Biofilm biomass disruption by natural substances with potential for endodontic use

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Abstract: This study evaluated the *in vitro* effects of four natural substances on the biomass of bacterial biofilms to assess their potential use as root canal irrigants. The following substances and their combinations were tested: 0.2% farnesol; 5% xylitol; 20% xylitol; 0.2% farnesol and 5% xylitol; 0.2% farnesol, 5% xylitol, and 0.1% lactoferrin; 5% xylitol and 0.1% lactoferrin; and 20 mM salicylic acid. The crystal violet assay was used to evaluate the effects of these substances on the biomass of biofilms formed by *Enterococcus faecalis* and *Staphylococcus epidermidis*. All substances except for 20 mM salicylic acid and 20% xylitol reduced biofilm mass when compared to controls. The combination of farnesol and xylitol was the most effective agent against *E. faecalis* ATCC 29212 ($p < 0.05$). Farnesol combined with xylitol and lactoferrin was the most effective against biofilms of the endodontic strain of *E. faecalis* MB35 ($p < 0.05$). Similarly, combinations involving farnesol, xylitol, and lactoferrin reduced the biomass of *S. epidermidis* biofilms. In general, farnesol, xylitol, and lactoferrin or farnesol and xylitol reduced biofilm biomass most effectively. Therefore, it was concluded that combinations of antibiofilm substances have potential use in endodontic treatment to combat biofilms.

Descriptors: Farnesol; Xylitol; Lactoferrin; *Enterococcus faecalis*.

Introduction

Current evidence indicates that apical periodontitis is a disease caused by biofilm infection.[1] Bacteria organized in biofilm communities are often observed in the apical root canal system of teeth with primary or post-treatment apical periodontitis.[1] Therefore, treatment of apical periodontitis involves targeting the biofilm with specific substances and delivery strategies.

In root canal treatment, mechanical debridement is of utmost importance to remove biofilms and organic matter that might hinder the potency of antimicrobials or serve as nutrients for residual bacteria. However, studies have demonstrated that although instrumentation and irrigation are effective in substantially reducing the bacterial bioburden in infected canals, in many cases bacteria remain in the main root canal even when sodium hypochlorite (NaOCl) is used as the irrigant.[2] In addition to exhibiting a clinical performance that does not match its *in vitro* antibacterial potential, NaOCl has many disadvantages, including cytotoxicity to

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vital tissues,[3] reduced efficacy in the presence of organic matter,[4] bad smell and taste, and interference with pulp regeneration procedures and bonding of adhesive materials by altering the dentin surface.[5] These undesirable influences therefore warrant the search for alternative irrigants that are safer and more effective.

Naturally occurring substances with antibiofilm effects have been suggested for treatment of biofilm-related diseases, including caries and chronic wound infections.[6,7] Examples of these substances include those that target bacterial attachment (lactoferrin and salicylic acid) and those that block formation or cause degradation of the biofilm matrix (xylitol and farnesol). By partially disrupting the biofilm structure, the remaining bacteria can become more vulnerable to antimicrobial agents. Therefore, substances that affect biofilm biomass may be of great utility for the treatment of biofilm infections.

Given their specific mechanisms of action, these antibiofilm substances have the potential to be used as endodontic irrigants or interappointment medications. Trans-trans farnesol (*tt*-farnesol) is a sesquiterpene alcohol commonly found in propolis and in essential oils of citrus fruits and has been reported to have antibiofilm effects, either by preventing biofilm formation or by attacking biofilms already established.[8,9] Xylitol is a five-carbon alcohol sugar found naturally in small quantities in fruits and vegetables, and it has been shown to inhibit biofilm formation[10,11] and disrupt biofilm structure.[12] Lactoferrin is a large, multifunctional iron-binding glycoprotein of the innate immune system that has been shown to exhibit antibiofilm effects.[12,13] Combinations involving farnesol/xylitol[11,14] and lactoferrin/xylitol[12,15] act synergistically against biofilms. Lastly, salicylic acid is produced by many plants as part of their defense mechanisms against infection and also inhibits biofilm formation.[16,17] The present study was conducted to evaluate the potential of these substances and their combinations to reduce the biomass of biofilms formed by two strains of *Enterococcus faecalis* and one of *Staphylococcus epidermidis*.

Methodology

The following antibiofilm substances/combinations were used in this study:

- 0.2% *tt*-farnesol (FAR; Sigma-Aldrich, St. Louis, USA);
- 5% xylitol (XYL; Sigma-Aldrich);
- 20% xylitol (XYL; Sigma-Aldrich);
- 0.2% *tt*-farnesol and 5% xylitol (FAR-XYL);
- 0.2% *tt*-farnesol, 5% xylitol and 0.1% lactoferrin (FAR-XYL-LAC; Sigma-Aldrich);
- 5% xylitol and 0.1% lactoferrin (XYL-LAC); and
- 20 mM salicylic acid (Sigma-Aldrich).

Saline was used as a control. Biofilm biomass was visualized and quantified with a modified crystal violet binding assay.[18-21] The following bacterial strains were used in this experiment:

- *E. faecalis* ATCC 29212,
- *E. faecalis* MB35 isolated from a human root canal–treated tooth with post-treatment disease,[22] and
- *S. epidermidis* ATCC 35984.

A 0.5 McFarland standard of an overnight culture of each bacterial strain was prepared in Tryptic Soy Broth (Difco, Detroit, USA) supplemented with 1% glucose (Merck, Whitehouse Station, USA). After agitation by vortex, 200-µL aliquots of cultures were distributed in wells of a 96-well microtiter plate (tissue culture–treated polystyrene, flat bottoms, model 92096 TPP "Techno Plastic Products", Trasadingen, Switzerland) and incubated for 24 h at 35°C. The content of each well was then aspirated, and the wells were rinsed three times with 200 µL of phosphate-buffered saline (pH 7.2) to remove loosely attached cells. Each test substance was applied at 200 µL per well for 5 min at 37°C. After washing three times with phosphate-buffered saline, adherent bacteria were stained for 20 min with 200 µL of 0.1% crystal violet solution at room temperature. Excess stain was rinsed off by copious washing with distilled water. Plates were overturned and air-dried, and the dye bound to the adherent cells was solubilized with 150 µL of 95% ethanol for 5 min. To quantify biofilm mass remaining after treatment, absorbance (590 nm) of the crystal violet solution was measured using an ELISA reader (Model

680, Bio-Rad Laboratories, Hercules, USA). For the positive control, saline was used instead of the test substance. For the negative control, sterile culture broth was used. All assays were performed with four repetitions on three separate occasions. The cut-off value for optical density (OD) measurements was defined as three standard deviations above the mean OD of the negative control.[18] Therefore, final OD values were expressed as average OD value reduced by the cut-off value.

Data were statistically evaluated via analysis of variance and the Student-Newman-Keuls test for multiple comparisons with the significance level established at 5% ($P < 0.05$). The statistical analysis was performed using SPSS 17.0 computer software (IBM, New York, USA).

Results

All test strains formed biofilms as assessed with the crystal violet assay. The most substantial biofilms were produced by *S. epidermidis*, followed by *E. faecalis* strain MB35 and then ATCC 29212. All the test substances significantly reduced biofilm biomass compared with controls ($p < 0.05$); exceptions were 20% XYL against *E. faecalis* MB35 biofilms and salicylic acid and 20% XYL against *S. epidermidis* biofilms. Analysis of the antibiofilm effects against *E. faecalis* ATCC 29212 biofilms revealed that FAR-XYL was significantly more effective than all other substances ($p < 0.05$) (Figure 1). FAR-XYL-LAC was the next most effective, also being significantly more potent than all the other substances ($p < 0.05$). FAR-XYL-LAC was the most effective against biofilms of the endodontic strain of *E. faecalis* MB35 ($p < 0.05$) (Figure 2). FAR-XYL was the second most effective, being significantly more potent than all the other agents except for FAR. As for the strong biofilm producer *S. epidermidis,* FAR-XYL, FAR-XYL-LAC, XYL-LAC, and 5% XYL were the most effective, with no significant difference between them ($p > 0.05$) (Figure 3). All of them were significantly more effective than the other agents, except for the comparison between FAR-XYL and FAR. In general, FAR-XYL-LAC and FAR-XYL were the most effective substances for reducing biofilm biomass (Figure 4).

Discussion

The present study evaluated the ability of several potential endodontic irrigants and medicaments to disrupt the biomass of single-species biofilms. Concentrations of the test substances were based on previous studies.[7,11,12,23] Despite some variations of effectiveness depending on the bacterial source of the biofilm, our overall findings revealed that the combinations of farnesol, xylitol and lactoferrin or farnesol and xylitol had the best outcomes among the substances tested.

Antibiofilm substances can inhibit biofilm formation (preventive effect) or alternatively act on biofilms already formed (therapeutic effect). The mechanism of action against established biofilms may be through disruption of biofilm biomass and/or direct killing of the biofilm bacteria. It is important for an

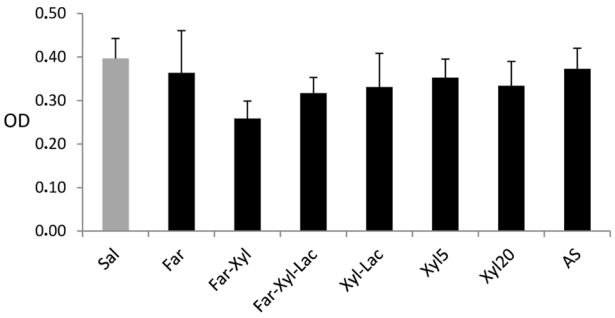

Figure 1 - Effects of the candidate antibiofilm substances on the biomass of biofilms produced by *Enterococcus faecalis* ATCC 29212 as measured by the crystal violet assay ($p < 0.05$).

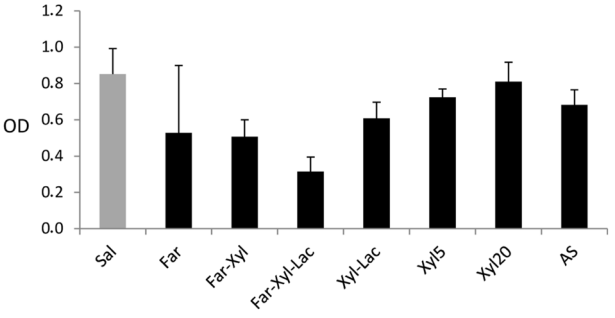

Figure 2 - Effects of the test substances on the biomass of biofilms produced by *Enterococcus faecalis* strain MB35 as measured by the crystal violet assay ($p < 0.05$).

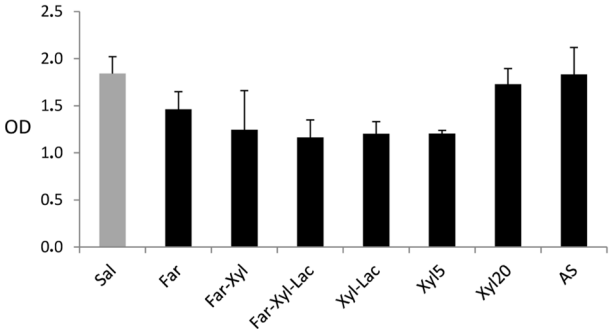

Figure 3 - Effects of the test substances on the biomass of biofilms produced by *Staphylococcus epidermidis* ATCC 35984 as measured by the crystal violet assay ($p < 0.05$).

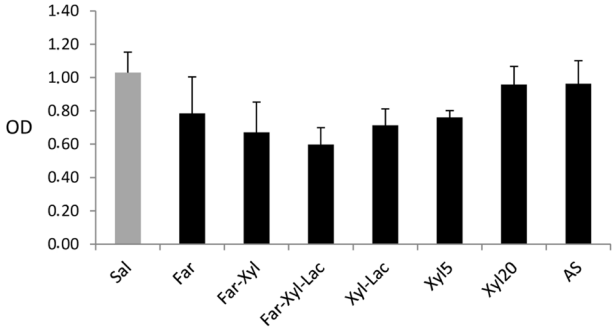

Figure 4 - Overall antibiofilm results. Sal, saline; Far, farnesol; Xy, xylitol; Lac, lactoferrin; AS, salicylic acid.

endodontic irrigant or medicament to act primarily on established biofilms attached to the root canal walls so as to promote their elimination.

Farnesol has been shown to have both effects on biofilms, i.e., by inhibiting biofilm formation and disrupting already-formed biofilms.[8,24,25] Indeed, topical application of farnesol reduces the biofilm matrix content.[24,26] In addition to disrupting biofilm biomass, farnesol also directly kills biofilm bacteria.[9]

Xylitol also inhibits biofilm formation and disrupts the structure of established biofilms.[10,12] However, xylitol only minimally reduces bacterial viability in biofilms.[12] Xylitol can act synergistically with farnesol, and this combination can selectively inhibit the growth of *Staphylococcus aureus*.[11,14] The present findings confirm the potential synergy between farnesol and xylitol.

Lactoferrin also has antibiofilm effects,[12,13] but the mechanisms are not well established. Lactoferrin has great potential to act synergistically with xylitol to disrupt biofilm structure and reduce bacterial viability.[12,15] Specifically, xylitol disrupts biofilm integrity whereas lactoferrin permeabilizes bacterial membranes.[12] Our present findings demonstrate that the combination of farnesol, xylitol and lactoferrin reduces biofilm biomass most effectively compared with the other agents tested. Further studies are required to evaluate the effects of these substances and their combinations on bacterial viability in endodontic biofilms.

Salicylic acid, another substance tested in this study, prevents bacterial attachment to medical devices[27] and inhibits biofilm formation.[16,17] Specifically, salicylic acid–based polymers interfere with *Salmonella enterica* biofilm formation.[28] In a study using mixed biofilms, salicylic acid specifically inhibited *S. aureus*, consequently increasing the ratios of *Pseudomonas aeruginosa* and *E. faecalis* within the same biofilm.[23] This indicates that salicylic acid preferentially affects certain species, which may help explain its poor results against the three strains (two species) tested in the present study. Another possible explanation for our observed poor performance of salicylic acid is that, in most other studies, the compound was applied prior to biofilm formation whereas we evaluated its effects on established biofilms.

The crystal violet assay used in this study remains among the most frequently used assays for investigating biofilm formation or testing the effects of substances on biofilm biomass.[18] Crystal violet is a basic dye that not only stains bacterial cells but also binds to negatively charged surface molecules and polysaccharides in the biofilm extracellular matrix.[29] The main advantages of the method are its robust reproducibility and rapid analysis of biofilm reduction, permitting a screen of potential antibiofilm substances prior to performing labor-intensive confocal microscopic quantification. One limitation of the method is that there is no relationship between the reduction of biofilm biomass and the potential to kill biofilm bacteria.[30] Because crystal violet stains viable and dead cells and also the biofilm matrix, it cannot be used to specifically evaluate the killing of biofilm bacteria.[30] Thus, even though the crystal violet assay provides useful information on the efficacy of substances to remove biofilm remnants, oth-

er assays must be used to evaluate the ability to kill viable sessile bacterial cells. Taking this information into consideration, a poor result in the crystal violet assay does not necessarily mean that the substance has not killed the bacteria composing the biofilm.

Antimicrobial effectiveness is one of the most important properties required for endodontic irrigant solutions.[2] Other important properties for selecting an irrigant include tissue compatibility, substantivity to dentin, and soft-tissue dissolving ability, and therefore the substances tested in our study must be further evaluated with respect to these properties.

Conclusion

In conclusion, our findings demonstrate that the combination of farnesol, xylitol and lactoferrin significantly reduces the biomass of biofilms produced by two *E. faecalis* strains and one *S. epidermidis* strain. Therefore, a combination of these antibiofilm substances has the potential to be used in endodontic treatment to combat biofilms.

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