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# Effect of MUC7 peptides on the growth of bacteria and on *Streptococcus mutans* biofilm

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***Objectives*: To investigate the susceptibility of selected bacteria as well as *Streptococcus mutans* biofilm to MUC7 peptides and compare the activities with those of other known antimicrobial peptides.**

***Methods*: MIC and MBC of peptides for *S. mutans, Escherichia coli*, *Streptococcus gordonii, Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Pseudomonas aeruginosa* were determined using the microdilution method. For *S. mutans*, the effects of the peptides on the kinetics of growth inhibition, time-killing, and on biofilm formation and reduction were also examined. For biofilm studies, polystyrene microtitre plates, Calgary Biofilm Device (CBD) and hydroxylapatite (HA) discs, along with Crystal Violet and Alamar Blue dyes, and/or EM observations, were employed.**

***Results*: *S. mutans* was the most susceptible to all peptides tested (MICs of 9.4–25.0 μM), compared with the other species (MICs of 3.1–>100 μM). MUC7 peptides (except MUC7-12-mer-L4) exerted 2-fold higher activity against *S. mutans* than Hsn5-12-mer and magainin-II, and faster killing of *S. mutans* than Hsn5-12-mer. The MUC7 peptides also had an effect on *S. mutans* biofilm. On the polystyrene plates, they suppressed the biofilm formation, with $MBIC_{50}$ of 6.25–12.5 μM, and reduced the 1 day developed biofilm in a batch culture, with $MBRC_{50}$ of 25–50 μM. On the CBD pegs, the viabilities of the biofilm were suppressed by >95% in the presence of MUC7 peptides at 4× MIC (50 μM). One day developed biofilm viabilities were inhibited by 49–75%. On HA, the formation of biofilm (as observed by EM) was also considerably reduced.**

***Conclusions*: MUC7 peptides present somewhat preferential antimicrobial activity against *S. mutans*. They also have an effect on *in vitro* formation and reduction of the preformed *S. mutans* biofilm.**

Keywords: susceptibility, saliva, mucins, antimicrobial peptides, CBD

## Introduction

Dental caries and periodontitis are common oral diseases. *Streptococcus mutans* is the main pathogenic agent of dental caries, and *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are the bacteria associated with periodontitis. There is a great interest in the use of antimicrobial agents for the prevention and treatment of caries and periodontitis. The prevention of dental caries and periodontal disease requires the control of these pathogens that exist in an oral biofilm know as dental plaque. Chlorhexidine is a potent antiplaque chemical agent. However, its clinical application is limited because it has a bitter taste and can cause teeth to stain on frequent use. Thus, it is important to develop alternative antiplaque agents from natural sources that exhibit few or no side-effects. Cationic antimicrobial peptides (CAMPs) have appeared as a promising novel group of antibiotics.[1]

Histatins and mucins are two families of human salivary proteins that are the components of the innate immunity, a first-line of the host defence system against pathogens. Histatins and histatin-derived peptides have been shown to possess a significant *in vitro* antimicrobial activity against fungi,[2–6] bacteria[7–11] and bacterial biofilms.[12] MUC7, the low molecular mass human salivary mucin (357 amino acid residues), protects the oral cavity from microbial infections through more general protective mechanisms (such as binding to and clearance of various microorganisms)[13] rather than the direct killing of microorganisms. Our previous studies showed that the peptides derived from

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the N-terminal region of MUC7, including MUC7 51-mer, MUC7 20-mer and MUC7 12-mer, cationic peptides with a positive charge of 8, 7 and 6 respectively, have a significant fungicidal activity *in vitro*.[14–17] MUC7 12-mer possesses antifungal activity in low salt RPMI 1640 medium and also exhibits synergic antifungal effects *in vitro* with histatin-5 12-mer (Hsn5 12-mer) or with miconazole.[18] Our most recent study showed that the antifungal activity of MUC7 12-mer-L in human whole saliva was enhanced in the presence of protease inhibitors and EDTA, and MUC7 12-mer-D isomer exerted higher antifungal activity than MUC7 12-mer-L in human whole saliva.[19]

To date, however, information on antibacterial activity of the MUC7 peptides is limited. In one of our earlier studies we demonstrated, by an *in vitro* killing assay, that MUC7 20-mer exerts antibacterial activity.[14] In the present study, we have further investigated the susceptibility of selected bacteria to MUC7 12-mer-L, MUC7 12-mer-D and MUC7 20-mer, using a broth microdilution assay method. To examine the potential of MUC7 and other antimicrobial peptides as therapeutic agents to control the oral biofilm *in vivo*, we also examined the effect of these peptides on the formation and reduction of *S. mutans* biofilm. For these experiments we employed polystyrene microtitre plates as a primary substratum and Calgary Biofilm Device (CBD) and hydroxylapatite (HA) discs as supplementary substrata.

## Materials and methods

### Peptides and chemicals

MUC7 12-mer-L (RKSYKCLHKRCR, amino acids 40–51 of the parent human salivary mucin, MUC7), MUC7 12-mer-L4 (RKSYKALHKRAR, the two C residues substituted with A), MUC7-12-mer-D (D amino acid isomer), MUC7-20-mer (LAHQKPFIRKSYKCLHKRCR, amino acids 32–51 of the parent human salivary mucin, MUC7), Hsn5 12-mer (AKRHHGYKRKFH, amino acids 4–15 of the parent Hsn5, also known as P113)[20] and magainin-II (GIGKFLHSAKKFGKAFVGEIMNS, a 24 amino acid peptide from frog skin) were custom-synthesized by Bio-Synthesis (Lewisville, Texas). The company analysed the prepared peptides by HPLC and mass spectrometry. The purity (>70%) was taken into consideration in preparing the stock solution of each peptide for antibacterial assays. The peptides were dissolved in sterile de-mineralized water at 1 mg/mL; aliquots were freeze-dried and stored at –20°C. For each experiment, the freeze-dried peptides were re-dissolved at 1 mg/mL in sterile dd-water. Chlorhexidine digluconate solution (Sigma Chemical Co., St Louis, MO, USA) was diluted to 0.04 mg/mL with sterile dd-water.

### Bacterial strains and growth media

The bacteria used in this study included *S. mutans* ATCC10449, *S. mutans* AU 159, *S. mutans* GS-5, *S. mutans* GS-5 mutant (^gtf BCD), *Streptococcus gordonii* Challis, *P. gingivalis* W50, *P. gingivalis* 381, *A. actinomycetemcomitans* NCTC 9710, *Pseudomonas aeruginosa* ATCC 17648 and *Escherichia coli* HB101. The strains were kindly provided by Kuramitsu's lab, the Department of Oral Biology, University at Buffalo. One-quarter strength (25%) of brain–heart infusion broth (BHI; DIFCO Laboratories, Detroit, MI, USA) was used for the growth of *S. mutans*, *S. gordonii*, *A. actinomycetemcomitans*, *P. aeruginosa* and *E. coli*. BHIS (25% BHI supplemented with 2% sucrose) was used for the growth of *S. mutans* biofilm. For the growth of *P. gingivalis*, tryptic soy broth-yeast extract medium (TSBY; DIFCO Laboratories) supplemented with cysteine hydrochloride (0.05%), menadione (0.02 μg/mL), haemin (5 μg/mL) and potassium nitrate (0.02%) was used. All bacteria were cultured at 37°C; *P. aeruginosa* and *E. coli* were grown aerobically and the other bacteria anaerobically. To prepare bacterial cell suspension for antibacterial activity assays, each overnight culture was harvested by centrifugation (3783 **g**, 10 min), washed once with 10 mM sodium phosphate-buffered saline (PBS, pH 7.2), resuspended in PBS and adjusted to a concentration of $1 \times 10^6$ cells/mL.

### Bacterial susceptibility assay

MICs of the peptides were determined using the broth microdilution method as described previously.[18] Briefly, 2-fold serial dilutions of each peptide were prepared with 25% BHI medium at a volume of 200 μL per well in 96-well flat-bottom microtitre plates (Costar, Cambridge, MA, USA). The final concentrations of each peptide ranged from 0.78 to 100 μM, and that of chlorhexidine, used as a positive control, from 0.16 to 20 μM (0.078–10 mg/L). The microtitre plate wells were inoculated with 20 μL per well of bacterial cell suspension, at a final concentration of $5 \times 10^6$ cfu/mL for *P. gingivalis* and $5 \times 10^5$ cfu/mL for the other bacterial species. After incubation at 37°C for 48 h, the absorbance was measured at 595 nm using a microplate reader (Model 3550, Bio-Rad) to assess the cell growth. The MIC endpoint was defined as the lowest concentration of the test agent that completely inhibited growth or produced at least 90% reduction in absorbance compared with that of the drug-free control. The MIC value represents the median of at least three independent experiments. To measure the MBC, an aliquot (100 μL) of the cell suspension was taken from two wells above the MIC, centrifuged and washed three times with PBS. Then each cell suspension was plated on a TSBY agar plate, and bacterial cells were enumerated after incubation at 37°C for 48 h. MBC was defined as the lowest concentration of the peptide at which more than 99.9% of the cells were killed compared with a non-treated control.

### Growth curve assay

The effect of the peptides on the growth of *S. mutans* was examined as follows: a bacterial culture was grown in 25% BHI at 37°C to an OD of 0.1 at 600 nm and then equally allocated into 50 mL tubes (20 mL/tube). Each peptide was added to one culture tube to a final concentration of 25 μM. Chlorhexidine (1.25 mg/L, 2.5 μM) was used as a positive control, and the culture without the agents was used as a bacterial growth control. The cultures were grown for 10 h and the absorbance at 600 nm of 1 mL aliquots recorded at 1 h intervals.

### Time–kill assay

The time–kill kinetic studies against *S. mutans* were performed using the broth macrodilution method.[21] *S. mutans* GS-5 ($1 \times 10^6$ cfu/mL) and antibacterial agents (12.5 μM for MUC7 12-mer-L, MUC7 12-mer-D or MUC7 20-mer; 50.0 μM for MUC7 12-mer-L4; 25.0 μM for Hsn5 12-mer or magainin-II; and 2 μM for chlorhexidine) were incubated in 25% BHI at 37°C. At 0, 1, 2, 4, 8 and 24 h, samples were taken and viable counts determined as follows: the samples were serially diluted in sterile 10 mM PBS (pH 7.2) and 50 μL aliquots were plated onto TSBY agar. The plates were incubated anaerobically at 37°C for 48 h, followed by enumeration of the cfu. Killing curves were constructed by plotting the $\log_{10}$ cfu/mL versus time over 24 h. All assays were performed in duplicate on at least two occasions.

*Biofilm susceptibility assay*

The following three models were used:

*(1) Polystyrene tissue culture plate.* The effect of the peptides on *S. mutans* biofilm formation was examined using the microdilution method. This method was similar to the MIC assay for planktonic cells. Two-fold serial dilutions of each peptide were prepared in the wells of a 96-well polystyrene tissue culture plate (Falcon 3072, Becton Dickinson and Company, NJ) containing BHIS at a volume of 200 µL per well. The final concentration of the peptide ranged from 0.78 to 100 µM for each peptide. Chlorhexidine (0.078–10 mg/L) was used as the positive control, the medium without the agents as the non-treated control and the medium alone as the blank control. The cell suspension of *S. mutans* GS-5 was prepared as described in the MIC assay, and 20 µL aliquots (final concentration of cells $5 \times 10^5$ cfu/mL) were inoculated in the wells of the polystyrene tissue culture plate except in the wells with the medium alone as the blank control. After incubation at 37°C for 24 h, absorbance at 595 nm was recorded to assess the culture growth. The culture supernatants from each well were then decanted and planktonic

**Table 1.** MIC of cationic antimicrobial peptides against selected bacteria[a]

| Species | Strain | Test agent | MIC | | | |
|---|---|---|---|---|---|---|
| | | | range ($n$[b] = 6) | | median | |
| | | | (µM) | (mg/L) | (µM) | (mg/L) |
| *S. mutans* | GS-5 | MUC7 12-mer-L | 6.25–25 | 9.9–39.5 | 12.5 | 19.7 |
| | GS-5 ˆgtf BCD | MUC7 12-mer-L4 | 25–50 | 39.5–79 | 50.0 | 79.0 |
| | AU 159 | MUC7 12-mer-D | 6.25–12.5 | 9.9–19.7 | 12.5 | 19.7 |
| | ATCC10449 | MUC7 20-mer | 6.25–12.5 | 15.7–31.4 | 9.4 | 23.6 |
| | | Hsn5 12-mer | 12.5–50 | 19.7–79 | 25.0 | 39.5 |
| | | magainin II | 25–50 | 61.7–123.5 | 25.0 | 61.7 |
| | | chlorhexidine | 0.62–2.5 | 0.313–1.25 | 2.6 | 1.3 |
| *S. gordonii* | Challis | MUC7 12-mer-L | 12.5–50 | 19.7–79.0 | 25.0 | 39.5 |
| | | MUC7 12-mer-L4 | 50–50 | 79–79 | 50.0 | 79.0 |
| | | MUC7 12-mer-D | 12.5–50 | 19.7–79 | 25.0 | 39.5 |
| | | MUC7 20-mer | 6.25–12.5 | 15.7–31.4 | 6.3 | 15.7 |
| | | Hsn5 12-mer | 12.5–100 | 19.7–158.0 | 100.0 | 158.0 |
| | | magainin II | 6.25–100 | 15.4–246.9 | 25.0 | 61.7 |
| | | chlorhexidine | 0.62–2.5 | 0.313–1.25 | 2.6 | 1.3 |
| *A. actinomycetemcomitans* | NCTC 9710 | MUC7 12-mer-L | 50–>100 | 79–>158 | 100.0 | 158.0 |
| | | MUC7 12-mer-D | 25–100 | 39.5–158 | 75.0 | 118.5 |
| | | MUC7 20-mer | 50–100 | 125.6–251.3 | 50.0 | 125.6 |
| | | Hsn5 12-mer | 50–>100 | 79–>158 | >100 | >158 |
| | | magainin II | 1.56–40 | 3.9–98.8 | 3.1 | 7.7 |
| | | chlorhexidine | 1.25–20 | 0.63–10 | 2.6 | 1.3 |
| *P. gingivalis* | W 50 | MUC7 12-mer-L | >100–>100 | >158–>158 | >100 | >158 |
| | 381 | MUC7 12-mer-D | 25–100 | 39.5–158.0 | 75.0 | 118.5 |
| | | MUC7 20-mer | >100–>100 | >251.3–>251.3 | >100 | >251 |
| | | Hsn5 12-mer | >100–>100 | >158–>158 | >100 | >158 |
| | | magainin II | >100–>100 | >246.9–>246.9 | >100 | >246.9 |
| | | chlorhexidine | 1.25–2.5 | 0.63–1.25 | 2.6 | 1.3 |
| *P. aeruginosa* | ATCC 17648 | MUC7 12-mer-L | 25–100 | 39.5–158.0 | 100.0 | >158 |
| | | MUC7 12-mer-D | 50–100 | 79.0–158.0 | 50.0 | 79.0 |
| | | MUC7 20-mer | 100–>100 | 251.3–>251.3 | >100 | >251 |
| | | Hsn5 12-mer | >100–>100 | >158–>158 | >100 | >158 |
| | | magainin II | 25–100 | 61.7–246.9 | 62.5 | 154.3 |
| | | chlorhexidine | 2.5–9.9 | 1.25–5 | 9.9 | 5.0 |
| *E. coli* | HB101 | MUC7 12-mer-L | 12.5–25 | 19.7–39.5 | 12.5 | 19.7 |
| | | MUC7 12-mer-D | 3.13–6.25 | 4.9–9.9 | 6.3 | 9.9 |
| | | MUC7 20-mer | 6.25–50 | 15.7–125.6 | 37.5 | 94.2 |
| | | Hsn5 12-mer | 12.5–50 | 19.7–79.0 | 50.0 | 79.0 |
| | | magainin II | 1.56–6.25 | 3.9–15.4 | 4.7 | 11.6 |
| | | chlorhexidine | 1.25–2.50 | 0.63–1.25 | 2.6 | 1.3 |

[a]Four *S. mutans* strains and two *P. gingivalis* strains were tested.
[b]*n*, the number of observations.

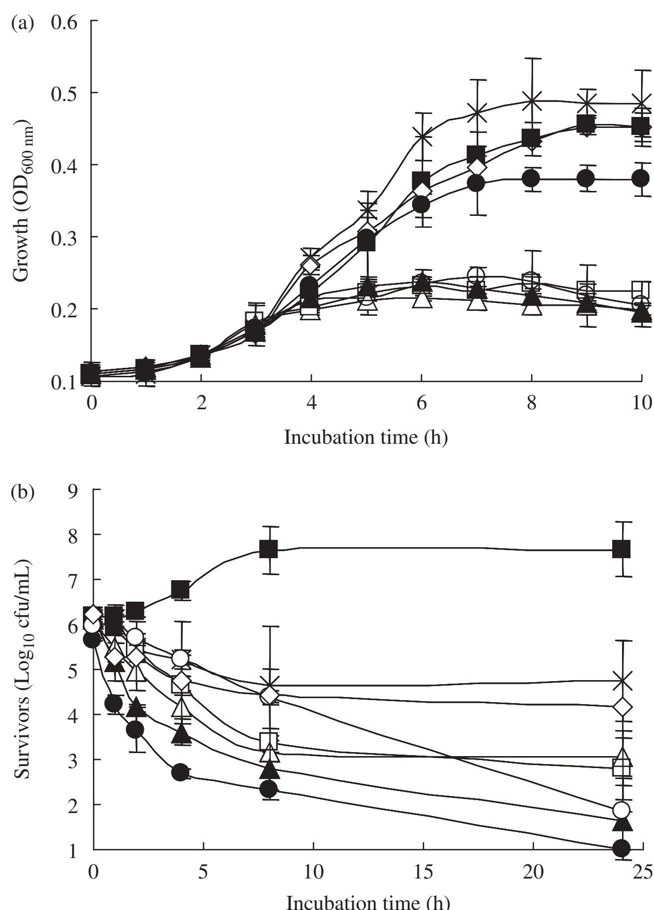


**Figure 1.** Effect of MUC7 peptides on the growth of *S. mutans* (a) and time-killing curves for *S. mutans* (b). For growth inhibition (a), the peptides were used at 25 μM and chlorhexidine at 2.5 μM concentration, a control had no agent added. For the time-killing test, each agent was used at its MIC. The assays were performed as described in the text. Values are the means of two experiments. MUC7 12-mer-L, open triangles; MUC7 12-mer-D, open squares; MUC7 20-mer, filled triangles; MUC7 12-mer-L4, open diamonds; Hsn5 12-mer, crosses; magainin-II, filled circles; chlorhexidine, open circles; and control, filled squares.

cells were removed by washing with PBS, pH 7.2. The biofilm was fixed with methanol for 15 min and then air dried at room temperature. It was then stained with 0.1% (w/v) Crystal Violet (Sigma) for 5 min and rinsed thoroughly with water until the control wells appeared colourless. Biofilm formation was quantified by the addition of 200 μL of 95% ethanol to each Crystal Violet-stained well. The plate was rocked at room temperature for 30 min and the absorbance at 595 nm ($A_{595}$) was determined using a microplate reader (Bio-Rad Model 3550). The percentage of inhibition was calculated using the equation (1 – $A_{595}$ of the test/$A_{595}$ of non-treated control) × 100. The minimum biofilm inhibition concentration ($MBIC_{50}$) was defined as the lowest agent concentration that showed 50% or more inhibition on the formation of biofilm.

To examine the effects of the peptides on the 1 day developed biofilm, *S. mutans* GS-5 cells (20 μL aliquots of $5 \times 10^5$ cfu/mL) were inoculated into the wells of a polystyrene microtitre plate containing BHIS at a volume of 200 μL per well. After incubation at 37°C for 24 h, the culture supernatant from each well was decanted and planktonic cells were removed by washing with PBS. BHIS containing 0.78–100 μM peptides, prepared in another microtitre plate, was then transferred to the 1 day biofilm plate, and the plates were further incubated at 37°C for 24 h. The cell growth was then assessed by measuring the absorbance at 595 nm, and the biofilm was fixed, stained and quantified as described above. Minimum biofilm reduction concentration ($MBRC_{50}$) was defined as the lowest drug concentration that showed reduction of the biofilm by 50% or more.

*(2) Calgary biofilm device.* To examine the effects of the peptides on the biofilm formed on the vertical surface, which is closer to natural oral conditions than on a (horizontal) polystyrene surface, CBD (MBEC Bioproducts, Calgary Canada) Assay System was used. CBD was described earlier,[22] and has essentially 96 pegs mounted on the inside surface of the lid of a 96-well microtitre plate. When the CBD is placed over a microtitre plate, each peg is lowered into its corresponding well, but without contacting the well surface. The peg-studded lid fits over the 96-well microtitre plate to permit cells from the culture medium to colonize on the surface of the pegs.

To examine the effect of the peptides on the formation of biofilm on the peg surface, *S. mutans* (200 μL of $10^6$ cfu/mL), in BHIS, was inoculated to each well of a 96-well microtitre plate. The plate was covered with the peg-studded lid and incubated at 37°C anaerobically

**Table 2.** Effects of peptides on the formation ($MBIC_{50}$[a]) and reduction ($MBRC_{50}$[b]) of *S. mutans* biofilm ($n$[c] = 6)

| Test agent | $MBIC_{50}$ | | | | $MBRC_{50}$ | | | |
|---|---|---|---|---|---|---|---|---|
| | range | | median | | range | | median | |
| | (μM) | (mg/L) | (μM) | (mg/L) | (μM) | (mg/L) | (μM) | (mg/L) |
| MUC7 12-mer-L | 6.25–12.5 | 9.9–19.7 | 12.5 | 19.7 | 12.5–>50 | 19.7–>79.0 | 50 | >79.5 |
| MUC7 12-mer-L4 | 25–50 | 39.5–79.0 | 50 | 79 | 50–>50 | 79.0–>79.0 | >50 | >79.5 |
| MUC7 12-mer-D | 3.1–12.5 | 4.9–19.7 | 6.25 | 9.9 | 6.25–25 | 9.9–39.5 | 25 | 39.5 |
| MUC7 20-mer | 3.1–12.5 | 7.9–31.4 | 6.25 | 15.7 | 6.25–25 | 15.7–62.8 | 25 | 62.8 |
| Hsn5 12-mer | 25–25 | 39.5–39.5 | 25 | 39.5 | 12.5–>50 | 19.7–>79.0 | >50 | >79.5 |
| Magainin II | 25–25 | 123.5–123.5 | 25 | 123.5 | 25–>50 | 61.7–>123.5 | >50 | >123.5 |
| Chlorhexidine | 0.625–1.25 | 0.313–0.625 | 1.25 | 0.625 | 2.5–>20 | 1.25–>10 | >20 | >10 |

[a]$MBIC_{50}$, minimum biofilm inhibition concentration was defined as the lowest drug concentration that showed 50% or more inhibition on the formation of biofilm.
[b]$MBRC_{50}$, minimum biofilm reduction concentration was defined as the lowest drug concentration that showed 50% or more reduction of biofilm.
[c]$n$, the number of observations.

for 24 h. Then, the lid with pegs, to which cells adhered, was transferred to a challenge plate, in which a series of 2-fold dilutions of each test agent was made in BHIS medium. For the control, no agent was added. After further incubation at 37°C anaerobically for 24 h, the culture medium was discarded and the pegs were washed twice by submerging the lid for 5 s in 200 μL of PBS in each well of a 96-well microtitre plate. The presence of a heavy- and light-formed biofilm on the non-treated control pegs and drug-treated pegs, respectively, was confirmed by scanning electron microscopy (SEM). The viability of the biofilm on the individual pegs was determined by an assay using the redox indicator Alamar Blue according to the manufacturer's instructions. Briefly, Alamar Blue (Biosource, Camarillo, CA, USA) was diluted to 5% in BHIS and 200 μL was then added to each well of a clean 96-well plate. Also included on each plate was a blank containing Alamar Blue in the medium and a blank containing the medium alone. The lid with pegs with the attached biofilm was transferred to the Alamar Blue plate, and the plate was then protected from light and incubated at 37°C for 4 h. The plates were read in a microplate reader AD340 (Beckman Coulter, CA, USA) at a wavelength of 570 nm and a wavelength of 595 nm. The relative absorbance for the samples was converted into the percentage increase over background absorbance, as determined by the blank sample on each plate.

To test the reduction effects of peptides on the 1 day developed biofilm, the lid with pegs to which the cells adhered was transferred to a plate containing fresh BHIS and the plate was further incubated for 24 h. The preformed biofilm was then transferred to the challenge plate containing the test agents for the determination of biofilm reduction using Alamar Blue viability dye, as described above.

*(3) HA discs.* To examine the pattern of the biofilm formed on the surface of HA discs by SEM, biofilms were grown in BHIS on the surface of HA discs that were deposited in the wells of a 48-well polystyrene tissue culture plate (Falcon 3047, Becton Dickinson, NJ, USA). *S. mutans* ($10^6$ cfu/mL) in 0.5 mL of BHIS in the presence or absence of MUC7 12-mer-L at a concentration of 4× MIC was inoculated to each well of the plate. After incubation at 37°C anaerobically for 24 h, the HA discs were removed for SEM analysis.

### *SEM*

Biofilms grown on the surfaces of HA discs and CBD pegs for 24 h in the presence or absence of antimicrobial agents were rinsed once with distilled water, fixed using 10% buffered formaldehyde (VWR) at 20°C overnight. Fixed samples were then dehydrated through a graded series of ethanol concentrations, air dried, mounted and sputter coated with carbon. Samples were analysed by SEM

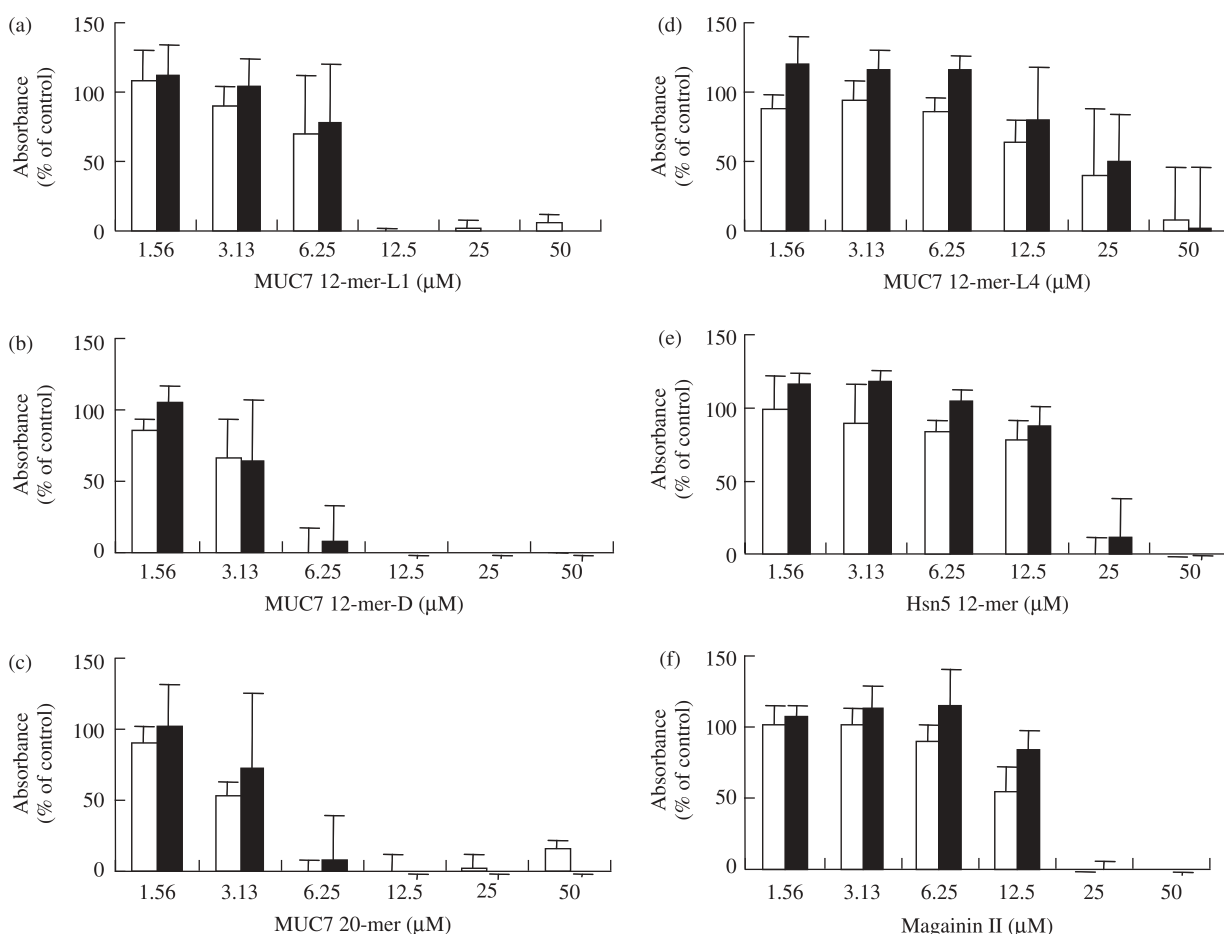


**Figure 2.** Effect of peptides on *S. mutans* biofilm formation. The bacterial cells were inoculated in a 96-well microtitre plate containing 25% BHIS medium with different concentrations of each agent. Culture without the agents was used as the no-treatment control. After incubation at 37°C for 24 h, absorbance at 595 nm was recorded to assess the cell growth; the results are expressed as a percentage of the no-treatment control cell growth and depicted by the clear bars. The biofilm assay was performed by discarding the supernatants, washing with PBS, fixing with methanol and staining with CV. The absorbance at 595 nm was recorded to assess the amount of biofilm formed; the results are expressed as the percentage of the no-treatment control formed biofilm and depicted by the solid bars. Data represent the mean and SD of two independent tests with duplicates for each.

(Hitachi S-4000; Hitachi Instruments Inc., San Jose, CA, USA) at several magnifications (×1000 to ×10 000 for HA discs, ×200 to ×5000 for CBD pegs) in the EM laboratory, located in the School of Dental Medicine, University at Buffalo.

## Results

### *MIC and MBC*

The concentrations of the peptides required to inhibit and to kill planktonic bacteria (MIC) are summarized in Table 1. Among the test bacterial species, *S. mutans* was the most susceptible to the peptides, followed by *E. coli*, *S. gordonii, A. actinomycetemcomitans, P. gingivalis* and *P. aeruginosa*. MUC7 12-mer-L, 12-mer-D and 20-mer exerted 2-fold higher antibacterial activities against *S. mutans* (median MICs of 9.4–12.5 μM) than Hsn5-12-mer and magainin-II (MICs of 25 μM) and 4-fold higher activities than MUC7 12-mer-L4 (Table 1, MICs of 50 μM). MBC values were also determined (not shown). They were equal to the corresponding MIC values except the following: 2-fold higher values were found for Hsn 5 12-mer and magainin-II against *S. mutans* and for all tested agents against *S. gordonii*. This indicates that MUC7 peptides exert bactericidal rather than bacteriostatic activity against *S. mutans.*

### *Growth inhibition and time-killing*

As shown in Figure 1(a), upon addition of the peptides or chlorhexidine to the exponentially growing *S. mutans* cell cultures, all cultures continued to grow approximately at the same rate, which was comparable to the non-treated control culture growth, for approximately 1 h. A decrease in bacterial growth rate was observed only after a longer exposure to some agents. MUC7 12-mer, MUC7 12-mer-D and MUC7 20-mer at equal molar concentrations (25 μM), all exhibited inhibitory effect on the *S. mutans* growth, similar to that achieved by chlorhexidine (2.5 μM) (represented in Figure 1a by the lower four curves). On the other hand, low inhibition or no inhibition was found in the presence of MUC7 12-mer-L4, Hsn5 12-mer and magainin-II (represented in Figure 1a by the upper curves).

The results of the time–kill kinetic studies are shown in Figure 1(b). The killing was time-dependent within 24 h

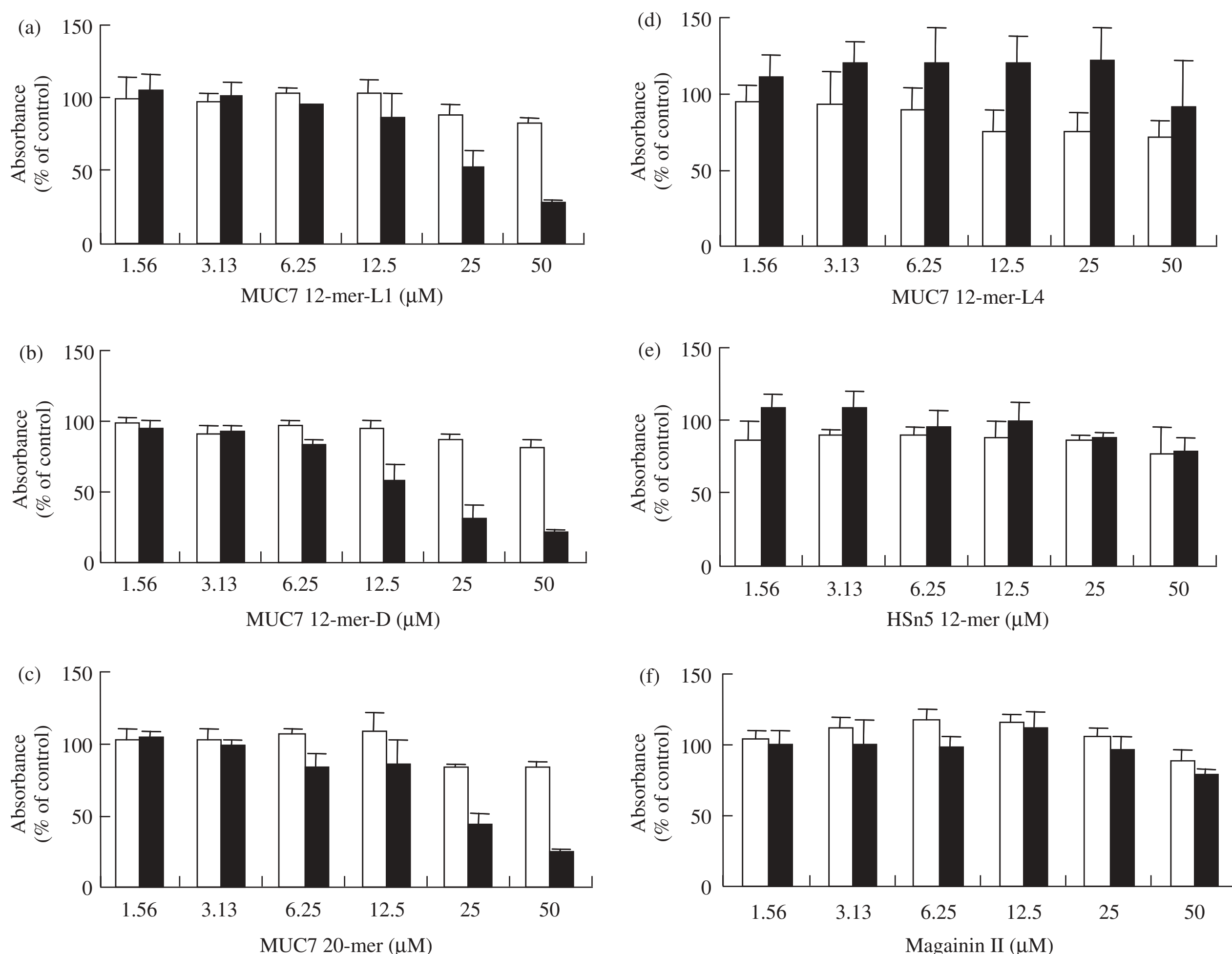


**Figure 3.** Effect of peptides on *S. mutans* 1 day developed biofilm. One day biofilm was prepared as described in the Materials and methods section. BHIS medium with different concentrations of each agent, prepared in another microtitre plate, was then transferred to the biofilm plate. After further incubation at 37°C for 24 h, the biofilm was fixed, stained and quantified as described in the legend to Figure 2. Data represent the mean and SD of two independent tests performed in duplicate. The symbols for the growth and biofilm are the same as in Figure 2.

of incubation. At the MIC (equal potent concentration), magainin-II (25 μM or 61.7 mg/L) and MUC7 20-mer (12.5 μM or 31.4 mg/L) showed the fastest killing rate for *S. mutans*, followed by MUC7 12-L and MUC7 12-D (12.5 μM or 19.7 mg/L). MUC7 12-mer-L4 (50 μM or 79.0 mg/L), Hsn5 12-mer (25 μM or 39.5 mg/L) and the positive control agent chlorhexidine (2 μM or 1.3 mg/L) showed a slow killing rate compared with the other agents. After 4 h of incubation, magainin-II reduced the viable counts of *S. mutans* by 3 $\log_{10;}$ MUC7 20-mer, MUC7 12-mer-L and MUC7 12-mer-D by 2 $\log_{10}$; and MUC7 12-mer-L4, Hsn5 12-mer and chlorhexidine by 1 $\log_{10}$, respectively.

### *Biofilm inhibition*

MUC7 peptides, with the exception of MUC7 12-mer-L4, exhibited an inhibitory effect on the formation of *S. mutans* biofilm, with $MBIC_{50}$ from 6.25 to 12.5 μM, and reduced activity against 1 day developed *S. mutans* biofilm, with $MBRC_{50}$ from 25 to 50 μM (Table 2). Consistent with the activity against planktonic bacterial cells, MUC7 12-mer-L, 12-mer-D and 20-mer showed 2- or 4-fold higher activities against *S. mutans* biofilm formation than Hsn 5 12-mer or magainin-II (Table 2 and Figure 2). MUC7 12-mer-L4 exerted low activity against the biofilm formation, with $MBIC_{50}$ of 50 μM (Table 2).

Generally, the inhibition of the *S. mutans* biofilm formation corresponds to the inhibition of bacterial growth. However, for the 1 day developed biofilm (Figure 3), the reduction of the biofilm was observed in the presence of MUC7 12-mer, MUC7 12-mer-D or MUC7 20-mer at $MBRC_{50}$, while the growth did not decrease in parallel. This indicates that MUC7 peptides exerted reduction activity on the 1 day developed biofilm. The reduction was not observed in the presence of MUC7 12-mer-L4, Hsn5 12-mer or magainin-II.

In addition, MUC7 peptides also exhibited inhibitory effects on the formation of the biofilm on the vertical surface of CBD pegs. As shown in Figure 4, compared with the no-drug growth control, the viabilities of the biofilm were suppressed by more than 95% in the presence of MUC7 peptides at 4× MIC (50 μM). The other tested agents suppressed the viability of the biofilm to a similar extent. The 1 day developed biofilm was also inhibited by MUC7 12-mer-L, 12-mer-D and 20-mer peptides, as demonstrated by biofilm viability reduction by 49, 50 and 75%, respectively (Figure 4). On the other hand, the other two peptides showed much lower biofilm viability reduction. The positive control, chlorhexidine, showed 97% reduction in terms of viability.

SEM photographs of biofilms formed on the pegs or HA discs and the effect of MUC7 12-mer-L, a representative MUC7 peptide, on the biofilm formation are shown in Figure 5. For the non-treated controls, a biofilm formed on the HA discs consists of nearly uniform, thick layer of cells (Figure 5j–l), while the biofilm formed on the pegs is much less dense, and individually formed colonies are seen (Figure 5d–f). The formation of the biofilm on both surfaces was prevented or greatly reduced in the presence of the MUC7 12-mer-L peptide. More specifically, on the pegs, the biofilm contained only a few cells, with bacterial cells or colonies being spread around the pegs (Figure 5a–c) or discs (Figure 5g–i), and large parts of the pegs or discs were uncolonized.

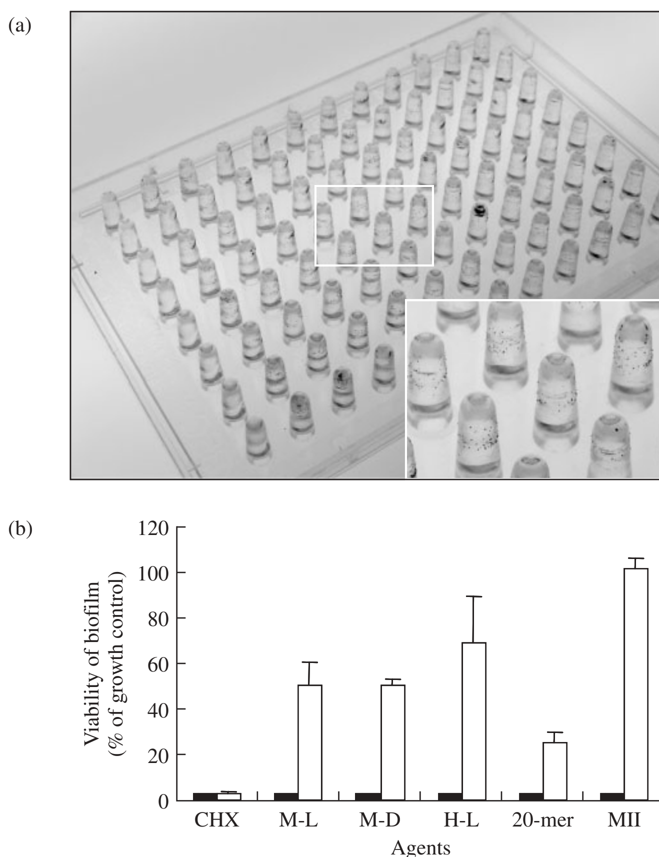


**Figure 4.** Effect of peptides on the formation of and reduction of 1 day developed *S. mutans* biofilm on CBD pegs. The biofilms were grown on the surface of CBD pegs (a) and the biofilm viabilities (b) were determined by an assay using Alamar Blue as described in the text. Data represent the mean and SD of four tests. CHX, chlorhexidine; M-L, MUC7 12-mer-L; M-D, MUC7 12-mer-D; 20-mer, MUC7 20-mer; H-L, Hsn5 12-mer; and MII, magainin-II. The agent concentrations were 10 μM for CHX and 50 μM for all the peptides. Filled bars, biofilm formation; open bars, reduction of 1 day biofilm.

## Discussion

In this study, we investigated the susceptibility of selected bacteria and *S. mutans* biofilm to several MUC7 peptides, along with two other antimicrobial peptides Hsn5 12-mer and magainin-II. The major findings of this study are that (i) MUC7 peptides exhibit antibacterial activity, and among the tested bacteria, *S. mutans* is the most susceptible to all peptides tested; (ii) MUC7 peptides exert higher antibacterial activities against *S. mutans* than the well-known antimicrobial peptides Hsn5-12-mer and magainin-II with lower MIC values and faster killing rate; (iii) MUC7 peptides inhibit formation of *S. mutans* biofilm and are also effective in reduction of the 1 day developed biofilm; and (iv) MUC7-12-mer-L4, a peptide lacking cysteine residues, exhibits low antibacterial activity against *S. mutans.*

*S. mutans* is considered one of the primary causative agents of dental caries and can also be a source of infective endocarditis.[23] The main virulence factors associated with cariogenicity include adhesion, acidogenicity and acid tolerance inside the dental biofilm. The prevention of dental caries is targeted at the control of dental plaque.[24] Studies have shown that chlorhexidine, hexetidine, delmopinol, amine fluoride/stannous fluoride, triclosan,

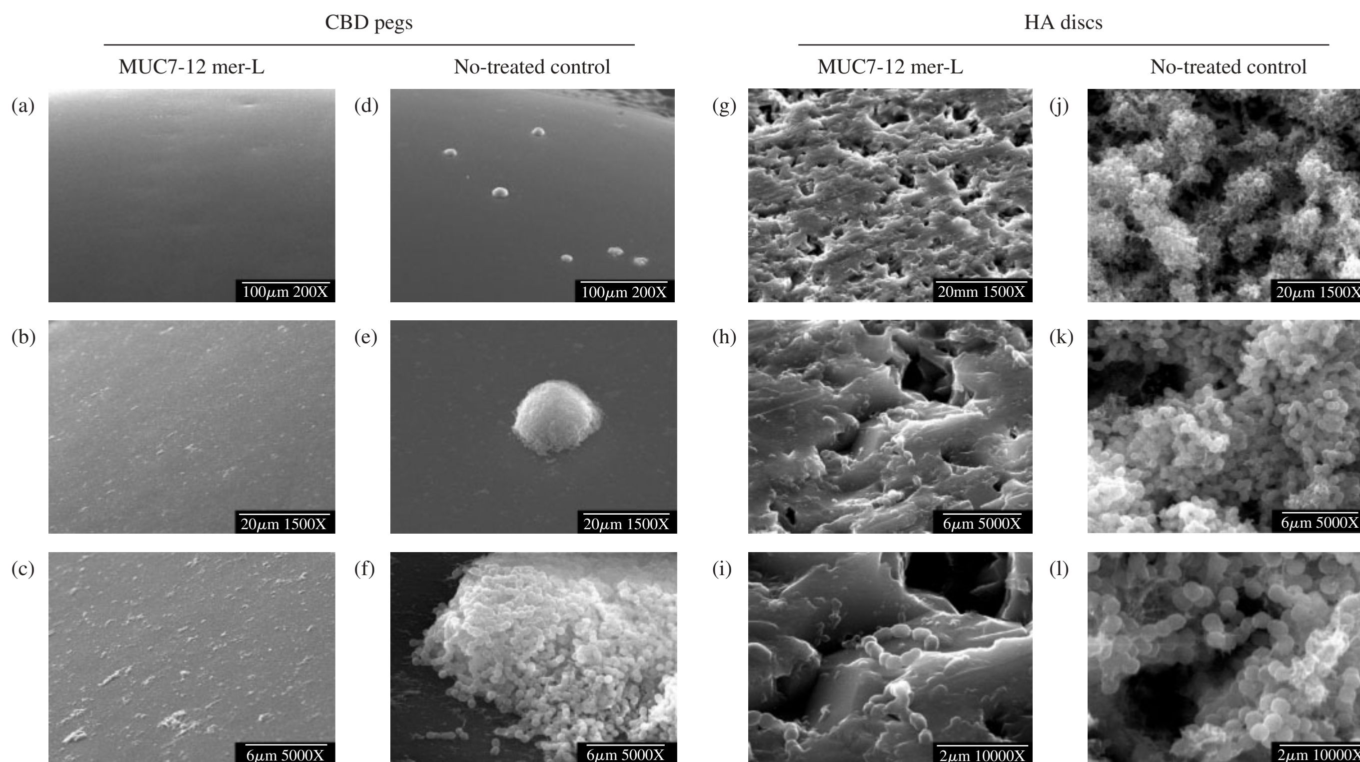


**Figure 5.** SEM micrographs of *S. mutans* biofilm formation on CBD pegs and HA disc surfaces. Biofilms were grown in BHIS in the presence of or absence of MUC7 12-mer-L at 4× MIC (50 μM). All images shown were taken at magnifications (×1000 to ×10 000 for HA discs, ×200 to ×5000 for CBD pegs). The selected images were chosen as the best representatives of the amount of biofilm on the surface of the HA disc and CBD peg.

phenolic compounds, among others, may inhibit biofilm development and maturation as well as affect bacterial metabolism.[24]

CAMPs exhibit killing activity against numerous oral bacteria.[8–10,25–28] Synthetic histatin analogue dhvar4,[29] human B-defensin-2 (hBD-2),[25] hBD-3[28] and gaegurin 6[27] have been reported to have inhibitory effects against oral bacteria including *S. mutans* and *Streptococcus sanguis*. Our study showed that MUC7 peptides exhibit a preferential antibacterial activity against *S. mutans*, compared with the other species tested.

Mechanisms of the antimicrobial peptide activity include three pore-forming models, and several modes of intracellular killing, though their relevance to how peptides damage and kill microorganisms still needs to be clarified.[30] Regardless of the specific antimicrobial mechanism, three specific steps (attraction, attachment and peptide insertion) must occur to induce bacterial killing. Our previous study showed that cysteine residues are not important for fungicidal activity against both *Candida albicans* and *Candida neoformans*.[17] In the present study, however, antibacterial activity of MUC7 12-mer lacking cysteine residues decreased remarkably (Table 1). It has been demonstrated that the cysteine residues located in the N-terminal region of MUC7 are important components of a domain that binds bacterial surface of *S. mutans*.[31–33] Our results suggest that cysteine residues may play an important role in bactericidal activity against *S. mutans*. MUC7 cationic peptides are likely to be attracted to *S. mutans* bacterial surfaces by the electrostatic bonding between their cationic charges and anionic structures (such as teichoic acid) on the *S. mutans* bacterial surface or by cysteine binding domain. The peptides then may traverse capsular polysaccharides, teichoic acids and lipoteichoic acids before interaction with the *S. mutans* cytoplasmic membrane. MUC7 20-mer has been shown to be able to cross the fungal cell membrane and accumulate inside the cells,[14] suggesting that MUC7 peptides may also exert intracellular killing activity against *S. mutans*. Interestingly, *A. actinomycetemcomitans, P. gingivalis* and *P. aeruginosa*, which are all Gram-negative bacteria, exhibited low susceptibility to the test peptides.

The oral bacteria are protected by the formation of biofilms. Bacteria in a biofilm are invariably less susceptible to antimicrobial agents than their planktonic counterparts.[34] For biofilm studies presented, we employed three different substrata, polystyrene microtitre plates with a horizontal surface, CBD with pegs that provided a vertical surface and HA discs, also a horizontal surface but more relevant to *in vivo* situation in terms of tooth surfaces. The effect of MUC7 peptides and of other agents on biofilm formation and reduction was studied in most detail on the polystyrene plates. Because the biofilm showed less susceptibility to the agent compared with planktonic cells, we used $MBIC_{50}$ or $MBRC_{50}$ (the lowest drug concentration that showed 50% or more inhibition on the formation of biofilm and 50% or more reduction of biofilm, respectively) to evaluate the activity of the agent against the biofilm. Unlike MIC and MBC for planktonic cells, the test agents did not exhibit 90% or more reduction of the biofilm even at the highest concentration of the agent. However, in terms of $MBIC_{50}$ or $MBRC_{50}$, we found that MUC7 peptides exhibited not only an inhibitory effect on the formation of *S. mutans* biofilm, but also reduction activity against 1 day *S. mutans* biofilm developed in batch culture on a polystyrene microtitre plate.

The biofilm-forming potential of a strain would depend on the extent of its growth, i.e. the better a strain grows, the better it forms a biofilm. The inhibition by the test agents on the formation of the biofilm may be the result of the inhibition of the bacterial growth since the growth and biofilm varied in parallel. However, this did not occur for 1 day *S. mutans* biofilm developed in batch culture on a polystyrene microtitre plate. The inhibition of the biofilm was higher than that of the growth as shown in Figure 3, indicating that MUC7 peptides exerted reduction effect on the developed biofilm. Similar results, but measured in terms of viability of the biofilm, were also obtained on the reduction of 1 day biofilm preformed on a vertical surface of the CBD pegs, as shown in Figure 4. In order to get some information regarding the mechanism of inhibition or reduction of the biofilm by MUC7 peptides, we have examined MUC7 peptide effect on glucosyltransferases (GTF) or fructosyltransferases (FTF) activity (one time only) using the PAS reagent stain method. However, no inhibition was found against GTF or FTF activity (data not shown). This suggests that the inhibition or reduction of the biofilm may not be as the result of inhibition of extracellular polysaccharides.

Some factors, such as the starvation of the bacteria, pH and sucrose may affect the growth of the biofilm in the batch culture. To solve or minimize these problems, we set up appropriate controls in parallel and replaced the medium when the culture was grown for more than 24 h.

We cannot claim in the present study that MUC7 peptides at the test concentration exhibited inhibitory activity against the mature biofilm. However, early biofilm is more important than mature biofilm because once the early biofilm is inhibited, the mature biofilm may in turn be prevented.

In conclusion, our findings that MUC7 peptides exhibit a preferential antibacterial activity for *S. mutans* compared with other species tested, and the prevention of biofilm formation and the reduction of 1 day developed *S. mutans* biofilm by MUC7 peptides strongly suggest that MUC7 peptides may have a potential for clinical use. However in the *in vivo* situation, *S. mutans* does not occur in monoculture, but rather in the microbial plaque or biofilm, a complex ecosystem. The *in vitro* biofilm assays employed in this study are essentially preliminary screens. *In vitro* assessment of activity against biofilms in a more clinically relevant, brief exposure model system will be needed in order to extrapolate the MUC7 peptide activities to caries inhibiting potential or for use as anti-biofilm agents.

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## Transparency declarations

None to declare.

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