peptides instead of antibiotics is that bacteria may have a lower chance of developing drug resistance due to active membrane mechanisms such as membrane pore formation or membrane lysis induced by these peptides (Rodriguez-Rojas, Moreno-Morales, Mason, & Rolff, 2018; Zasloff, 2002). Another added advantage is that only a few substitutions in the amino acid residues of these antimicrobial peptides can effectively modify their antibacterial properties, thus making it harder for bacteria to develop resistance (Kim, Iwamuro, Knoop, & Conlon, 2001). It has previously been reported that AmyI-1-18, a peptide derived from rice α-amylase, as well as its analog peptide G12R, with a Gly12Arg substitution, show antimicrobial properties against planktonic *P. gingivalis* (Taniguchi, Ochiai, Takahashi et al., 2015; Taniguchi, Ochiai, Takahashi et al., 2016) Although the antibiofilm properties of these peptides would be useful in periodontal therapy, they have not been elucidated yet. We studied the antimicrobial properties of AmyI-1-18 and G12R against experimental biofilms of two periodontal bacteria: *P. gingivalis* and *F. nucleatum.*

2. Materials and methods

2.1. Peptides and reagents

The amino acid sequences of peptides[1] used in this study are summarized in Supplementary Table 1. Chemically synthesized AmyI-1-18 and its arginine-substituted analog G12R were designed as described in a previous report (Taniguchi, Ochiai, Takahashi et al., 2016). Chlorhexidine (CHX)[2] was used as the reference medium.

2.2. Bacterial culture

P. gingivalis strain FDC 381 and *F. nucleatum* strain ATCC 25586 were cultured in modified Gifu anaerobic medium broth[3] in an anaerobic jar[4] in the presence of AnaeroPack™ at 37 °C for 48 h. Using our established growth curves, we determined the number of colony-forming units (CFUs) by measuring optical density at 600 nm.

2.3. Biofilm quantification

To assess the effects of the peptides on *P. gingivalis* and *F. nucleatum* biofilm formation, bacterial cultures were diluted to 1×10^8 CFU/mL. A total of 100 μL of bacterial suspension was transferred to 96-well flat plates[5] with each peptide at final concentrations of 0 μM–400 μM and incubated for 48 h (*P. gingivalis*) or 72 h (*F. nucleatum*), and then the biofilm was quantified. The biofilm quantification was assessed with the crystal violet[6] staining method as described previously (Onozawa et al., 2015; Zhang et al., 2017).

Also, 1×10^8 bacterial CFU/mL were incubated anaerobically at 37 °C for 24 h (*P. gingivalis*) or 48 h (*F. nucleatum*) to establish biofilms. Then the peptides were added to the bacterial suspensions; this was followed by another 24 h incubation to assess the effects on the established biofilms. Phosphate-buffered saline (PBS) served as the control medium.

2.4. Determination of the minimum inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the peptides were determined with the use of a microplate dilution assay, as described previously (Wang et al., 2015).

2.5. Propidium iodide assay

P. gingivalis and *F. nucleatum* biofilms were formed in a Lab-Tek™ Chamber Slide[7] at a density of 1×10^8 CFU/mL. Bacteria were incubated with peptides at 12.5 % MBIC for 48 h (*P. gingivalis*) or 72 h (*F. nucleatum*) in anaerobic conditions. Biofilm samples were stained with a LIVE/DEAD BacLight™ Bacterial Viability Kit[8] according to the manufacturer's instructions. Samples were observed using a BZ-X710 microscope,[9] and the images were analyzed with imaging software (ImageJ 1.52k[10]).

2.6. Transmission electron microscopic imaging

P. gingivalis and *F. nucleatum* (1×10^9 CFU/mL) were treated with peptides at 50 % MBIC for 2 h in anaerobic conditions. Then the bacteria were centrifuged and fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH, 7.4) at room temperature for 1 h. They were subsequently post-fixed with 1 % osmium tetroxide in 0.1 M phosphate buffer at 4 °C for 1 h.

After dehydration in a graded ethanol series, the bacteria were embedded in Epon 812[11] and cut into ultrathin (70 nm) sections with an ultramicrotome (Ultracut-N).[12] The sections were stained with uranyl acetate and lead citrate on 150-mesh copper grids and observed under a transmission electron microscope (H-7650).[13] The transmission electron microscopy (TEM) images were quantified; approximately 100 randomly selected bacteria from each sample were captured, and the number of bacteria with aggregation of inner structure or membrane destruction was measured in a blind manner.

2.7. Statistical analysis

The data are expressed as means ± standard errors of the mean (SEMs). Statistical analyses were performed with GraphPad Prism 5 graphing and statistical software.[14] One-way analysis of variance was performed. A P value of less than 0.05 was considered statistically significant. All experiments were independently repeated at least twice, on separate days.

[1] Eurofins Genomics, Tokyo, Japan.
[2] FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan.
[3] Nissui, Tokyo, Japan.
[4] Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan.
[5] Techno Plastic Products AG, Trasadingen, Switzerland.
[6] Chroma-Gesellschaft Co. Ltd., Münster, Germany.
[7] Nunc, Rochester, NY, USA.
[8] Thermo Fisher Scientific, San Diego, CA, USA.
[9] Keyence, Osaka, Japan.
[10] National Institute of Health, Bethesda, MD, USA
[11] Nisshin EM Co. Ltd., Tokyo, Japan.
[12] Reichert-Jung, Vienna, Austria.
[13] Hitachi, Tokyo, Japan.
[14] GraphPad Software, Inc., San Diego, CA, USA

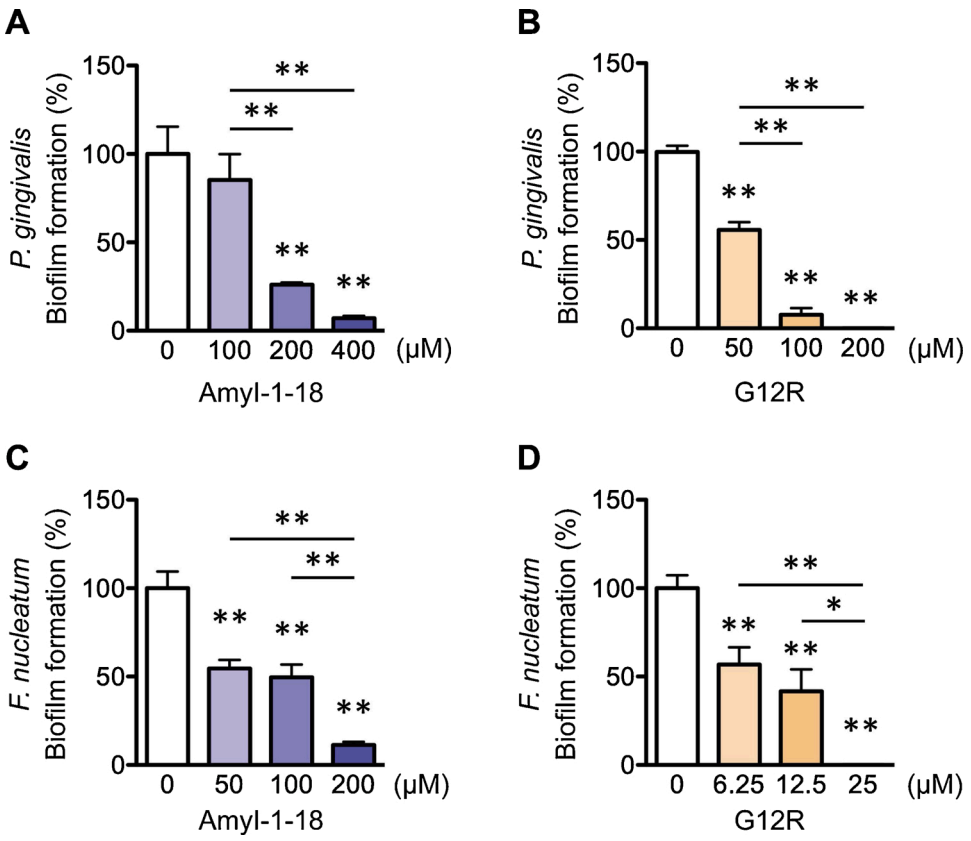

Fig. 1. AmyI-1-18 and G12R peptides suppressed formation of single-species biofilm of *P. gingivalis* or *F. nucleatum*.
The effects of peptides on single-species biofilm formation of *P. gingivalis* (A: AmyI-1-18 and B: G12R) or *F. nucleatum* (C: AmyI-1-18 and D: G12R). After incubation with each peptides, the remaining amount of biofilm was evaluated with crystal violet stain (n = 4). The results are presented as means ± standard errors of the mean (SEMs). $^{**}P < 0.01$, versus 0 μM or as indicated, according to analysis of variance.

Table 1
The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of peptides with regard to *P. gingivalis* and *F. nucleatum*.

(μM)	*Porphyromonas gingivalis*			*Fusobacterium nucleatum*		
	MIC	MBC	MBIC	MIC	MBC	MBIC
AmyI-1-18	200	>1600	400	200	200	200
G12R	100	200	200	25	25	25
Chlorhexidine	35	35	35	70	70	70

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

3. Results

3.1. AmyI-1-18 and G12R peptides suppressed formation of single-species biofilm of P. gingivalis or F. nucleatum

First, we investigated the effect of AmyI-1-18 and G12R peptides on single-species biofilm formation. These peptides significantly inhibited biofilm formation by *P. gingivalis* (Fig. 1A and B) and by *F. nucleatum* (Fig. 1C and D) in a dose-dependent manner. The minimum biofilm inhibitory concentration (MBIC) of G12R was lower than that of AmyI-1-18 (Table 1), which indicates that G12R inhibited the biofilm formation of both bacteria more effectively than did AmyI-1-18. Moreover, the MBIC of G12R against *F. nucleatum* (25 μM) was much lower than that against *P. gingivalis* (200 μM), which suggests that G12R had a stronger antibiofilm effect against *F. nucleatum* than against *P. gingivalis*.

3.2. AmyI-1-18 and G12R peptides did not eradicate established biofilm of P. gingivalis or F. nucleatum

Next, we investigated whether AmyI-1-18 and G12R eradicated the established biofilm. There were significant differences in the amount of *P. gingivalis* biofilms at 200 μM G12R but not at 400 μM compared with 0 μM as the control (Fig. 2B). Therefore, neither AmyI-1-18 nor G12R eradicated the established biofilms of *P. gingivalis* (Fig. 2A and B) or *F. nucleatum* (Fig. 2C and D).

3.3. G12R showed stronger bactericidal activity against P. gingivalis and F. nucleatum than did AmyI-1-18

In previous studies, several agents inhibited biofilm formation by suppressing the bacterial growth through bactericidal or bacteriostatic properties (Baehni & Takeuchi, 2003). To clarify the mechanisms of the antibacterial effects of AmyI-1-18 and G12R, we determined their MIC and MBC (Maezono et al., 2011) (Table 1). The large difference between the MIC and MBC of AmyI-1-18 against *P. gingivalis* suggested bacteriostatic property. In contrast, the MIC and MBC of G12R were similar, which indicates that G12R has bactericidal activity against *P. gingivalis*. These results suggested that the antibacterial mechanisms of AmyI-1-18 against *P. gingivalis* are different from those of G12R. On the other hand, the MIC values of both peptides were same as their MBC values against *F. nucleatum*, indicating that both peptides have bactericidal activity against *F. nucleatum*. These results suggest that G12R has stronger bactericidal activity against *P. gingivalis* and *F. nucleatum* than does AmyI-1-18. Also, the MBC value of G12R against *F. nucleatum* was lower than that of CHX, the positive control. The bactericidal properties of G12R against *F. nucleatum* may be stronger than those of CHX. The antimicrobial kinetics of AmyI-1-18 and G12R, as shown in Supplementary Fig. 1, suggests that the killing rate of G12R is comparable to CHX.

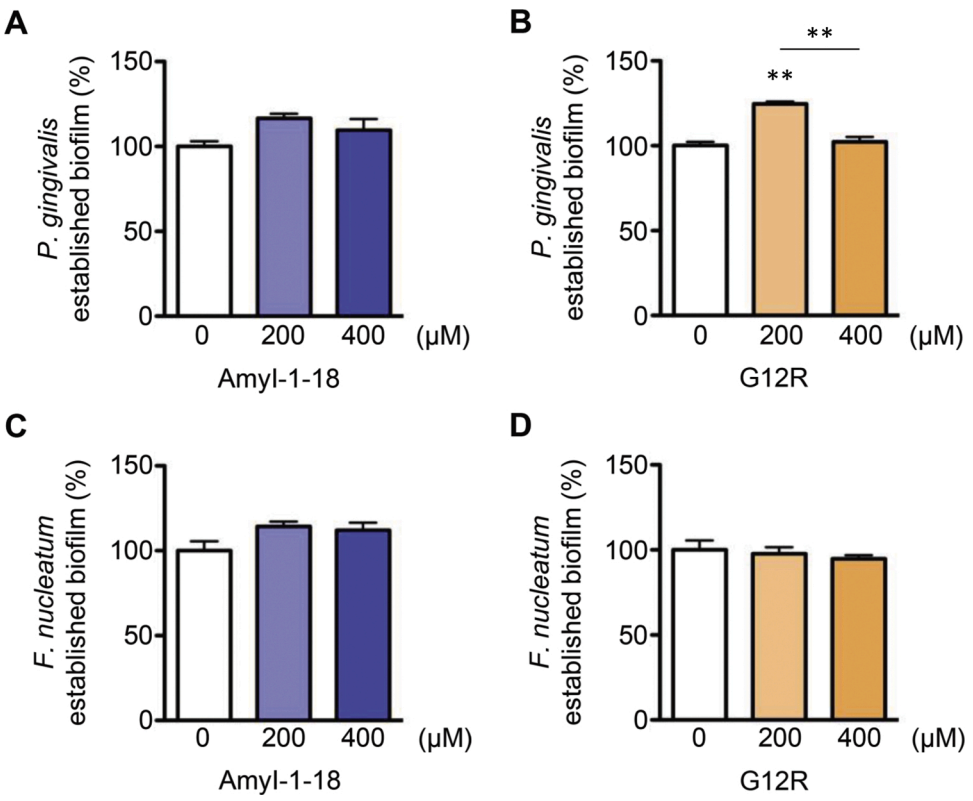

Fig. 2. AmyI-1-18 and G12R peptides did not eradicate established biofilm of *P. gingivalis* or *F. nucleatum*.
The effects of peptides on established biofilm of *P. gingivalis* (A: AmyI-1-18 and B: G12R) or *F. nucleatum* (C: AmyI-1-18 and D: G12R). Both peptides were added after biofilms were established. The remaining amount of biofilm was evaluated with crystal violet stain (n = 4). The results are presented as means ± standard errors of the mean (SEMs). $^{**}P < 0.01$ versus 0 μM or as indicated, according to analysis of variance.

3.4. Membrane permeability in both *P. gingivalis* and *F. nucleatum* was enhanced by G12R

Several studies have shown that antimicrobial peptides bind to and disrupt the integrity of bacterial membranes (Leontiadou, Mark, & Marrink, 2006). Propidium iodide (PI) enters the bacterial membrane only when the membrane has been damaged; therefore, uptake of extracellular PI was performed to determine the extent of membrane damage (Zhang et al., 2017). PI-positive cells were not observed in *P. gingivalis* and *F. nucleatum* incubated with PBS as control (Fig. 3A and E). G12R-treatment (Fig. 3C) enhanced PI influx into bacteria more than did AmyI-1-18 (Fig. 3B) in *P. gingivalis*. PI-positive *F. nucleatum* was observed in both AmyI-1-18- and G12R-treated samples (Fig. 3F and G). The quantified intensity of PI influx was significantly higher in G12R-treated samples (Fig. 3D and H), which suggests that G12R disrupted the membrane integrity of both bacteria more strongly than did AmyI-1-18.

3.5. G12R destroyed bacterial membranes of *P. gingivalis* and *F. nucleatum*

As with PI influx, TEM analysis showed that bacterial membrane integrity, cell structure, and cell nuclei were clearly intact in the negative control group incubated with PBS (Fig. 4A and E). In *P. gingivalis* samples, the addition of AmyI-1-18 did not destroy membrane structure (Fig. 4B); with G12R, the membrane was destroyed, and inner structures were observed to be flowing out (Fig. 4C). The cell walls of *F. nucleatum*, were stripped down and destroyed after addition of AmyI-1-18 and G12R, and cell structures leaked out (Fig. 4F and G). The addition of G12R significantly increased the percentage of bacteria whose membrane was destroyed or that were dead (Fig. 4D and H).

3.6. G12R peptide inhibited formation of dual-species biofilm of *P. gingivalis* and *F. nucleatum*

Finally, we tested the inhibitory effect of these peptides on biofilm formation by both *P. gingivalis* and *F. nucleatum*. G12R inhibited this dual-species biofilm formation in a dose-dependent manner (Fig. 5B), whereas AmyI-1-18 did not inhibit biofilm formation (Fig. 5A). Neither AmyI-1-18 nor G12R eradicated biofilm established by both species (Fig. 5C and D). These results suggested that G12R inhibited polymicrobial biofilm formation but not eradicate it.

4. Discussion

P. gingivalis is anaerobic bacteria that colonizes in periodontal deep pockets in patients with severe chronic periodontitis (Noiri, Li, Yoshimura, & Ebisu, 2004), and *F. nucleatum* plays a key role in subgingival biofilm formation by bridging between early and late colonizing bacteria in biofilm maturation process (Kolenbrander & London, 1993). Therefore, suppressing these periodontopathic bacteria is an effective approach for preventing or controlling periodontal disease, especially in a bacteria-specific manner to improve the situation of AMR currently prevailing world wide.

In this study, we found that AmyI-1-18 and G12R peptides inhibit single-species biofilm formation by *P. gingivalis* and *F. nucleatum* (Fig. 1). In addition, G12R peptide inhibited the dual-bacterial biofilm (Fig. 5), presumably because G12R more strongly inhibits *F. nucleatum* biofilm

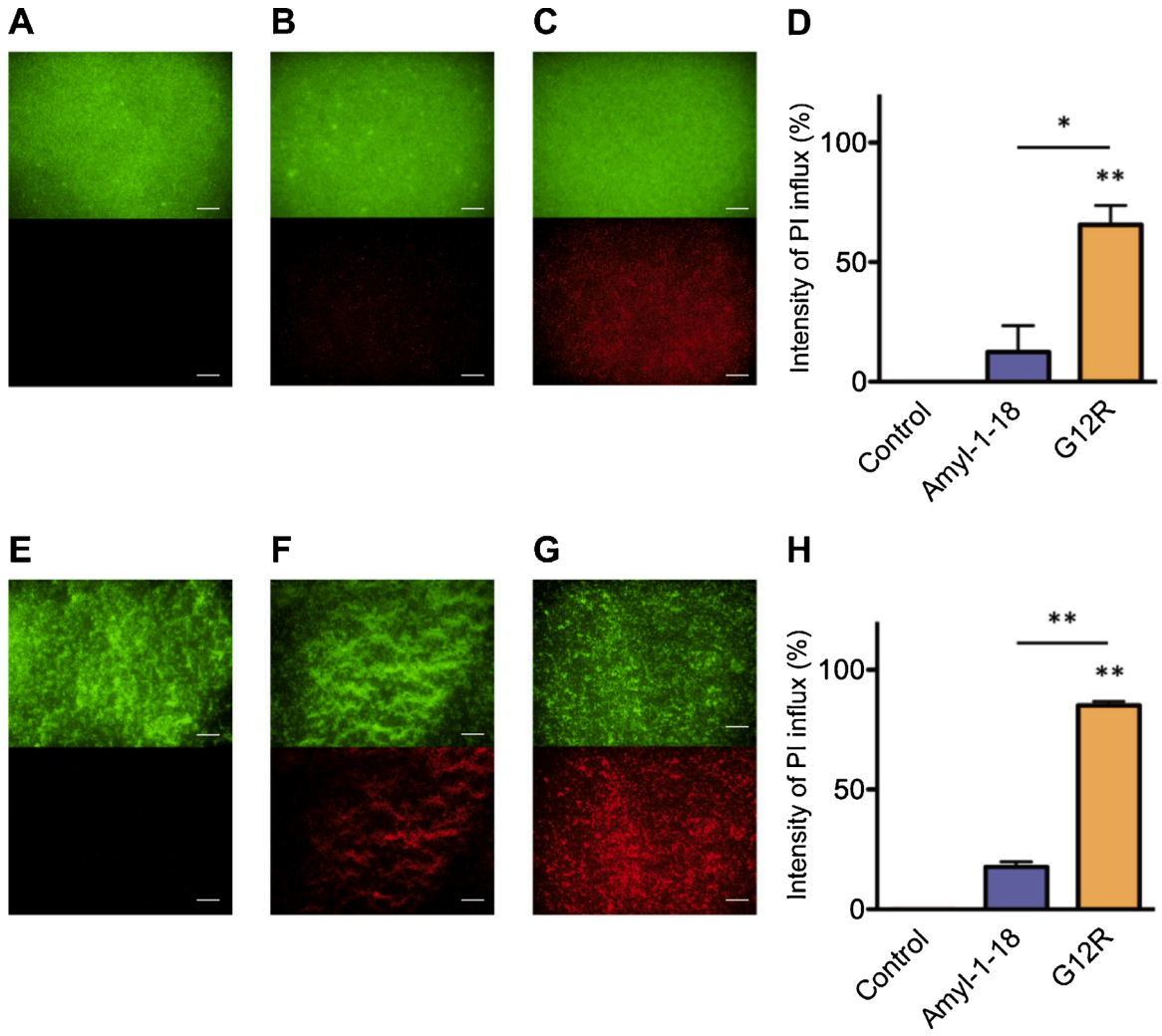

Fig. 3. Membrane permeability was enhanced by G12R in both *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.
SYTO 9 positive staining (green) shows live bacteria, and propidium iodide (PI) positive staining (red) shows membrane damage in *P. gingivalis* (A: control, B: AmyI-1-18, and C: G12R) and *F. nucleatum* (E: control, F: AmyI-1-18, and G: G12R). Scale bars: 100 μm. The intensity of PI influx was quantified and normalized to SYTO 9–positive intensity (D: *P. gingivalis* and H: *F. nucleatum*; n = 3). The results are represented as means ± standard errors of the mean (SEMs). $*P < 0.05$ (control) and $**P < 0.01$ (as indicated) according to analysis of variance.

formation than does AmyI-1-18 (Fig. 1D). This finding indicates that controlling biofilm of *F. nucleatum* may help prevent periodontal disease because the congregation ability of *F. nucleatum* is strong. Although these peptides were unable to remove established biofilm (Fig. 2), the clinical use of these peptides in periodontal therapy may be advantageous because, according to previous reports, resistant bacteria generally do not arise in the presence of antibacterial peptides (Rodriguez-Rojas et al., 2018; Zasloff, 2002).

According to data in Table 1 and Fig. 3B, analyses by MBC determination, and the results of PI staining, AmyI-1-18 inhibited *P. gingivalis* biofilm by bacteriostatic function without membrane disruption. This is concordant with a previous observation that antimicrobial activity of AmyI-1-18 against *P. gingivalis* is independent of membrane destabilization, according to a study of membrane depolarization with a 3,3′-dipropylthiadicarbocyanine iodide (DiSC3[5]) probe (Taniguchi, Ochiai, Takahashi et al., 2015). In addition, inhibition of protein translation and synthesis by AmyI-1-18, observed in a cell-free rapid translation system, indicates the bactericidal effect is independent of membrane destabilization (Taniguchi, Ochiai, Fukuda et al., 2016).

The outer membrane of Gram-negative bacteria, a barrier that maintains cell structure and includes lipopolysaccharide, is a target of antimicrobial peptide because of its hydrophobic moment and electrostatic interactions (Schmidtchen, Pasupuleti, & Malmsten, 2014; Torres et al., 2018; Ulmschneider et al., 2014; Yin, Edwards, Li, Yip, & Deber, 2012). Interestingly, AmyI-1-18 had a bactericidal effect on *F. nucleatum* owing to its membrane-disrupting properties, which was in contrast to effects observed on *P. gingivalis* (Table 1 and Fig. 3F). We speculate that this could be because the outer membrane of *F. nucleatum* carries a stronger negative charge than that of *P. gingivalis*, leading to the cationic charge of AmyI-1-18 acting more effectively on the outer menbrane of *F. nucleatum*. In support of our observations, previous reports have suggested that *P. gingivalis* is relatively resistant to polymyxin B, which is

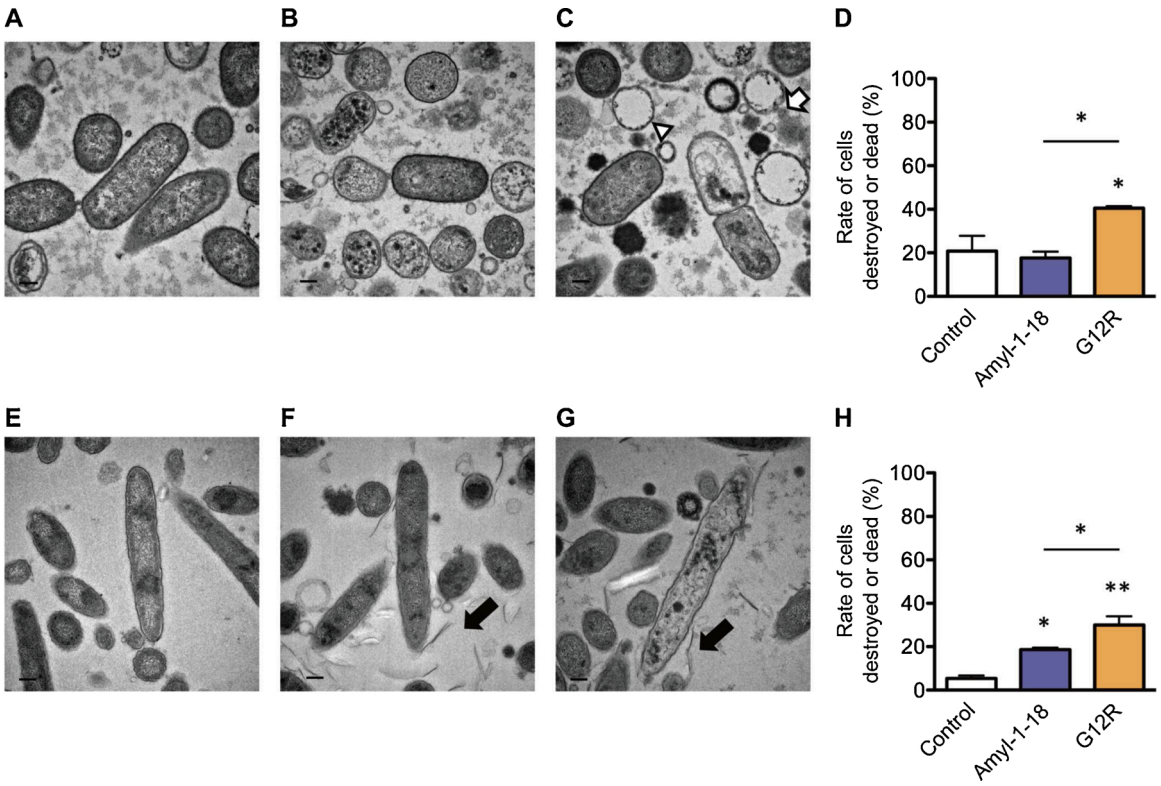

Fig. 4. G12R destroyed bacterial membranes of *P. gingivalis* and *F. nucleatum.* Transition electron microscopy (TEM) representative images of *P. gingivalis* (A: control, B: AmyI-1-18, and C: G12R) and *F. nucleatum* (E: control, F: AmyI-1-18, and G: G12R). Scale bars: 200 nm. Bacterial membranes were destroyed, and inner structures leaked out (C: white arrow), and dead cells (C: white triangle) were observed. Black arrow indicates cell walls stripped down (F and G). The number of bacteria with destroyed membranes and the number dead were counted, and the percentage is shown in the graph (n = 3) (D: *P. gingivalis* and H: *F. nucleatum*). The results are represented as means ± standard errors of the mean (SEMs). $^{*}P < 0.05$ (control) and $^{**}P < 0.01$ (as indicated), according to analysis of variance.

also a cationic antimicrobial peptide, due to lower negative charge on the lipopolysaccharide as well as the lack of lipid A phosphorylation (Coats et al., 2009; Jain & Darveau, 2010). In contrast, the lipid A of *F. nucleatum* has a chemical structure similar to that of the general lipid A as other bacteria has (Hase, Hofstad, & Rietschel, 1977). In summary, AmyI-1-18, a rice derived bactericidal peptide, operates by species specific mechanisms.

In this study, we found that G12R possessed bactericidal activity in a membrane-disruptive manner against both *P. gingivalis* and *F. nucleatum,* in contrast to AmyI-1-18. We speculate that the ability of G12R to disrupt celluler membranes was enhanced by the net charge of amino acids in G12R (+3), which was higher than that in AmyI-1-18 (+2) and indicates that the electrostatic interaction between the peptide and cellular membrane of bacteria was reinforced (Supplementary Table1). Moreover, higher α-helix content in G12R than in AmyI-1-18 was assumed to be another factor that promoted antimicrobial activity (Tossi, Sandri, & Giangaspero, 2000), whereas control peptide K18A, low cationic charge, and low α-helix content were not potent enough to inhibit biofilm formation (Supplementary Fig. 2).

We analyzed the cytotoxicity of these peptides by exposing them to Ca9-22, a human oral epithelial cell line (Supplementary Fig. 3). Amy1-1-18 and G12R didn't show any toxicity at MBIC. Furthermore, these peptides show lower cytotoxicity compared to CHX, which is cytotoxic at MBIC.

Further study is needed to understand the molecular mechanisms underlying bacterial membrane disruption, which may enable further fine-tuning of the hydrophobic moment and charge distribution, as well as improve specific antibacterial action through amino acid substitution. One limitation of this study is that the analyses were performed using a dual-species biofilm; therefore, the effectiveness of the peptides as medication in oral application has to be validated in future clinical studies for more complexed biofilms. Another limitation of this study is that the stability of these peptides in saliva or gingival crevicular fluid was not ascertained and thus further studies are required to enable future clinical use. The identification of peptides and analog peptides with amino acid substitutions that have antimicrobial actions against periodontopathic bacteria might enable personalized treatment of biofilm-related diseases; these peptides would represent potential alternatives to existing antibiotics that may overcome antimicrobial resistance.

5. Conclusion

AmyI-1-18 and G12R inhibited biofilm formation of *P. gingivalis* and *F. nucleatum.* The amino acid substitution in the latter reinforced antibacterial effects, which suggests that these peptides could be effective in controlling oral biofilm.

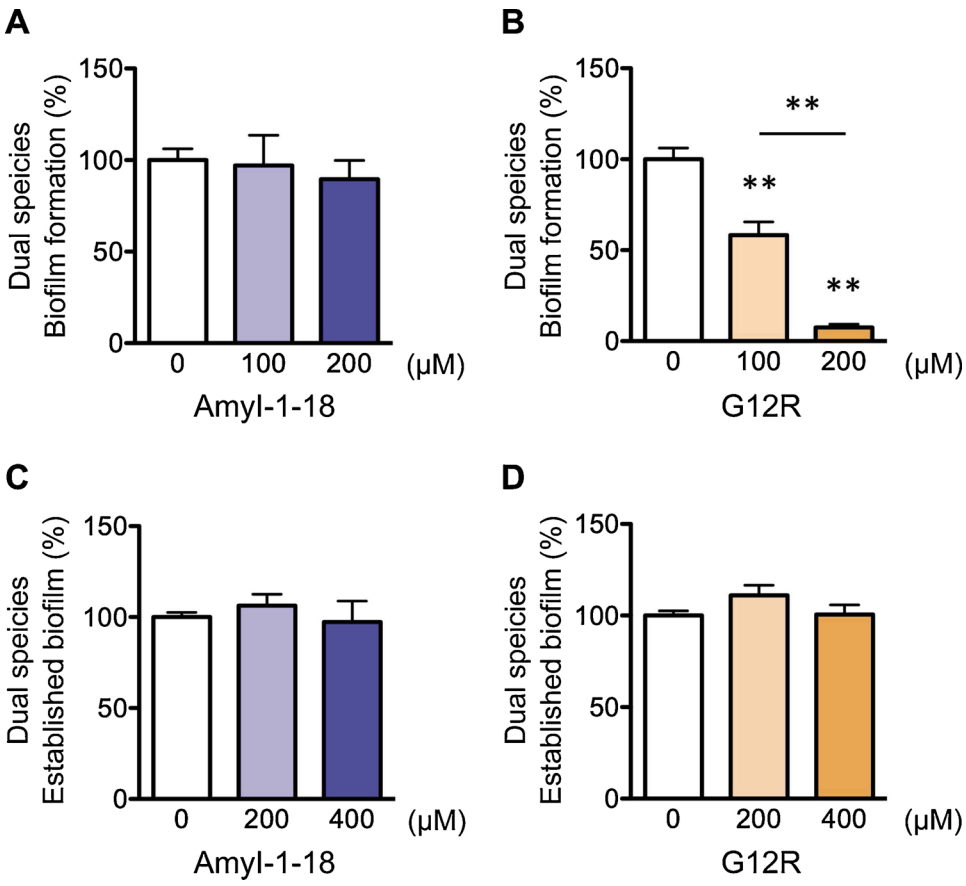

Fig. 5. G12R peptide inhibited formation of dual-species biofilm of *P. gingivalis* and *F. nucleatum*. The effects of peptides on dual-species biofilm formation (A: AmyI-1-18 and B: G12R) and established biofilm eradication (C: AmyI-1-18 and D: G12R). *P. gingivalis* (1×10^8 CFU/mL) and *F. nucleatum* (1×10^8 CFU/mL) were incubated together in anaerobic conditions. Peptides were added at 0 h (A and B) or 24 h (C and D). The amount of biofilm was evaluated with crystal violet stain (n = 4). The results are represented as means ± standard errors of the mean (SEMs). $^{**}P < 0.01$ for control or as indicated, according to analysis of variance.

Declaration of Competing Interest

The authors report no declarations of interest.

Ethics

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

Author contribution statement

Y.A-N. and K.T. designed the research and wrote the manuscript with the support of Y.M., M.H., Y.T., H.D., M.T., N.T., and K.Y. drafting the manuscript and revising it critically; A.M., Y.A-N., M.Y-T., M.Y-H., and M.H. have been involved in data collection and data analysis and interpretation. All authors have given final approval of version to be published.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.archoralbio.2020.104956.

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Supplementary Table 1. The properties of peptides.

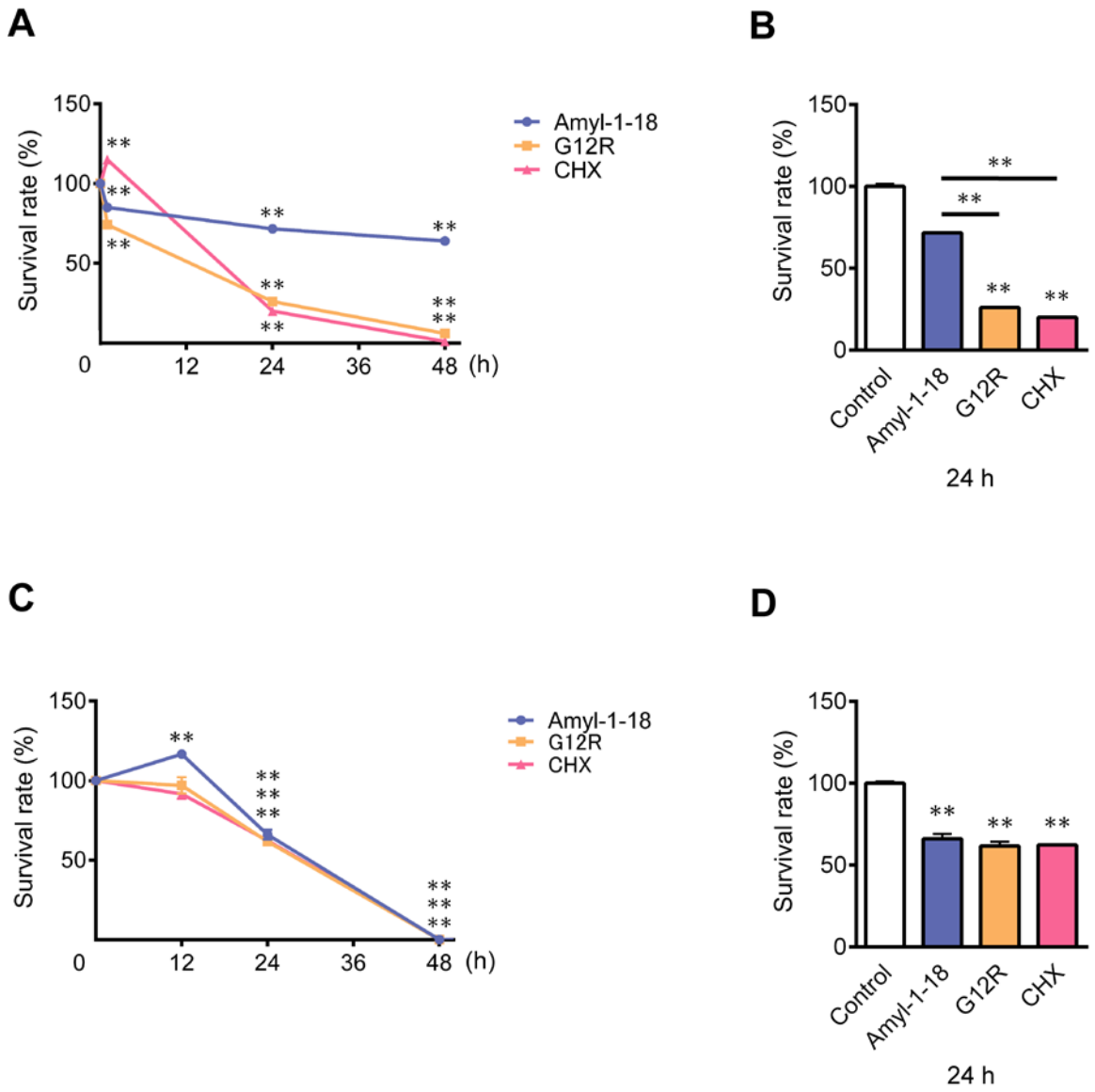
Peptide	Sequence	Molecular Weight (g/mol)		α-Helix Content[a] (%)	pI (−)	MH[b]	Net Charge[c]
		Measured	Calculated				
AmyI-1-18	HLNKRVQRELIGWLDWLK	2306.10	2304.69	61.1	10.8	−0.68	+2
G12R	HLNKRVQRELI**R**WLDWLK	2405.47	2403.83	66.7	11.6	−0.91	+3
K18A	HLNKRVQRELIGWLDWL**A**	2247.75	2247.6	55.6	9.7	−0.37	+1

[a]α-Helix content and α-helical region (indicated by an underline) were estimated using MLRC secondary prediction, available at the Network Protein Sequence @nalysis (NPS@) website (https://npsa-prabi.ibcp.fr).

[b]The mean hydrophobicity (MH) values of the peptides were calculated with the Kyte and Doolittle scale.

[c]The net charge of the peptides were calculated with the help of the Peptide Property Calculator (https://pepcalc.com).

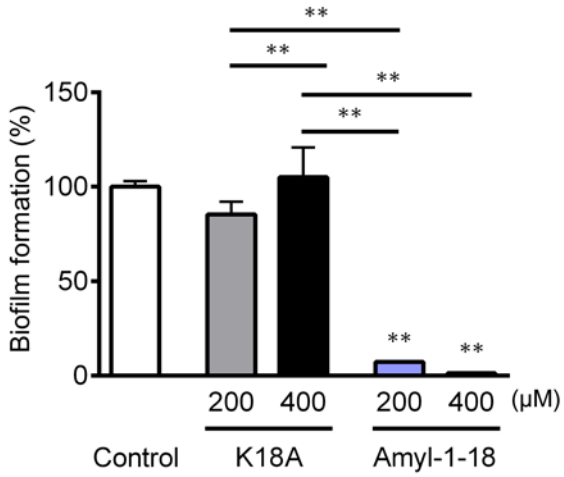
Supplementary Figure 1.



Supplementary Figure1.
The antimicrobial kinetics of Amy Ⅰ-1-18 and G12R peptides.
Comparison of antimicrobial activity kinetics of Amy Ⅰ-1-18 and G12R peptides to CHX(A: *P. gingivalis,* B: *F. nucleatum*) . Time-killing curve was plotted against percentage of live cells and time point. Similar to CHX, G12R caused bacterial death at 24h (C: *P. gingivalis,* D: *F. nucleatum*). Amy Ⅰ-1-18 displayed the same killing rate with CHX and G12R at 24 h in case of *F. nucleatum*, however, the killing rate for *P. gingivalis* was relatively lower.
The time-killing curve (A and C) are expressed as the mean ± SEM (***p* < 0.01 vs. 0 h, by paired-t test). The killing rate at 24 h are expressed as the mean ± SEM (***p* < 0.01 vs. Control or as indicated, by ANOVA, n = 4).

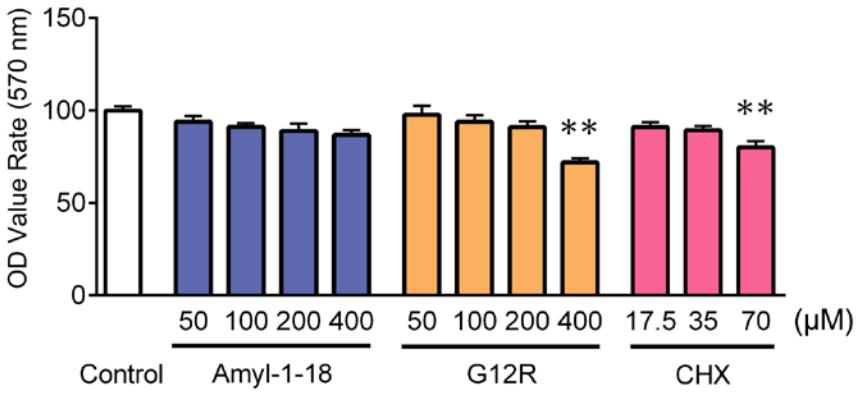
Materials and Methods
Time-killing assay
The time-killing kinetic studies against *P. gingivalis* and *F. nucleatum* were determined by ATP assay[1]. Briefly, culture of *P. gingivalis* was diluted to $1x10^8$ CFU/mL, and culture of *F. nucleatum* was diluted to $1x10^9$ CFU/mL. The diluted bacteria cultures were treated with peptides and CHX (*P. gingivalis*: at 1xMBIC, *F. nucleatum*: at 2xMBIC). The killing curves were constructed by plotting the survival rate versus control.
[1] Madison, Wisconsin, USA

Supplementary Figure 2.



Supplementary Figure2.
Control sequence peptide didn't show to inhibit biofilm formation of *P. gingivalis*.
The effects of peptides on biofilm formation of *P. gingivalis* (control (PBS), K18A, and Amy Ⅰ-1-18). *P. gingivalis* ($1x10^8$ CFU/mL) was incubated with peptides for 48h under anaerobic condition. The amount of biofilm was evaluated by CV stain (n=4).
The results are presented as the mean ± SEM (***p* < 0.01, versus 0μM or as indicated, ANOVA).

Supplementary Figure 3.



Supplementary Figure3.
The peptides didn't show any toxicity at MBIC towards human oral epithelial cells.
Effect of peptides on cell viability was evaluated using the MTT assay. The Amy Ⅰ-1-18 peptide did not show any toxicity at maximum concentration that we tested i.e. at 400μM. The G12R peptide showed cytotoxicity towards Ca9-22 at 400uM; however, the MBIC is below than this toxic concentration. Lastly, these peptides have lower cytotoxicity compare to CHX, which shows toxicity at MBIC. The results are expressed as the mean ± SEM (**p < 0.01 vs. Control, by ANOVA, n=4).

Materials and Methods
Cell Viability Tests
The effect of peptides on cell viability was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay[1]. Briefly, human oral epithelial cell line, Ca9-22 cells were plated in 96-well plates at a concentration of $5x10^4$ cells/well. Peptides and CHX were added to each well, and the plate was incubated for 24 h. Absorbance was measured using the MORECULAR DEVICES SpectraMAX ABS plus[2] at a wavelength of 570 nm.
[1] Sigma, St. Louis, MO, USA
[2] MORECULAR DEVICES, San Jose, CA, USA