

A MODEL SYSTEM TO STUDY ANTIMICROBIAL STRATEGIES IN ENDODONTIC BIOFILMS

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ABSTRACT

The purpose of this work was to develop a model system to study antimicrobial strategies in endodontic biofilms. *Enterococcus faecalis* suspension was colonized in 10 human root canals. Five milliliters of Brain Heart Infusion (BHI) were mixed with 5 mL of the bacterial inoculum (*E. faecalis*) and inoculated with sufficient volume to fill the root canal during 60 days. This procedure was repeated every 72 h, always using 24-h pure culture prepared and adjusted to No. 1 MacFarland turbidity standard. Biofilm formation was analyzed by scanning electron microscopy (SEM). *E. faecalis* consistently adhered to collagen structure, colonized dentin surface, progressed towards the dentinal tubules and formed a biofilm. The proposed biofilm model seems to be viable for studies on antimicrobial strategies, and allows for a satisfactory colonization time of selected bacterial species with virulence and adherence properties.

Key words: Biofilm. Endodontic biofilm. *Enterococcus faecalis*. Intracanal medicaments. Antimicrobial strategies.

INTRODUCTION

Root canal is an extraordinary microenvironment for several microbial species to attach on dentin surface and form dense bacterial biofilms²⁰, which are prevalent on most wet surfaces and can cause environmental problems, representing a common cause of persistent infections^{2,30}.

The stages of structural organization of biofilm, the composition and activities of the colonizing microorganisms in various environments may be different, although the establishment of a micro-community on a surface seems to follow essentially the same series of developmental stages, including deposition of a conditioning film, adhesion and colonization of planktonic microorganisms in a polymeric matrix, co-adhesion of other organisms, and detachment of biofilm microorganisms into surroundings³⁰. The success of infected root canal treatment is dependent on inactivation of microorganisms present in biofilm and planktonic ambience. Root canal preparation with antimicrobial agents, like chemical irrigants and/or intracanal dressings, can contribute for endodontic microbiota reduction⁵.

Sundqvist and Fidgor²⁹ (2003) reported that "root canal infection is not a random event". Species that establish a persistent endodontic infection are selected by the

phenotypic traits that they share and that are suited to the modified environment. Some of these shared characteristics include the capacity to penetrate and invade dentin, a growth pattern of chains or cohesive filaments, resistance to antimicrobials used in endodontic treatment, as well as an ability to grow in mono-infections, to survive periods of starvation and to evade the host response²⁹.

E. faecalis has proved a potentially important microorganism to the colonization or overgrowth in endodontic infections, being the dominant microorganism in post-treatment apical periodontitis, and has often been isolated from the root canal in pure culture. However, it is usually also found together with other bacteria and yeasts. In mixed infections, *E. faecalis* is typically the dominant isolate. The pathogenicity of *E. faecalis* in endodontic infections is well documented^{28,29}. Several studies have been developed with different bacterial biofilm models to test the antimicrobial efficacy of endodontic medicaments^{4,6,9,27}. Investigations have shown either antimicrobial efficacy²⁷, or inefficacy^{5,9,20} of intracanal medicaments against bacterial biofilm. The planktonic or biofilm ambience can be of influence on antimicrobial efficacy^{5,20}.

*Bacteria growing on a surface display a novel phenotype*¹⁵. A consequence is an increased resistance to

antimicrobial agents that can result from restricted inhibitor penetration, slower bacterial growth rates, transfer of resistance genes, suboptimal environmental conditions for inhibitor activity, and the expression of a resistant phenotype¹⁸.

The large number of published studies on bacterial biofilm can certainly bring in its context contradictory positions on antimicrobial strategies, mainly due to the variability between the methodologies. Therefore, the purpose of this work was to develop a model system to study antimicrobial strategies in endodontic biofilms.

MATERIAL AND METHODS

Biofilm Model

Tooth Preparation

Fourteen extracted human mandibular premolars with intact cementum obtained from the tooth bank of the Brazilian Dental Research and Learning Center (CEPOBRAS, Goiânia, GO, Brazil) were selected for this study. The teeth were removed from storage in 0.2% thymol solution and were immersed in 5% sodium hypochlorite (NaOCl, Fitofarma, Goiânia, GO, Brazil) for 30 min to remove organic tissues. Following periapical radiographs, standard access cavities were prepared and the coronal root canal third was enlarged using sizes 2 and 3 Gates Glidden drills (Dentsply Maillefer, Ballaigues, Switzerland). The teeth were prepared up to a size 50 K-file (Dentsply Maillefer) 1 mm short of the apical foramen, using a crown-down preparation technique. During instrumentation, the root canals were irrigated with 3 mL of 1% NaOCl at each change of file. Thereafter, the crowns were removed and tooth length was standardized to 17 mm (from root apex to coronal border). Root canals were dried and filled with 17% EDTA (pH 7.2) for 3 min for smear layer removal. This management was repeated twice. Next, the teeth were autoclaved at 120°C for 30 min.

Biofilm Development

A reference strain of Gram-positive facultative anaerobic cocci (*E. faecalis*; ATCC 29212) obtained from the American Type Culture Collection was used for biofilm formation. The bacterial strain was inoculated in 7 mL of Brain Heart Infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 24 h. The experimental suspensions were prepared by cultivating the biological marker on the surface of Brain Heart Infusion agar (BHIA; Difco Laboratories), following the same incubation conditions. The bacterial cells were resuspended in saline to reach a final concentration of about 3×10^8 cells/mL, adjusted to No. 1 MacFarland turbidity standard.

In the experimental model, a split platform was used during the period of inoculation with the biological indicator. The coronal portion of the root canal of each tooth was connected to the cut end of a 1.5 mL polypropylene Eppendorf tube (Cral, São Paulo, SP, Brazil) using a cyanoacrylate adhesive (Super Bonder, Itapevi, SP, Brazil) and epoxy resin (Durepoxi, São Paulo, SP, Brazil). The tooth-tube connections were entirely coated with two layers of nail polish (Max Factor, Cosmetics

and Fragrances, London, UK). The specimens (teeth plus polypropylene tubes) were sterilized in 5% NaOCl for 30 min and rinsed with sterile water for 30 min. The specimens were placed into BHI broth and the test apparatus was incubated at 37°C for 24 h to ensure sterilization. No bacterial growth was observed after this period.

Five milliliters of sterile BHI broth were mixed with 5 mL of the bacterial inoculum containing *E. faecalis* and were inoculated using sterilized syringes of sufficient volume to fill the root canal during a 60-day period. This procedure was repeated every 72 h, always using 24-hour pure culture prepared and adjusted to No. 1 MacFarland turbidity standard. The teeth were maintained in a humid environment at 37°C.

At 60 days, each tooth was removed from its apparatus under aseptic conditions and irrigation was done with 5 mL of sterile distilled water with sterile syringe. The root canals were dried and refilled with sterile distilled water. Thereafter, sterile paper points (Tanari, Tanariman Ind. Ltda., Manacaru, AM, Brazil; size 45) were introduced into the root canals and maintained for 3 min for sample collection. Each sample was collected using three paper points, which were individually transported and immersed in 7 mL of Lethen Broth (LB; Difco Laboratories), followed by incubation at 37°C for 48 h in a reduced oxygen atmosphere.

Microbial growth was analyzed by turbidity of the culture medium. Thus, after assessing LB changes, inoculums of 0.1 mL obtained from the medium was transferred to 7 mL of BHI, and subsequently incubated at 37°C for 48 h. Microbial growth was also checked by turbidity of the culture medium. Gram staining of BHI cultures was used for verification of contamination by *E. faecalis*. The negative control was used to test sterility and the positive control was used to check bacterial viability throughout the experiment. Therefore, during the 60-day period of contamination of the root canals, 2 non-inoculated teeth were kept incubated at 37°C, as an aseptic control, and 2 teeth were inoculated with *E. faecalis*, incubated and analyzed under identical conditions.

Preparation for SEM Analysis

Ten teeth were submitted to metallographic preparation for SEM analysis (JED – JSM -6360LV, Tokyo, Japan), Federal University of Paraná, Curitiba, PR, Brazil at x1,500 and 3,000 magnifications to observe biofilm formation. Root canal length was measured and divided equally into coronal, middle and apical thirds, which were evaluated separately. Two SEM micrographs were obtained from each third, totalizing 60 SEM micrographs. Longitudinal grooves were made along the entire length of each root sectioned and then split with a hammer and chisel.

The images were then analyzed for the presence of *E. faecalis* biofilm on root dentin surface by three previously calibrated observers, using the following scores: 1. few areas covered by *E. faecalis* biofilm colonizing root dentin surface without invasion of dentinal tubules; 2. few areas covered by *E. faecalis* biofilm colonizing root dentin surface with invasion on dentinal tubules; 3. most areas covered by *E. faecalis* biofilm colonizing root dentin surface and invading dentinal tubules; 4. all areas covered by *E. faecalis* biofilm, no dentinal tubule

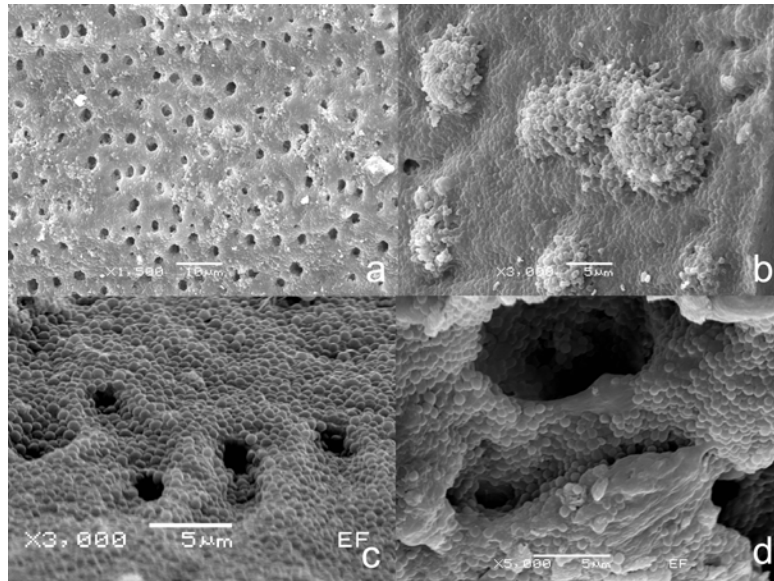


FIGURE 1- Scanning electron microscopy of (a) Various areas covered by *E. faecalis* biofilm on root dentinal surface (X1,500; Score #3). (b) Root dentinal surface completely covered by *E. faecalis* biofilm, and no dentinal tubule openings (X3,000; Score #4). (c) Evidence of root dentinal surface all covered by *E. faecalis* biofilm (X3,000; Score #4). (d) Bacterial penetration in dentinal tubules (X5,000)

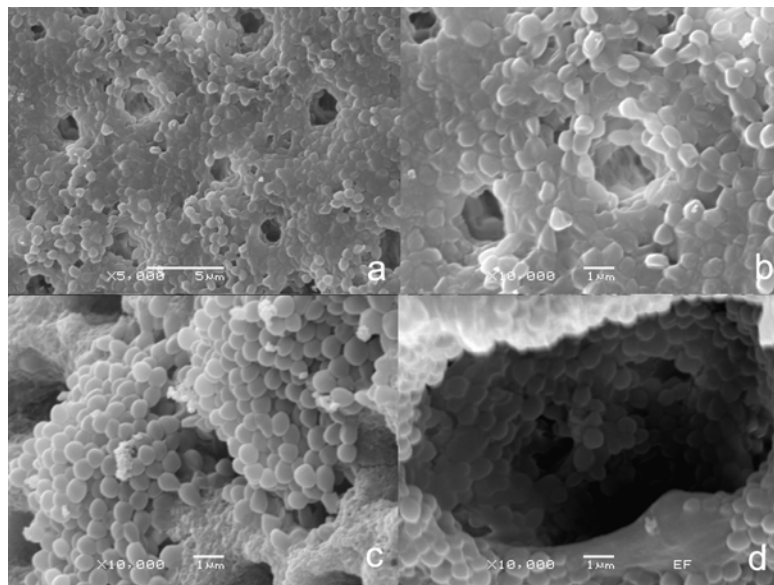


FIGURE 2- Scanning electron microscopy of (a) Root dentinal surface covered by *E. faecalis* biofilm (X5,000). (b) Magnification area of previous picture (X10,000). (c,d) Aggregated bacterial cells on and in dentinal tubules (X10,000)

openings.

Statistical analyses of data were performed by Kruskal-Wallis test, to assess significant differences among thirds, and Mann-Whitney U-test, to verify significant differences between the x1,500 and 3,000 magnifications. The level of significance was 5%.

RESULTS

The analysis of the SEM micrographs showed that the *E. faecalis* biofilm model is a promising model to study the

antimicrobial potential of endodontic medicaments. There was no statistically significant difference comparing image magnifications ($p > 0.05$). The coronal and middle thirds showed significantly more areas covered by *E. faecalis* than the apical third ($p < 0.05$). Figures 1 and 2 show *E. faecalis* biofilm formed on root dentin surface.

DISCUSSION

Bacteria elaborate a variety of different surface structure appendages that are involved in host colonization. They mediate

bacterial adhesion to host surfaces, promote invasion of host cells, allow bacterial cell-cell contact and communication, or protect bacteria from host immune defenses¹⁵.

Numerous biofilm models have been described for the study of intracanal medicaments^{6,9,22,24}. The experimental models involve infected human teeth *in vivo*^{1,20}, infected human teeth *in ex vivo*^{5,6,24}, infected dog's teeth *in vivo*¹⁰, infected bovine teeth *ex vivo*^{9,22}, biofilm model in membrane filters^{8,27}. However, there is limited number of biofilm models with validated studies of antimicrobial strategies. Considering a logical analysis of the facts, three aspects must be considered to discuss a biofilm model to study antimicrobial strategies on endodontic biofilm: the bacterial colonization structure, the biological indicator, and the time necessary for biofilm formation.

The biofilm study model that reflects the real environment is an adequate human root canal with variations and anatomic complexity^{6,24}. The invasion of root dentinal tubules by root canal bacteria is a multi-factorial event and a limited number of oral bacterial species have the necessary properties to participate in¹⁷. The penetration of microorganisms in infected root dentin has shown variations in the experimental model, the biological indicator and the time of incubation employed in the studies^{7,9,24}. The development and modification of *E. faecalis* biofilm on root canal and its penetration into the dentinal tubules have been associated with the prevailing environmental conditions⁷. The bacterial colonization structure on dentinal collagen constitutes the natural biological representation. Dentinal tubules contain a considerable amount of unmineralized collagen³, and type I collagen serves as an adhesion substrate to oral streptococci¹⁶. Certainly, bacterial adhesion and plaque formation on artificial surfaces and natural oral hard tissues are different.

The biofilm produced by membrane filter has been employed in some investigations^{8,27}. This model may not provide an accurate representation of the antimicrobial efficacy of the tested substance, but has some merits, to which may also be added the lack of need for extracted teeth and time-consuming preparation²⁷.

E. faecalis has been used as a biological marker in great part of endodontic studies and its importance for endodontic research is well documented^{4,7,8,12,14,19,29}. *E. faecalis* is a Gram-positive coccus, facultative anaerobe, present in human gastrointestinal infections and common in secondary apical periodontitis. Enterococci are variably classified as the second to third most common organisms found in hospital-acquired infections, and 85-95% of these isolates are *E. faecalis*^{12,19}. The virulence of enterococci involves the need of adherence to host tissues, which encounter an environment vastly different during the process of tissue invasion, with higher redox potentials and limited essential nutrients. Infecting enterococci likely express genes favoring growth under these environmental conditions and express factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, interfere with immunomodulation, and cause toxin-mediate damage¹².

The microbial flora of teeth with persistent apical periodontitis presents mainly simple species of Gram-positive organisms. *E. faecalis* represents the species most commonly recovered, and the overall healing rate of re-treatment was

74%²⁹. Adhesion to the dentin surface is an essential step that determines the pathogenic potential of *E. faecalis*. The influence of *E. faecalis* proteases and collagen-binding protein have been studied^{11-14,21,23}. Collagen-binding protein, serine protein, Ace and possibly gelatinase are potential virulence factors that give *E. faecalis* the capacity to bind to dentin¹¹. Kristich, et al.¹⁴ (2004) used independent experimental approaches to characterize biofilm formation by *E. faecalis*, which forms robust biofilms and its development is modulated by the prevailing environmental conditions. Neither the *esp* gene product nor the remainder of the genes encoded by the known *E. faecalis* pathogenicity island is required for the development of biofilms with a complex architecture. A secreted metalloprotease, GelE, enhances biofilm formation of *E. faecalis* but, otherwise, the genetic determinants controlling biofilm development remain unknown. Enterococcal lipoteichoic acid may serve as a virulence factor by modulating inflammatory responses and through facilitation of plasmid transfer¹². The prevalence, virulence, phenotype and genotype of oral and endodontic enterococci have recently been studied^{25,26}. Phenotypic and genotypic evidence of potential virulence factors were identified in endodontic Enterococcus spp., specifically production of gelatinase and response to pheromones.

It has been postulated that *E. faecalis* cells maintain the capability to invade dentinal tubules and adhere to collagen in the presence of human serum¹⁷. This bacterium persists resistant even in critical environments where nutrients are reduced and in the presence of antimicrobial agents like calcium hydroxide, sodium hypochlorite, chlorhexidine, ozone, etc.^{1,5,6,9,29}. The use of *E. faecalis* as a biological indicator in several studies is well documented. Bacterial colonization and invasion of surface and root dentinal tubules add important factors, like nutrient supply and time for colonization.

The ability of *E. faecalis* to invade dentinal tubule has been reported^{9,22,24}. The establishment of the ideal time to contaminate dentin surface and root canal deep layers to form the bacterial biofilm is uncertain. Unquestionably, in an adequate microenvironment, the bacterial biofilm will have a better structural organization within a longer period. In several studies, the time taken for biofilm formation to occur showed varied considerably (15 min to 60 days)^{5,7,9,22,24,27}. *E. faecalis* has shown its important role in endodontic infections, with particular strategies to form biofilm, substantial virulence factors^{11-14,19,21,23,25,29}, adherence capacity to dentin collagen^{11,13,14,17}, survival in critical environment³, and resistance to endodontic therapy^{15,20,28}.

Haapasalo and Ørstavik⁹ (1987), employing an *in vitro* model for dentinal tubule infection using bovine teeth, evaluated antimicrobial effect of camphorated paramonochlorophenol (CMCP) and a calcium hydroxide compound (Calasept®) on tubule infection by *E. faecalis* within 28 days. Liquid CMCP proved highly effective inside the tubules, while Calasept® was quite ineffective. Estrela, et al.⁵ (2007) determined the antimicrobial efficacy of ozonated water, gaseous ozone, sodium hypochlorite and chlorhexidine in endodontic biofilm developed on human teeth within 60 days. The antimicrobial medicaments were not sufficient to inactivate *E. faecalis*. Based

on this investigations^{5,9} and analyzing various biofilm models^{5,6,9,22,27}, the purpose of this investigation was to review biofilm models and to develop a model system to study antimicrobial strategies.

CONCLUSION

Problem solving in endodontic biofilm formation involves the establishment of a standard model that compares the antimicrobial efficacy of endodontic materials under several conditions. A model of mature *E. faecalis* biofilm on human root dentin with 60 days to develop under low oxygen and nutrient-rich environment was suggested in the present study. The proposed biofilm model seems to be viable for studies on antimicrobial strategies, and allows for a satisfactory colonization time of the selected bacterial species with virulence and adherence properties.

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