Comparative Analysis of Enterococcus faecalis Biofilm Formation on Different Substrates

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Abstract

Introduction: The aim of this study was to compare Enterococcus faecalis biofilm formation on different substrates. Methods: Cell culture plates containing growth medium and E. faecalis (ATCC 29212) were used to grow biofilm on bovine dentin, gutta-percha, hydroxyapatite, or bovine bone. Substrates were incubated at 37°C for 14 or 21 days, and the medium was changed every 48 hours. After the growth induction periods, specimens (n = 5 per group and per induction period) were stained by using Live/Dead, and the images were analyzed under a confocal microscope. The total biovolume (μm³), live bacteria biovolume (μm³), and substrate coverage (%) were quantified by using the BioImage_L software. Results obtained were analyzed by nonparametric tests (P = .05). Results: Biofilm formation was observed in all groups. Gutta-percha had the lowest total biovolume at 14 days (P < .05) and hydroxyapatite the highest at 21 days (P < .05). No significant difference was observed in green biovolume at 14 days. At 21 days, however, hydroxyapatite had the highest volume (P < .05). The percentages of coverage were similar among all substrates at 21 days (P > .05), but at 14 days, bovine bone presented the highest coverage (P < .05). Conclusions: E. faecalis was capable of forming biofilm on all substrates during both growth periods; hydroxyapatite presented the highest rates of biofilm formation. The type of substrate influenced the biofilm characteristics, according to the parameters evaluated. (J Endod 2013;39:346–350)

Key Words

Biofilm, confocal microscopy, Enterococcus faecalis

Microorganisms and their products are etiologic factors of pulp and periapical disease (1). Enterococcus faecalis are facultative anaerobic gram-positive bacteria frequently isolated from root canals in cases of endodontic treatment failure (2–4). Resistant endodontic microbiota includes bacteria capable of adhesion and biofilm formation. The ability of a microorganism to form a biofilm depends on the nature and surface of the substrate, because microbial interaction occurs at this interface. Biofilm formation includes several steps, namely bacterial attachment, formation of microcolonies, and microbial growth. The substrate surface determines the composition of the pellicle and microbial attachment (5).

Several microorganisms are recognized for their ability to adhere, colonize, and form biofilms on the surface of root canal dentin and apical cementum (6, 7). E. faecalis, Streptococcus mutans, Streptococcus sanguinis, Candida albicans, and Prevotella nigrescens are able to adhere to gutta-percha or endodontic sealers (8), allowing biofilm formation (8–11).

Hydroxyapatite and bovine dentin are among the substrates more commonly used for analyses of biofilm formation (12–16). Biofilms grown on hydroxyapatite present regular morphology and considerable adherence to the substrate and are resistant to displacement during laboratory procedures. However, considering that the characteristics of the dentin surface influence the formation and elimination of biofilms (17, 18), the use of hydroxyapatite for antimicrobial testing may present limitations, and other substrates should be taken into consideration.

Development of E. faecalis biofilm has been commonly evaluated by scanning electron microscopy (8, 10, 11), but this method does not provide quantitative information regarding the structural architecture of the biofilms. On the other hand, confocal laser scanning microscopy has been used (9, 15) for analysis of multiple sections of the biofilm at different focal planes, with ease of observation and preparation of samples. Use of reactive dye kits, such as Live/Dead (Molecular Probes, Inc, Eugene, OR), and of imaging software allow identification and quantification of viable and nonviable cells (12, 15, 19).

The goal of the present study was to evaluate the influence of different substrates—bovine dentin, gutta-percha, hydroxyapatite, and bovine bone—on the development of E. faecalis biofilm during 2 evaluation periods.

Materials and Methods

Sample Preparation

Dentin blocks were obtained from bovine central incisors with fully developed roots. After removal of the crowns at the cementoenamel junction, dentin segments measuring 5 mm × 5 mm × 0.7 mm (width × length × thickness) were made with the aid of a diamond disk attached to a low-speed saw (Isomet; Buehler, Lake Bluff, IL) under abundant irrigation. The blocks obtained were kept in a test tube containing distilled water and sterilized by autoclaving at 121°C for 20 minutes.

Gutta-percha disks were fabricated from conventional gutta-percha cones (Dentsply-Maillefer, Petrópolis, RJ, Brazil). The cones were placed in water at 70°C for 60 seconds in a laboratory water bath (Righetto e Cia, Campinas, SP, Brazil). Then the heated material was placed in a metallic mold measuring 10 mm × 1.5 mm (internal diameter × thickness) and compressed between 2 glass slabs under controlled and
constant pressure of 0.5 N for 1 minute. Specimens were stored at room temperature (25°C–27°C) for 24 hours. Disks were sterilized by ultraviolet light in a laminar flow hood for 30 minutes per side. Hydroxyapatite disks (Bioengineering, Biomaterials, and Biological Mineralization Laboratory, Federal Fluminense University, Niterói, RJ, Brazil) measuring 10 mm × 1 mm (diameter × thickness) were also sterilized by UV light in a laminar flow hood for 30 minutes per side.

Bone disks measuring 10 mm × 5 mm (diameter × thickness) were obtained from bovine mandibles. The buccal cortical bone was perforated with a trephine bur (260-801-810; Bicon Dental Implants, Boston, MA), attached to a contra-angle under constant irrigation. The resulting bone cylinder was sliced with a diamond disk at low speed (Isomet) under abundant irrigation. The disks obtained were placed in a test tube containing distilled water and sterilized by autoclaving at 121°C for 20 minutes.

**Biofilm Growth**

The microbiological procedures and manipulation of the substrates were conducted under aseptic conditions in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil). A standard strain of *E. faecalis* (ATCC 29212) was used for biofilm formation. After confirmation of the strain purity by Gram staining and colony morphology, cells were grown in 4.0 mL sterile brain-heart infusion (BHI) broth (Difco, Detroit, MI) overnight at 37°C. Cell density was 3.2 × 10^7 colony forming units per milliliter.

All substrate samples had one of the surfaces identified with a pencil. The marked surface was placed toward the surface of the plate, and the other side was used for biofilm growth. The substrate samples were placed in 24-well culture plates. Each well contained 1.8 mL sterile BHI medium and 0.2 mL inoculum, in which the samples were kept submerged. The culture plates were placed in an anaerobic culture chamber and kept in an oven (model Q816M2; Quimis Aparelhos Científicos Ltda, Diadema, SP, Brazil) at 37°C for 14 or 21 days under agitation. To prevent nutrient deficiency, the BHI culture medium was completely replaced every 48 hours, without addition of new microorganisms.

**Biofilm Verification by Confocal Microscopy**

After the growth induction periods, substrate samples were washed twice in saline to remove traces of culture medium and nonadherent cells. Next, specimens were placed on a glass slab, and the biofilm layer was stained with 50 μL Live/Dead reagent (BacLight Bacterial Viability Kit L7012; Molecular Probes, Inc). Then, they were incubated at room temperature for 10 minutes before analysis under a confocal microscope.

The Live/Dead BacLight reagent stains the viable cells in green and those with damaged membranes in red. The reagent was prepared immediately before use and protected from light and heat throughout the procedures. Each sample was processed and analyzed individually. All the substrates were examined under a confocal laser scanning microscope (Leica TCS-SPE; Leica Microsystems GmbH, Mannheim, Germany) at ×40 magnification. Five specimens were assigned for each group, and 4 fields were observed per specimen, totaling 20 evaluations per group per period. The image selected for each specimen was divided into 4 fields by using the analysis software. The determined fields in equidistant points were assessed by using a confocal laser scanning microscope. Evaluations were conducted by a calibrated examiner blinded to the groups. Images were captured at 1.0-μm intervals with resolution of 512 × 512 pixels by using the Leica Application Suite-Advanced Fluorescence software (LAS AF; Leica Microsystems GmbH). Each image was representative of a 275 × 275 μm^2 field.

Images were then transferred to the BioImage_L Software (20). The biofilm analysis tool was used to evaluate the 4 fields of each sample. At the end of the process, an Excel (Microsoft Corp, Redmond, WA) spreadsheet presented data identified as biovolume-stack and substratum. The results for each group yielded a single mean, representative of the 20 fields. The variables studied were total biovolume (μm^3), green biovolume (μm^3), and substrate coverage (%). The total biovolume refers to the volume occupied by all cells in the biofilm, whereas the green biovolume indicates the volume occupied by live cells only.

Data were not normally distributed according to Kolmogorov-Smirnov normality test. Therefore, nonparametric tests were used. Kruskal-Wallis tests with Dunn post hoc tests were used to compare substrates, and Mann-Whitney test was used to compare periods. Analyses were performed by the GraphPad Prism version 5.0 for Macintosh (San Diego, CA), with the significance level set at *P < 0.05*.

**Results**

Analysis of the substrates revealed that bacterial adhesion with subsequent biofilm formation occurred in all groups. Figure 1 represents images obtained from the biofilms on different substrates during the 2 induction periods. Figure 2 is a 3-dimensional reconstruction of the biofilm shown in Figure 1A (biofilm in bovine dentin at 14 days). The image,

![Figure 1](image_url)
acquired by means of the OsiriX v.3.9.4 software (Pixmeo, Geneva, Switzerland), shows presence of microorganisms throughout the entire extension of the substrate, with marked predominance of live cells. The mean, median, highest, and lowest values for the variables total biovolume (μm³), green biovolume (μm³), and substrate coverage (%) for each group are shown in Tables 1, 2, and 3, respectively.

After 14 days of induction, the greatest total biovolume was observed in hydroxyapatite, despite lacking significant difference from the remaining substrates (P > .05). Gutta-percha had the smallest total biovolume, with significant difference from bovine bone (P < .05). After 21 days of induction, hydroxyapatite presented the greatest total biovolume with significant difference (P < .05) from gutta-percha, which had the lowest median. The Mann-Whitney test did not reveal significant difference (P > .05) between days 14 and 21, regardless of the substrate.

When only the live cells (green biovolume) were considered, the hydroxyapatite samples had the greatest values, but no significant difference was observed among the substrates at 14 days (P > .05). At 21 days, significant difference was detected between hydroxyapatite and gutta-percha (P < .05). None of the substrates showed statistical difference between the induction periods (P > .05).

The substrate with the greatest percentage of surface coverage at 14 days was bovine bone, with significant statistical difference from gutta-percha (P < .05). At 21 days, the highest value was found for hydroxyapatite, despite lacking significant difference from the other substrates (P > .05). Comparison between the 2 induction periods revealed that only gutta-percha presented significant increase between 14 and 21 days (P < .05).

### Discussion

Development of biofilms in vitro has been conducted by using several different types of substrate: human dentin (21, 22), bovine dentin (16), bovine dental enamel (23), silicone disks (24), cellulose acetate membranes (25), and hydroxyapatite (14, 15). Substrates with complex anatomic configuration lead to formation of irregular biofilm, alternating thicker areas with others that contain sparse bacterial cells.

Ideally, in vitro studies should simulate in vivo conditions. Therefore, for studies related to the root canal system, the ideal substrate is dentin from extracted human teeth. Nevertheless, because of being readily available and their similarities to human teeth, enamel and dentin from bovine teeth have often been used as substrates (12, 13, 23).

Hydroxyapatite has been evaluated as a model because of its recognized ability to form regularly distributed biofilm throughout the majority of its surface (14, 15, 26). On the other hand, the literature does not mention induction of biofilm development on bovine bone. Considering that bovine bone contains hydroxyapatite and is readily available, in the present study we decided to use it as an experimental model.

Gutta-percha is the most widely used solid core obturating material in endodontics. Because of its lack of antimicrobial effect, substances such as iodoform, chlorhexidine, and calcium hydroxide have been incorporated into its composition (27). In this study, it was possible to observe development of biofilm on the surface of conventional gutta-percha. At 14 days, this material presented the smallest total biovolume, with significant difference compared with bovine bone. This may be a result of the presence of zinc oxide, which has slight antibacterial action, in the formulation of gutta-percha. However, this effect was not strong enough to prevent bacterial adhesion.

The biofilm formed on the different substrates evaluated had significant differences in all 3 variables. Hydroxyapatite presented the greatest total and green biovolume in both induction periods. Conversely, gutta-percha had the lowest values for these 2 variables. The other 2 substrates evaluated, bovine dentin and bovine bone, presented intermediate results.

Development of biofilm in vitro allows evaluation of the antimicrobial action of endodontic cements, irrigating solutions, and intracanal dressings against microorganism colonies, as opposed to planktonic cells. The choice of substrate should take into consideration factors inherent to the studies themselves in addition to the clinical implications of the results obtained. Deng et al (18) evaluated the effect of the substrate on the pH and susceptibility of S. mutans biofilms to 0.2% chlorhexidine. The authors concluded that the type of substrate affected not only the metabolic activity of the biofilm but also its susceptibility to the antimicrobial agent. Portenier et al (17) assessed the antibacterial action of chlorhexidine with or without cetrimide and of MTAD against 2 E. faecalis strains in presence or absence of dentin or bovine serum albumin. Presence of dentin or bovine serum albumin significantly reduced the antibacterial action of these substances. With this in mind, studies comparing products for use in the root canal system should preferably be tested by using dentin as the substrate.

### TABLE 1. Mean, Median, Highest, and Lowest Values for Total Biovolume (μm³)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>14 days</th>
<th>21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lowest</td>
<td>Highest</td>
</tr>
<tr>
<td>Dentin</td>
<td>1.31 × 10⁵</td>
<td>8.95 × 10⁶</td>
</tr>
<tr>
<td>Gutta-percha</td>
<td>2.08 × 10⁴</td>
<td>1.92 × 10⁶</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>1.87 × 10⁴</td>
<td>1.60 × 10⁷</td>
</tr>
<tr>
<td>Bovine bone</td>
<td>4.55 × 10⁵</td>
<td>7.13 × 10⁵</td>
</tr>
</tbody>
</table>

Different uppercase letters indicate statistically significant difference (P < .05) in the same row. Different lowercase letters indicate statistically significant difference (P < .05) in the same column.
progressively more resistant to antibiotics as they mature (28). There-
P
Different lowercase letters indicate statistically significant difference (<P

TABLE 2. Mean, Median, Highest, and Lowest Values for Green Biovolume (µm^3)

<table>
<thead>
<tr>
<th></th>
<th>Dentin</th>
<th>Gutta-percha</th>
<th>Hydroxyapatite</th>
<th>Bovine bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowest</td>
<td>8.13 × 10^4</td>
<td>9.30 × 10^3</td>
<td>1.53 × 10^4</td>
<td>1.55 × 10^5</td>
</tr>
<tr>
<td>Highest</td>
<td>8.51 × 10^6</td>
<td>1.86 × 10^6</td>
<td>1.12 × 10^7</td>
<td>4.60 × 10^6</td>
</tr>
<tr>
<td>Median</td>
<td>4.73 × 10^5 Aa</td>
<td>2.30 × 10^5 Aa</td>
<td>1.56 × 10^6 Aa</td>
<td>6.46 × 10^2 Aa</td>
</tr>
<tr>
<td>Mean</td>
<td>1.14 × 10^6</td>
<td>4.69 × 10^5</td>
<td>2.33 × 10^6</td>
<td>1.40 × 10^6</td>
</tr>
<tr>
<td>21 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowest</td>
<td>1.68 × 10^4</td>
<td>3.26 × 10^3</td>
<td>1.45 × 10^5</td>
<td>4.44 × 10^4</td>
</tr>
<tr>
<td>Highest</td>
<td>4.50 × 10^6</td>
<td>6.97 × 10^6</td>
<td>1.19 × 10^7</td>
<td>3.61 × 10^6</td>
</tr>
<tr>
<td>Median</td>
<td>1.06 × 10^6 ABa</td>
<td>1.97 × 10^5 Aa</td>
<td>1.56 × 10^6 Ba</td>
<td>6.02 × 10^1 ABa</td>
</tr>
<tr>
<td>Mean</td>
<td>1.19 × 10^6</td>
<td>1.24 × 10^6</td>
<td>2.31 × 10^6</td>
<td>8.55 × 10^5</td>
</tr>
</tbody>
</table>

Different uppercase letters indicate statistically significant difference (< P < .05) in the same row. Different lowercase letters indicate statistically significant difference (< P < .05) in the same column.

One of the factors that determines biofilm resistance is its development stage (22). It has been demonstrated that biofilms become progressively more resistant to antibiotics as they mature (28). Therefore, mature biofilms display greater resistance to sodium hypochlorite (29). Previous studies have used different induction periods. Biofilms incubated for short periods of time may not display the resistance of a mature biofilm. The present study aimed not only to evaluate bacterial adhesion but also to characterize mature biofilms. For this reason, the biofilms were allowed to develop for extended periods of time (14 or 21 days), because E. faecalis biofilms are poorly structured during the first few days (30).

Biofilm formation studies can also be performed in situ, directly in the oral cavity of patients wearing removable orthodontic appliances containing the substrate of interest (12). Under favorable environmental conditions, microorganisms are able to form mature biofilm in less time. However, when using this induction model, the biofilm will be polymicrobial, making it difficult to study a specific target microorganism.

Biofilm development and adhesion do not occur in the same manner in different environments. The substrate surface and features of the cell surface may influence the strain’s adhesive properties (5). Abundance or lack of nutrients in the medium also affects the development of E. faecalis biofilms. In nutrient-rich environments, the biofilm presents regular morphology, whereas under nutrient-deprived conditions, the biofilm is irregular and sparse (8). In the present study, for both induction periods the culture medium was replaced every 48 hours to provide a constant source of nutrition and remove dead bacterial cells.

Identification and characterization of biofilms have been performed by using different methodologies. Scanning electron microscopy is widely used for detection of microorganisms within the root canal system (7, 31). The confocal microscope emits a light beam that diffuses through the biofilm, detecting its structure and generating a sequence of 2-dimensional images. These 2-dimensional images can be superimposed, resulting in 3-dimensional images. Fluorescent markers may be added to detect and identify the microorganisms. Moreover, markers can provide information regarding biochemical, physiological, and physicochemical aspects of the medium and to distinguish between gram-positive and gram-negative microorganisms (32).

The BioImage_L software is a user-friendly tool with simple commands that allow collection of relevant information about the biofilms, such as total volume, green volume, red biovolume, mean thickness, and percentage of substrate coverage. The software is able to detect the different colors (red and green) and independently process the subpopulations, determining the cell viability of the biofilm (20).

Analysis of the viable cell ratio is used to assess the action of irrigating solutions against biofilms (15, 26). However, when comparing ratios, groups with similar results may reveal distinct realities when the absolute values are analyzed. In the present study, the absolute data referring to the total volume and to the volume of green cells were used to ensure better representation of the amount of cells present in the biofilm. This demonstrates the importance of careful study planning, with selection of experimental conditions and comparison of the results with those obtained from previous studies on the same topic (5).

**Conclusions**

Considering the methodology used and the results obtained, we concluded that E. faecalis was able to develop biofilm on all the substrates tested during induction periods of 14 days and 21 days; hydroxyapatite was the substrate with the best conditions for biofilm development. The type of substrate influenced the characteristics of the biofilm, according to the parameters evaluated.

**Acknowledgments**

The authors thank Dr Luiz Chaves de Paz (University of Connecticut Health Center) for providing the BioImage_L software for analysis of the biofilms and Dr José Mauro Granjeiro from the Bioengineering, Biomaterials, and Biological Mineralization Laboratory at the Federal Fluminense University for providing the hydroxyapatite disks.

The authors deny any conflicts of interest related to this study.

**References**


**TABLE 3.** Mean, Median, Highest, and Lowest Values for Substrate Coverage (%)

<table>
<thead>
<tr>
<th></th>
<th>Dentin</th>
<th>Gutta-percha</th>
<th>Hydroxyapatite</th>
<th>Bovine bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowest</td>
<td>1.81</td>
<td>0.70</td>
<td>0.19</td>
<td>3.93</td>
</tr>
<tr>
<td>Highest</td>
<td>24.72</td>
<td>36.24</td>
<td>55.05</td>
<td>40.88</td>
</tr>
<tr>
<td>Median</td>
<td>5.60 ABa</td>
<td>4.01 Aa</td>
<td>15.95 ABa</td>
<td>19.78 Ba</td>
</tr>
<tr>
<td>Mean</td>
<td>8.34</td>
<td>7.28</td>
<td>17.34</td>
<td>20.38</td>
</tr>
<tr>
<td>21 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lowest</td>
<td>1.00</td>
<td>0.18</td>
<td>1.55</td>
<td>1.50</td>
</tr>
<tr>
<td>Highest</td>
<td>44.68</td>
<td>51.30</td>
<td>67.16</td>
<td>42.69</td>
</tr>
<tr>
<td>Median</td>
<td>7.61 Aa</td>
<td>12.64 Ab</td>
<td>19.49 Aa</td>
<td>11.40 Aa</td>
</tr>
<tr>
<td>Mean</td>
<td>12.66</td>
<td>16.63</td>
<td>23.91</td>
<td>15.41</td>
</tr>
</tbody>
</table>

Different uppercase letters indicate statistically significant difference (< P < .05) in the same row. Different lowercase letters indicate statistically significant difference (< P < .05) in the same column.
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