

Effect of Long-term Exposure to Peptides on Mono- and Multispecies Biofilms in Dentinal Tubules

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ABSTRACT

Introduction: The aim of this study was to evaluate the antibiofilm effectiveness of 2% chlorhexidine (CHX) and peptides 1018 and DJK-5 used either alone or in a mixture (peptide and 2% CHX) against *Enterococcus faecalis* and multispecies biofilms in dentin canals after short-term and long-term exposure. **Methods:** One hundred eighty dentin blocks were prepared and filled with *E. faecalis* or multispecies bacteria by centrifugation. Three-week-old biofilms in dentin were subjected to 2% CHX, DJK-5 (10 µg/mL), 1018 (10 µg/mL), DJK-5 + 2% CHX, or 1018 + 2% CHX for short-term (1 or 3 minutes), short-term exposure after 24 hours, and long-term exposure (24 hours of exposure). The antibacterial efficacy was determined by live/dead bacterial viability staining and confocal laser scanning microscopy. **Results:** Peptide DJK-5 with or without CHX was the most effective agent against all the biofilms ($P < .05$), killing 77% of biofilm bacteria in 1 minute. No significant difference in bacterial killing was detected between the first 3 minutes of exposure (>81%) and after 24 hours of exposure (83%) to DJK-5 or DJK-5 + CHX. Chlorhexidine and peptide 1018 had a weaker antibiofilm effect than DJK-5, and their effect was time dependent ($P < .05$) with a maximum killing of 60% after 24 hours of exposure. **Conclusions:** Peptide DJK-5 alone and together with CHX had a rapid antibacterial effect against dentin infection. An additional antibacterial effect by CHX and peptide 1018 was achieved after a 24-hour long-term exposure. (*J Endod* 2019; ■:1–7.)

KEY WORDS

Biofilms; chlorhexidine; long-term exposure; peptides; short-term exposure

Irrigation is a key part of successful root canal treatment. It reduces friction between the file and dentin and improves the cutting effectiveness of the files, dissolves necrotic and inflamed tissue, and kills and removes microorganisms¹. Many different types of irrigants have been used in root canal irrigation, but none of them fulfill all of the criteria for an optimal irrigant². Traditional irrigants such as sodium hypochlorite have variable degrees of cytotoxicity and may cause severe pain if extruded to periapical tissues. Microorganisms on the canal wall and in dentinal tubules are organized in biofilms. Obviously, eradicating biofilms in the root canal system plays a critical role in endodontic treatment.

Antimicrobial peptides are part of the innate immune system. These agents are important because of their partly independent immunomodulatory and direct antimicrobial and antibiofilm activities. They can exert antimicrobial or antibiofilm activity against a broad spectrum of gram-positive and gram-negative bacteria³. As such, antibiofilm peptides are considered alternatives to traditional disinfecting agents used to eradicate biofilm through the permeabilization and breakdown of the cell membrane of the preexisting biofilm-embedded bacteria⁴. Peptides can also target the biofilm mode of growth by inhibiting bacterial adhesion to the growing surfaces⁵. They act in part to suppress the global bacterial-stringent stress response required for biofilm formation and mediated by the accumulation of the alarmones guanosine tetraphosphate and guanosine pentaphosphate, collectively known as (p)ppGpp^{6,7}. Recently, new broad-spectrum antibiofilm 12-mer peptides including L-enantiomeric peptide 1018 and D-enantiomeric, protease-resistant peptide DJK-5 were introduced and shown to bind to and trigger the degradation of ppGpp^{3,8,9}. Peptide 1018¹⁰ was able to inhibit oral plaque biofilm formation and kill bacteria in oral plaque biofilms. A recent study comparing the antibiofilm efficacy of DJK-5 and 1018 on oral biofilms showed

SIGNIFICANCE

An antibiofilm peptide DJK-5 alone and together with chlorhexidine has a quick and strong antibacterial effect against biofilms in dentin canals.

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that the former was more effective in killing biofilm bacteria than the latter¹¹. DJK-5 was also shown to be a potentially promising agent as a component in an endodontic irrigant^{12,13} in which a mixture of EDTA and DJK-5 proved effective in killing oral biofilms on hydroxyapatite (HA) disks¹⁴ and in dentin canals¹³. The combination of DJK-5 and 2% chlorhexidine (CHX) killed 83%–87% and 88%–90% of 3-day-old young biofilms on HA disks in 1 or 3 minutes¹¹, respectively, meaning that some of the bacteria remained alive. Therefore, we hypothesized that long-term exposure to disinfection solutions might kill more bacteria and even completely eliminate viable bacteria. The goal of the present study was to evaluate the antibiofilm effectiveness of 2% CHX, peptide 1018, and DJK-5 used either alone or in a mixture (peptide and 2% CHX) against *Enterococcus faecalis* and multispecies biofilms in dentin canals after short-term and long-term exposure.

MATERIALS AND METHODS

Ninety caries-free single-rooted human teeth extracted for orthodontic reasons were collected according to the protocol approved by the university clinical research ethics committee review boards (certificate H12-02430). Following a previously described protocol¹⁴, 180 dentin blocks (approximately 4 × 4 × 2 mm) were prepared from root dentin.

Dentin Canal Infections

Strain *E. faecalis* Gel-31, originally isolated from persistent apical periodontitis cases¹⁵, and dental clinical plaque samples from 2 different donors were used as test organisms. Written informed consent was obtained for collecting the plaque samples. *E. faecalis* Gel-31 was grown on brain-heart infusion (BHI) agar (Becton-Dickinson, Sparks, MD) plates under anaerobic conditions (Bactron300 Shellab Anaerobic Chamber; Sheldon Manufacturing, Inc, Cornelius, OR) at 37°C overnight. The bacteria were harvested and suspended in BHI broth (Becton-Dickinson). The cell density was standardized to an optical density at 405 nm of 0.05 (ELx808 Absorbance Reader; BioTek Instruments, Inc, Winooski, VT). Supragingival and subgingival plaque were collected fresh from 2 adult volunteers using single-use toothpicks, suspended and grown in BHI, and incubated under anaerobic conditions at 37°C overnight¹⁶. The bacteria were collected and prepared with fresh BHI to the same optical density as described for *E. faecalis* previously.

Dentin pieces were attached and sealed to the bottom of the upper chamber of the filter

tubes using a resin composite¹⁴. Following a previously described protocol¹⁴, *E. faecalis* and plaque suspensions were centrifuged into dentinal tubules by serial centrifugation as previously described¹⁴. The prepared dentin specimens were colonized either with 1 of the plaque cultures or with *E. faecalis* and incubated in BHI broth under anaerobic conditions at 37°C. Nutrient broth was changed once a week by replacing 500 µL fresh BHI in the upper chamber of the dentin specimen filter tubes to facilitate the growth of anaerobic bacteria and to prevent overgrowth by rapidly growing gram-positive facultative bacteria.

Three weeks later, the dentin specimens were detached and taken out of each tube, and the surrounding composite material was removed. The specimens were rinsed in sterile water for 1 minute and gently air-dried. The outer surfaces (cemental sides) of the specimens were closed using nail varnish.

Antibiofilm Effect of Peptides 1018 and DJK-5 and CHX

The 180 dentin blocks were infected with 3-week-old *E. faecalis*, or 1 of the 2 donor's plaque biofilms in the dentin canals were divided into 3 groups: a short exposure group, a short delayed exposure at 24 hours group, and a long-term 24-hour exposure group (Fig. 1). Peptides 1018 and DJK-5 were synthesized by CPC Scientific (Sunnyvale, CA) using solid-phase 9-fluorenylmethoxycarbonyl chemistry and purified to a purity of >95% using reversed-phase high-performance liquid chromatography as previously described^{10–13}. The final concentrations of peptide 1018 and peptide DJK-5 were diluted in phosphate-buffered saline from 1-mg/mL stock solutions.

Short-term Antibiofilm Effect

The 144 specimens in the short-term group were randomly exposed to 1 of the following 6 different antibacterial solutions with 4 specimens in each group: sterile water (control), 2% CHX (Sigma-Aldrich, St Louis, MO), 10 µg/mL peptide 1018, 10 µg/mL peptide DJK-5, a mixture of 2% CHX + 10 µg/mL peptide 1018 (final concentrations), and a mixture of 2% CHX + 10 µg/mL peptide DJK-5 (final concentrations). A droplet of 50 µL of each disinfecting solution was placed on the root canal wall of the dentin specimens for 1 or 3 minutes. Five microliters of 20% stock CHX solution (Sigma-Aldrich) was used in each 50-µL droplet of CHX and peptide combinations. Half of the treated specimens were examined with confocal laser scanning microscopy (CLSM) immediately after medicament exposure as described later. The other half

were kept at 37°C under 100% humidity and examined by CLSM after 24 hours.

Long-term Antibiofilm Effect

The 36 specimens for the long-term group were exposed to the same disinfecting solutions for 24 hours. Fifty microliters of fresh solution was added every 8 hours on the dentin specimens.

Examination with CLSM

The specimens were washed with sterile water for 1 minute and fractured vertically through the root canal into 2 halves to expose a fresh surface of longitudinally fractured dentinal tubules. Two semicylindrical dentin halves (4 samples after fracturing the halves) of each group were examined by CLSM (FV10i-LIV; Olympus, Tokyo, Japan) and viability staining to determine the proportions of live and dead bacteria as described previously^{14,16}. Five randomly selected sites were scanned in each dentin piece, totaling 20 scans per group ($n = 20$).

Statistical Analysis

The sample size was determined using G-Power 3.1 software (University of Düsseldorf, Düsseldorf, Germany; <http://www.gpower.hhu.de/en.html>). A power analysis with the F family of tests (analysis of variance) was applied, resulting in a required minimum sample size of 16 for each group.

Statistical analysis was performed with SPSS 16.0 software (SPSS Inc, Chicago, IL). One-way analysis of variance was implemented, and the post hoc Fisher least significant difference multiple comparison test was applied when necessary; significance was considered to occur at the $P < .05$ confidence level.

RESULTS

In the CHX groups, 3 minutes of exposure resulted in higher killing of bacteria (19%–24%) for *E. faecalis* and the 2 multispecies biofilms than 1 minute of exposure (14%–18%, Fig. 2A–C, $P < .05$). No significant increase in these values was observed when parallel short exposure samples were treated after a 24-hour waiting period. Similarly, in peptide 1018 groups, there was no significant difference in the proportion of killed bacteria by short-term exposure when examined immediately after the exposure or after 24 hours. The combination of peptide 1018 and CHX showed an additive effect after short-term exposures of 1 and 3 minutes and almost doubled the proportion of killed biofilm bacteria (27%–37%) when

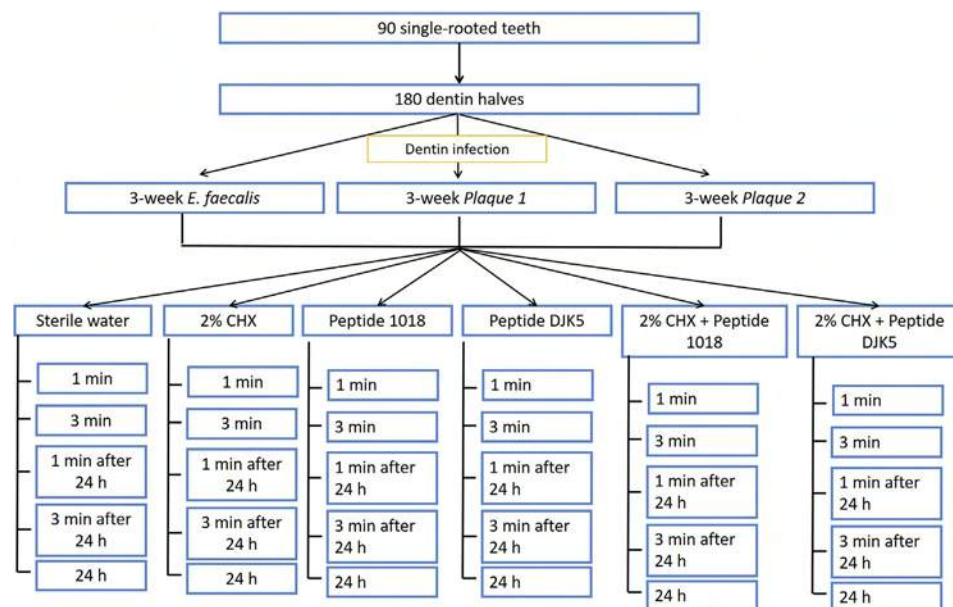


FIGURE 1 – The experimental workflow.

compared with each substance used alone (Fig. 2, $P < .05$). There was no continued killing after short-term exposure to the peptide 1018 + CHX cocktail as shown by measurements taken 24 hours later (Fig. 2). Peptide DJK-5 was the most efficient against all biofilms after only 1 and 3 minutes of exposure. Between 76%–82% of biofilm bacteria in all biofilms were killed after a short exposure to DJK-5. Measurements performed 24 hours after the short exposures did not show significant increases in bacterial killing (Fig. 2). Slightly more bacteria were killed by the combination of CHX and DJK-5 than by peptide DJK-5 alone, but the difference was not statistically significant in any of the groups.

Long-term exposure for 24 hours to the medicaments and their combinations resulted in higher killing than the short-term exposures to CHX and peptide 1018 and their combination (Fig. 2) in all 3 biofilms ($P < .05$). DJK-5 was by far the most effective antibiofilm agent also after the 24-hour exposure, although bacterial killing by DJK-5 alone or in combination with CHX did not increase substantially after the first 1 and 3 minutes (Figs. 2 and 3A–F). DJK-5 killed significantly more biofilm bacteria in all 3 biofilms after 1 minute of exposure than the other 2 compounds even after 24 hours of exposure.

No significant differences in killing were detected after short exposure or long-term

exposure to the medicaments between *E. faecalis* and the 2 multispecies biofilms.

DISCUSSION

The ideal prerequisite to successful antimicrobial treatments is exposure of all bacteria within the biofilm to an effective concentration for an adequate amount of time¹⁷. The optimal antibiofilm concentrations (10 $\mu\text{g}/\text{mL}$) of the peptides 1018 and DJK-5 have been determined in previous studies^{11,13}. The antimicrobial short-term effect of the combination of DJK-5 and CHX has been tested previously on 3-day-old biofilms on HA disks¹¹. The present study is the first report in which the effects of long-term exposure by the 2 peptides with CHX has been tested against mono- and multispecies biofilms in dentinal tubules of root dentin, which closely mimics the clinical situation. The present study showed that DJK-5 used alone or together with CHX for 1 minute is much more effective than killing previously reported for 6% sodium hypochlorite used for 3 minutes and equally effective as 6% sodium hypochlorite used for 30 minutes against the dentin microbes¹⁸ (an identical methodology was used in these 2 studies). Thus, the level of killing by the peptide DJK-5 is the highest ever reported against biofilms in dentin canals.

The exposure times, 1 and 3 minutes, were chosen to be clinically realistic. CHX has been reported to have substantivity (ie, it can

bind to a substrate such as dentin and continue its antimicrobial effect for a prolonged period of time)^{19,20}. Biofilm recovery studies^{19,20} have previously also reported an increase of the amount of dead bacterial cells 1 week after a 3- and 10-minute treatment by CHX using an open biofilm model on HA disks with no dentin. In the present study, killing in biofilm was measured right after the exposure and 24 hours later to detect the possibility of a continued CHX effect. Interestingly, there was no obvious extended residual activity over the 24 hours after the short-term of exposure to CHX or the peptides.

During the long-term treatments, the antimicrobial solutions were changed every 8 hours to maintain irrigant activity during the 24-hour period. Continued killing by CHX and 1018 could be explained by the active effect of solutions during the 24-hour period. Interestingly, DJK-5, even after 1 and 3 minutes of exposure killed many more biofilm microbes than 2% CHX, peptide 1018, or the 2 combined after 24 hours of exposure. Despite the highest killing ratio so far published, the current results showed that it is difficult or perhaps even impossible to kill all bacteria in the root canals. The incomplete killing of bacteria in the biofilms even after long-term exposure might be caused by the inability of the antimicrobial agents to penetrate effectively enough into the dentinal tubules or the metabolically inactive microbial cells and persist cells in the biofilms. A long-term exposure of 24 hours or more is possible in

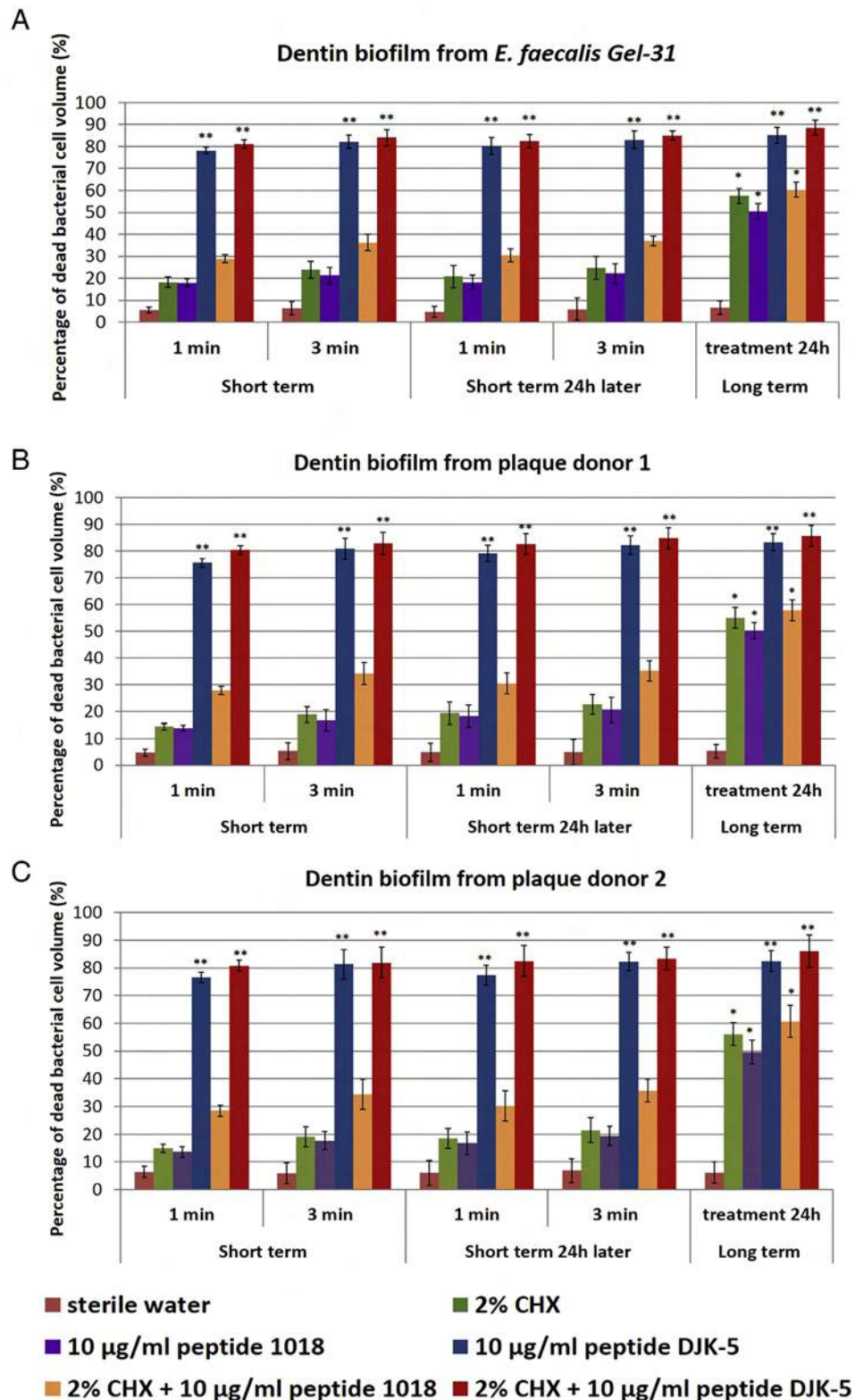


FIGURE 2 – The proportion of dead bacteria in dentinal tubules after short-term exposure, 24 hours after short-term exposure, and long-term exposure of (A) *E. faecalis* biofilms, (B) multispecies biofilms from donor 1, and (C) multispecies biofilms from donor 2.

multiappointment root canal treatments, but the present results clearly showed that the same (highest) killing by DJK-5 was already

achieved after 1 and 3 minutes of exposure (Fig 2). Furthermore, it is possible that the effectiveness of the antimicrobial agents in root

canals *in vivo* becomes reduced over time^{21,22}. A 50-µL droplet of the medicament was used in the present study on the dentin pieces; the

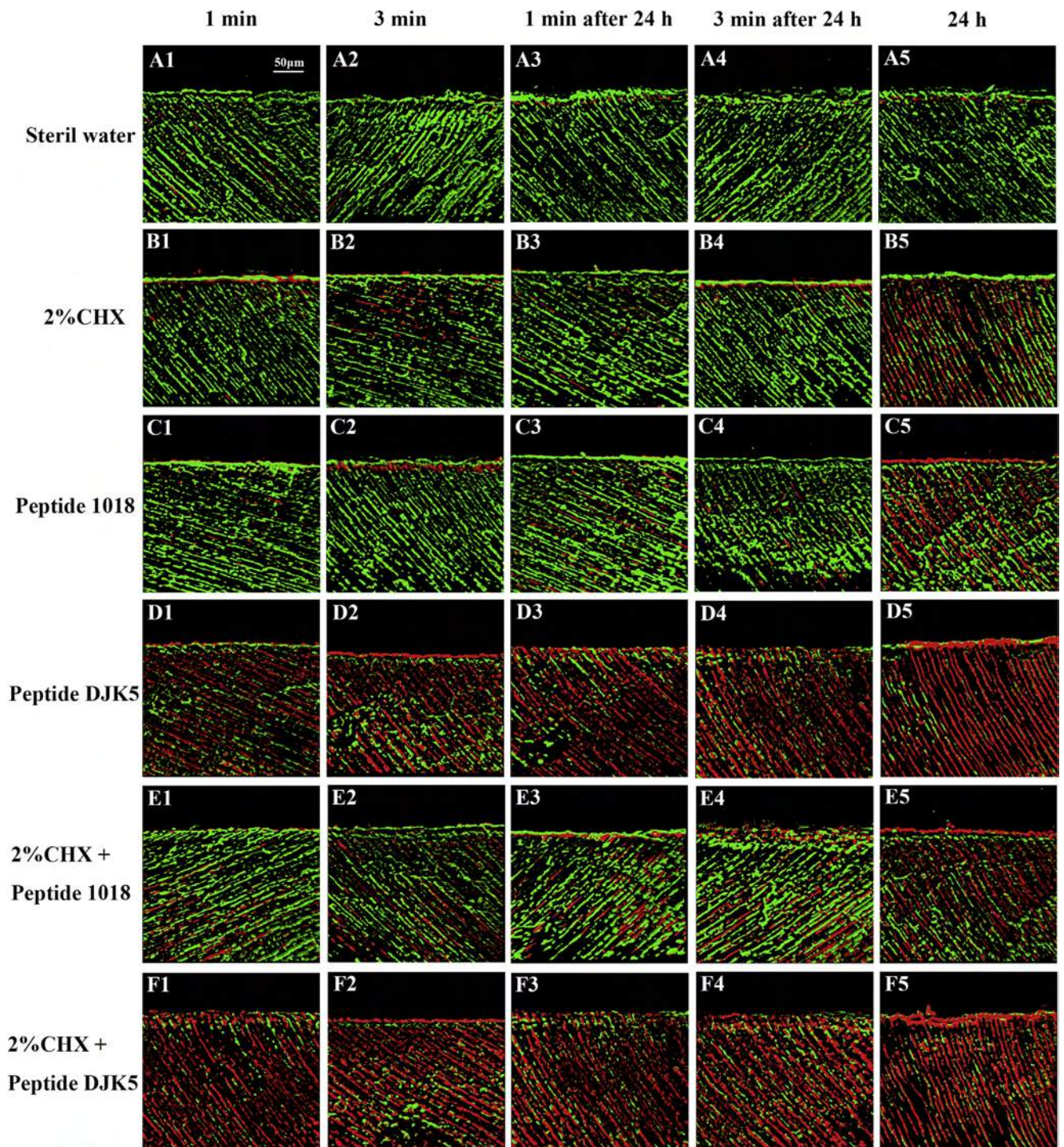


FIGURE 3 – Multispecies biofilms after short-term exposure, 24 hours after short-term exposure, and long-term exposure to (A) water, (B) 2% CHX, (C) 1018, (D) DJK-5, (E) 1018 + 2% CHX, and (F) DJK-5 + 2% CHX.

volume of an instrumented root canal is smaller and likely to be between 10–30 μ L.

Two percent CHX alone is known for its effectiveness in killing biofilm bacteria, but even more so for preventing biofilm growth on clean surfaces^{10,11}. The negative effects of CHX include staining of tooth surfaces and perception of bad taste²³. In the root canal, such negative effects are likely to be

meaningless. Nevertheless, the present results showed that although the combination of peptide 1018 and CHX had a strong additive effect in killing biofilm bacteria, the most effective killing was achieved by DJK-5 used alone; adding CHX to DJK-5 did not show any significant improvement in antibacterial activity. A rapid antibacterial effect of DJK-5 may be associated with its special design, which

allows the peptide to target the stringent stress response of microorganisms²⁴.

Only 1 concentration of each peptide was tested in the present study based on the results of several recent studies^{10–13}. Although the bactericidal action of 1018 is not as strong as that of DJK-5, peptide 1018 can modulate the host's immune system, allowing it to synergize with the host immune system while

decreasing inflammation, thereby potentially increasing its antibiofilm effectiveness⁹. Further study is needed to evaluate the peptides' antimicrobial effect against oral biofilm *in vivo*.

One of the interesting findings in the present study was that the 3 biofilms, a monospecies (*E. faecalis*) and 2 multispecies biofilms from different donors, showed similar sensitivity to the tested antimicrobial agents. This result is consistent with previous studies^{10–13} and supports previous findings that the source and possible differences in the species composition of the multispecies biofilm may not have a major impact on its susceptibility to antibiofilm treatment.

Within the limitations of this study, only CLSM was provided as the approach to evaluate the antibiofilm effects. Further investigations using a scanning electron microscope and/or polymerase chain reaction

can be considered as additional strategies to provide more evidence of biofilm killing on the morphologic and molecular biological levels. The high production cost has been recognized as another limitation of peptides to be used in the clinic⁹. However, peptides are aimed to be used in a very low concentration in irrigants, and more efforts have been made to simplify the manufacturing process for mass production.

More hurdles slowing down the success of the synthetic peptide application in clinics include unexplored toxicities, long amino acid sequence production, and degradation by host proteases²⁵. Efforts have been made to overcome some of the limitations by performing physicochemical modifications such as using D-enantiomer amino acids (DJK-5), sequence truncations, and incorporating computational methods²⁵.

In conclusion, peptide DJK-5 alone and together with CHX had a rapid antibacterial effect against mono- and multispecies biofilms against dentin infection. Long-term exposure to DJK-5 could not eradicate all bacteria in dentinal tubules. CHX and peptide 1018 had a weaker, time-dependent effect on the biofilms in dentin.

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R.E.W.H. is a coinventor of a patent application on the use of antibiofilm peptides DJK-5 and 1018 that is assigned to his employer (University of British Columbia) and has been licensed to ABT Innovations Inc, which is partially owned by R.E.W.H.

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