Analysis of *Bifidobacterium dentium* and *Fusobacterium nucleatum* biofilm formation on different substrates by confocal laser scanning microscopy

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ABSTRACT

Objective: This study aimed to evaluate the influence of different substrates (bovine dentin blocks and glass blocks) on biofilm development of *F. nucleatum* and *B. dentium* by confocal laser scanning microscopy (CLSM). **Methods:** A 24-well culture plate were used to induce biofilm on substrates with a strain of *F. nucleatum* ATCC 25586 and a strain of *B. dentium* ATCC 27534 during seven days. After the growth induction period, specimens were stained with the Live/Dead technique and analyzed by CLSM. Results obtained by CLSM were analyzed by the bioImage_L software. **Results:** All results were analyzed by a nonparametric test ($p < 0.05$). Biofilm formation occurred in all experimental groups. The total biovolume and the percentage of viable bacteria in biofilm of *B. dentium* on dentin and glass blocks did not show any statistic difference. Biovolume of viable bacteria did not show any difference between the substrates in biofilm of *F. nucleatum* on dentin and glass blocks. The total biovolume showed better results in biofilm formed on dentin blocks. *B. dentium* and *F. nucleatum* are capable of forming biofilm in all studied substrates. **Conclusion:** In the adopted methodology, the type of substrate influences the characteristics of the produced biofilm. Dentin blocks are more adequate to form biofilm of the microorganisms studied.

Keywords: Biofilm. Confocal microscopy. *Bifidobacterium dentium*. *Fusobacterium nucleatum*.

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Introduction

Microorganisms and their metabolic derivatives are the main etiologic factors of pulpal and periapical diseases.[1] *Bifidobacterium* is a genus of anaerobic gram-positive bacilli found in dental tubules of necrotic pulps[2] and it is also found as reminiscent microbiota after root canal treatment.[3] *Fusobacterium* is a genus of anaerobic fusiform gram-negative bacilli detected in 48% of microbiological cultures of infected teeth[4] and in 100% of root canals with primary lesion by molecular methods.[5] In infected root canals, bacteria are frequently found forming thick multi layer structures known as biofilms.[1,6] Biofilms are microbial communities attached to biotic or abiotic wet surfaces and they are involved by a matrix of polysaccharides.[7] The ability to form biofilm represents a common mechanism for the survival and virulence factor[7] of bacteria. Biofilm formation includes various stages, e.g., bacterial adhesion, microcolony formation and bacterial growth. The substrate surface determinates film composition and bacterial adhesion.[8]

Several microorganisms have the ability to adhere, colonize and form biofilm over dentin surfaces in root canals.[9,10] Various studies have evaluated biofilm development by confocal laser scanning microscopy (CLSM), analyzing multi-sessions of biofilm in different focal planes, with ease of preparation and based on sample observation.[11] Additionally, the efficiency of different irrigating solutions adopted in Dentistry has been proven using biofilm models induced over bovine dentin.[12,13] The use of staining that reveals viable and nonviable cells in biofilm, such as the Live/Dead technique, and the possibility of using software to analyze images obtained by CLSM, make this technique greatly applicable in studies on biofilm in Dentistry.[13,14,15]

This study aimed to evaluate the influence of different substrates (bovine dentin blocks and glass blocks) on the development of biofilm of *F. nucleatum* and *B. dentium* by means of CLSM.

Methods

Substrate preparation

Dentin blocks were obtained from bovine central incisors. After dental crown removal, small blocks measuring 4 mm x 4 mm x 2 mm were manufactured with a diamond blade (Isomet, Buehler, Lake Bluff, IL, USA) under copious irrigation. The dentin segments were treated with 1% sodium hypochlorite for 30 minutes and 17% EDTA for 5 minutes to eliminate organic residues and the potential presence of smear layer. After having their surfaces marked with a pencil, the dentin blocks were put in tubes filled with distilled water and autoclaved at 121 °C for 20 minutes.

Glass fragments were cut in 4 mm x 4 mm x 2 mm blocks and had their surfaces marked with nail polish. They were also autoclaved.

Biofilm formation

For biofilm formation, a strain of *Fusobacterium nucleatum subsp. nucleatum* (ATCC™ 25586™) and a strain of *Bifidobacterium dentium* (ATCC™ 27534™) were used. They were reactivated in liquid culture of pre-reduced Reinforced Clostridial Medium (RCM, Difco, USA), supplemented with hemin (5 µg/mL) and menadione (0.5 µg/mL), using Whitley A35 anaerobic workstation (Dom Whitley Scientific, Shipley, West Yorkshire, England) with an atmosphere of 80% N2, 10% H_2 and 10% CO_2 at 37 °C for 24 hours. The purity of strains was confirmed by Gram staining.

Inoculum standardization of strains was carried out with a spectrophotometer, resulting in a cell density of 3 x 10^8 colony-forming mL^{-1} units.

The substrates were placed in a 24-well culture plate to induce biofilm. Six dentin blocks and six glass blocks were used. Each block was placed in a well and each well was filled with 1 ml of standardized inoculum of each bacterium strain. The plate was placed in an anaerobic workstation for seven days, without renewal of the medium.

Biofilm analysis by confocal microscopy

After biofilm induction period, the substrates were washed with phosphate buffered saline (PBS) to remove residual culture media and non adherent cells. Subsequently, specimens were placed in Petri dishes and biofilm was stained with 50 µL of Live/Dead (Kit BacLight Bacterial Viability L7012, Molecular Probes, Eugene, OR, USA). After staining, the Petri

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dishes were sealed and wrapped in aluminum foil, so as to have stains diffused in the specimens, without light, at 37 °C for 20 minutes, according to the manufacturer's specifications.

The Live/Dead BacLight kit, which stains viable cells in green and cells with compromised membrane in red, was prepared immediately before use. During all procedures, the kit was protected from light and heat. Each sample was individually processed and analyzed.

All specimens were analyzed by confocal laser scanning microscopy (Leica TCS-SPE, Leica Microsystems GmbH, Mannheim, Germany) under 40X magnification. For each bacterial strain, six specimens were photographed, and from each specimen, four photographs were obtained: a total of 20 photographs were taken per biofilm. These images were captured by Leica Application Suite-Advanced Fluorescence software (LAS AF, Leica Microsystems, Mannheim, Germany).

Images were processed by bioImage_L software in order to analyze the structure of biofilm grown on dentin blocks, according to Chávez de Paz.[14]

Nonparametric Kruskal-Wallis test was used to analyze the groups tested, with a significance level of 5%.

Results

Confocal analysis

For both strains, analysis of the tested substrates showed adhesion and biofilm formation. Figure 1 discloses images of the formed biofilm on different substrates after seven days.

After the incubation period for biofilm formation, the highest volume of *F. nucleatum* biofilm was observed on dentin blocks, with statistical difference compared to the glass block. For the *B. dentium* strain, both substrates showed similar biofilm formation. For both strains, biofilm formation was similar on dentin blocks. Regarding the glass bock, biofilm formation showed to be statistically different, being a better substrate for *B. dentium* than for *F. nucleatum* (Fig 2).

When comparing the percentage of viable cells (stained in green), the results showed similarities on both tested substrates and on both strains.

Discussion

This study aimed to evaluate biofilm formation of a single species of *Bifidobacterium dentium* and *Fusobacterium nucleatum* in different substrates with a view to understanding biofilm structure better and determining the best substrate for its growth. *In vitro* biofilm development allows evaluation of antimicrobial activity of endodontic sealers, irrigation solutions and intracanal medication.[16] It also contributes to acquire greater knowledge of its mode of action and helps finding solutions for its elimination, since bacterial biofilm presents better resistance than bacteria in its planktonic form.[6]

Guerreiro-Tanomaru et al[19] evaluated *Enterococcus faecalis* biofilm formation in bovine dentin, gutta-percha, hydroxyapatite and bovine bone substrates. The best tested substrate was hydroxyapatite; however, *E. faecalis* was able to proliferate in all substrates.

To develop *in vitro* biofilm, different types of substrate have been used: human dentin, bovine dentin, hydroxyapatite, gutta-percha and cellulose membrane.[17,18,19] Ideally, these studies must simulate *in vivo* conditions. Therefore, for studies related to root canal systems, the ideal substrate is human dentin.[19] However, as it is easily found and resembles human teeth, bovine dentin has been often used as substrate.[12]

Glass surfaces have also been used in *in vitro* studies on biofilm formation.[20] Glass blocks were chosen because they are easily found and do not need Ethics Committee approval; therefore, it could be an alternative to prepare biofilm.

The results obtained by means of CLSM analysis showed that biofilm formation on bovine dentin was similar in both tested strains. When compared to formation on glass blocks, for *F. nucleatum*, dentin showed to be superior with a statistical difference, whereas for *B. dentium*, it showed to be similar. Therefore, glass blocks showed to be a better substrate for *B. dentium* than for *F. nucleatum* biofilm formation, in which it presented statistical difference. When related to cell viability, all substrates were similar in both strains.

Analysis with confocal laser scanning microscopy produces images with the aid of fluorescent

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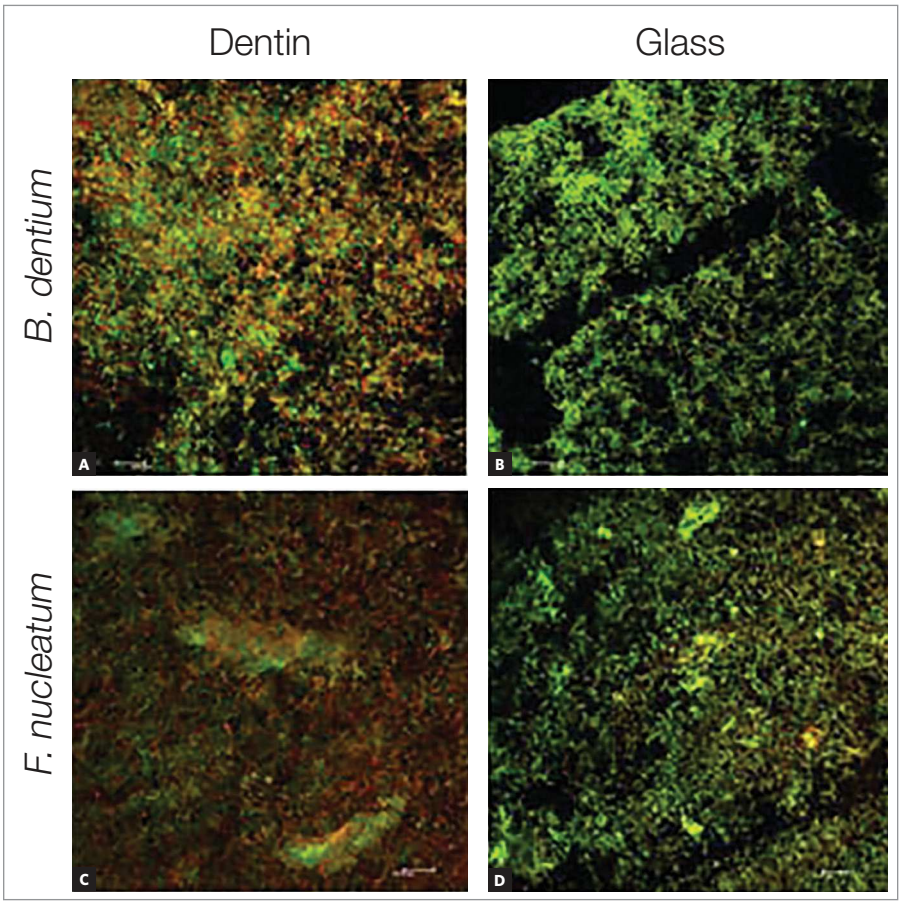

Figure 1. Biofilm formed on dentin and glass blocks: **A**) dentin, *B. dentium*; **B**) glass, *B. dentium*; **C**) dentin, *F. nucleatum*; **D**) glass, *F. nucleatum* (40x magnification).

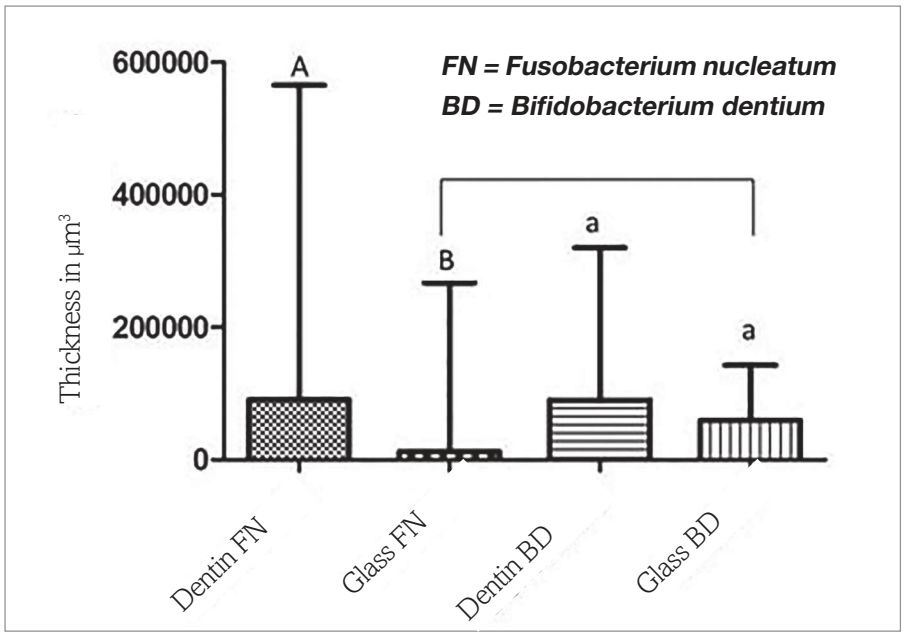

Figure 2. Chart representing thickness of biofilm formed on different substrates for the tested strains within seven days. Capital letters stand for statistical difference for *Fusobacterium nucleatum* (FN) in different substrates. Lowercase letters stand for similarity in biofilm formation of *Bifidobacterium dentium* (BD) in different substrates. Brackets represent statistical difference for biofilm formation in the same substrate of different strains.

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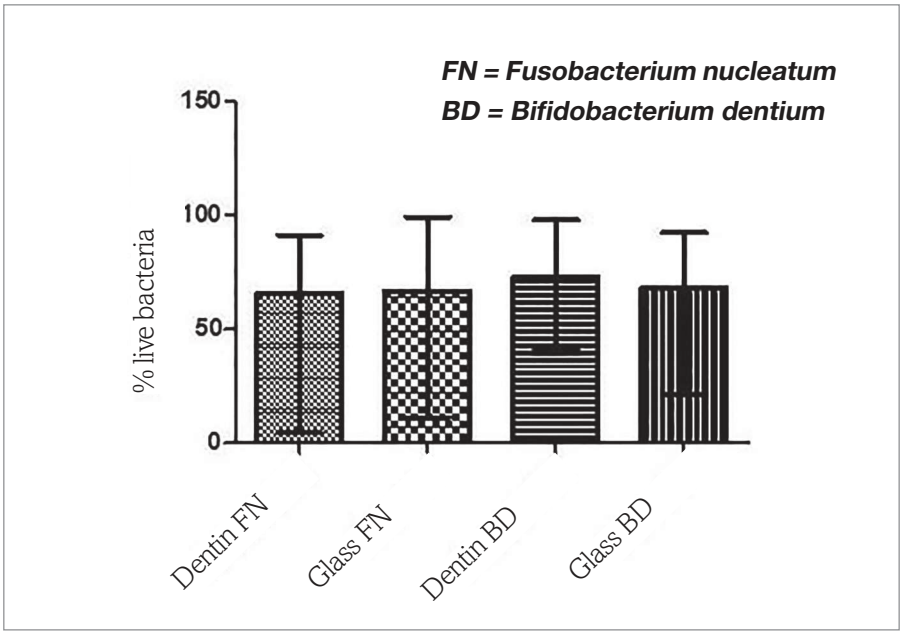

Figure 3. Chart representing the percentage of viable bacteria present in biofilm formed in different substrates for both strains.

Table 1. Median of viability and biovolume percentage (in μm^3) of biofilm formed in different substrates.

Strains	Substrates	Viable (%)	Biovolume μm^3
F. nucleatum	dentin	65.51	90.790
	glass	66.65	12.070
B. dentium	dentin	72.69	90.480
	glass	68.17	59.270

markers which detect the viability or nonviability of bacteria responsible for biofilm formation. Relevant information were acquired through image analysis with bioImage_L software: total biofilm volume, volume of alive (green) and dead (red) bacteria, thickness and the percentage cover of biofilm on the substrate.[14]

The genus *Bifidobacterium* has been extensively researched in the food area because of its beneficial effect as a probiotic bacterium in the intestine. However, *B. dentium* was reported as pathogenic when related to the dental cavity,[21] especially by producing and tolerating a highly acid environment.[22] *B. dentium* is a gram-positive anaerobic bacteria also found in root canals and dentinal tubules in teeth with necrotic pulps,[2,3] being part of the remaining microbiota of root canal after treatment.

Gram-positive bacilli are part of an important segment of endodontic microbiota, even after root canal filling.[23] *Bifidobacterium*, found in root canals and dentinal tubules in teeth with necrotic pulps, can be considered important in the transition of microbiota from dental cavities to pulp necrosis. Therefore, its resistance and pathogenicity should receive more attention and should be studied more extensively. Nevertheless, only a few species of this

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group of organisms have been recognized to be directly related to apical periodontitis.[24]

Fusobacterium nucleatum is a genus of gram-negative bacteria, strictly anaerobic, non-motile and non-spore-forming species, which is normally isolated from the oral cavity. Although there are up to seven known *Fusobacterium* species, *F. nucleatum* is one of the most common species isolated from human infections.[25] From the predominant species found in root canals, *F. nucleatum* has an elevated capacity to co-aggregate *in vitro* with many oral bacteria.[26]

Biofilm development and adhesion do not occur in the same way in different substrates. The substrate surface and the characteristics of the cell membrane can influence the adhesive properties of species. Similarly, availability or lack of nutrients in the media can influence its development.

The present study allows us to claim that glass blocks can be used as substitutes for bovine dentin in studies that aim to evaluate the antibiofilm activity of any material related to *B. dentium*. However, for *F. nucleatum*, bovine dentin is the best substrate to use in antimicrobial studies.

Within the methodology adopted, it is reasonable to claim that the type of substrate influences the characteristics of the biofilm formed, being the substrate of dentin blocks the most appropriate for biofilm formation of the used microorganisms.

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References

1. Ricucci D, Siqueira JF Jr. Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. J Endod. 2010 Aug;36(8):1277-88
2. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med. 2002;13(2):171-83.
3. Chávez de Paz LE, Molander A, Dahlén G. Gram-positive rods prevailing in teeth with apical periodontitis undergoing root canal treatment. Int Endod J. 2004 Sept;37(9):579-87.
4. Sundqvist G. Taxonomy, ecology, and pathogenicity of the root canal flora. Oral Surg Oral Med Oral Pathol. 1994 Oct;78(4):522-30.
5. Souza CA, Teles RP, Souto R, Chaves MA, Colombo AP. Endodontic therapy associated with calcium hydroxide as an intracanal dressing: microbiologic evaluation by the checkerboard DNA-DNA hybridization technique. J Endod. 2005 Feb;31(2):79-83.
6. Chávez de Paz LE. Redefining the persistent infection in root canals: possible role of biofilm communities. J Endod. 2007 Jun;33(6):652-62.
7. Mohammadi Z, Palazzi F, Giardino L, Shalavi S. Microbial biofilms in endodontic infections: an update review. Biomed J. 2013 Mar-Apr;36(2):59-70.
8. Hancock V, Witsø IL, Klemm P. Biofilm formation as a function of adhesin, growth medium, substratum and strain type. Int J Med Microbiol. 2011 Nov;301(7):570-6.
9. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med. 2004 Nov 1;15(6):348-81.
10. Leonardo MR, Rossi MA, Silva LA, Ito IY, Bonifácio KC. EM evaluation of bacterial biofilm and microorganisms on the apical external root surface of human teeth. J Endod. 2002 Dec;28(12):815-8.
11. Chávez de Paz LE. Development of a multispecies biofilm community by four root canal bacteria. J Endod. 2012 Mar;38(3):318-23.
12. Del Carpio-Perochena AE, Bramante CM, Duarte MA, Cavenago BC, Villas-Boas MH, Graeff MS, et al. Biofilm dissolution and cleaning ability of different irrigant solutions on intraorally infected dentin. J Endod. 2011 Aug;37(8):1134-8.
13. Ordinola-Zapata R, Bramante CM, Cavenago B, Graeff MS, Moraes IG, Marciano M, et al. Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. Int Endod J. 2012 Feb;45(2):162-8.
14. Chávez de Paz LE. Image analysis software based on color segmentation for characterization of viability and physiological activity of biofilms. Appl Environ Microbiol. 2009 Mar;75(6):1734-9.
15. Zapata RO, Bramante CM, Moraes IG, Bernardineli N, Gasparoto TH, Graeff MS, et al. Confocal laser scanning microscopy is appropriate to detect viability of Enterococcus faecalis in infected dentin. J Endod. 2008 Oct;34(10):1198-201.
16. Ordinola-Zapata R, Bramante CM, García-Godoy F, Moldauer BI, Gagliardi Minotti P, Tercília Grizzo L, et al. The effect of radiopacifiers agents on pH, calcium release, radiopacity, and antimicrobial properties of different calcium hydroxide dressings. Microsc Res Tech. 2015 Jul;78(7):620-5.
17. Norrington DW, Ruby J, Beck P, Eleazer PD. Observations of biofilm growth on human dentin and potential destruction after exposure to antibiotics. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008 Apr;105(4):526-9.
18. Müller P, Guggenheim B, Schmidlin PR. Efficacy of gasiform ozone and photodynamic therapy on a multispecies oral biofilm in vitro. Eur J Oral Sci. 2007 Feb;115(1):77-80.
19. Guerreiro-Tanomaru JM, Faria-Júnior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod. 2013 Mar;39(3):346-50.
20. Ohl SW, Iqbal K, Khoo BC, Neo J, Fawzy AS. High intensity focused ultrasound for Enterococcus faecalis biofilm. J Acoust Society Am. 2013;134(5):4182.
21. Ventura M, Turroni F, Zomer A, Foroni E, Giubellini V, Bottacini F, et al. The Bifidobacterium dentium Bd1 genome sequence reflects its genetic adaptation to the human oral cavity. PLoS Genet. 2009 Dec;5(12):e1000785.
22. Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol. 2005 Aug;33(4):248-55.
23. Molander A, Reit C, Dahlén G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. Int Endod J. 1998 Jan;31(1):1-7.
24. Happonen RP. Immunocytochemical diagnosis of cervicofacial actinomycosis with special emphasis on periapical inflammatory lesions. Proc Finn Dent Soc. 1986;82 Suppl 7-8:1-50.
25. Jacinto RC, Montagner F, Signoretti FG, Almeida GC, Gomes BP. Frequency, microbial interactions, and antimicrobial susceptibility of Fusobacterium nucleatum and Fusobacterium necrophorum isolated from primary endodontic infections. J Endod. 2008 Dec;34(12):1451-6.
26. Sundqvist G. Associations between microbial species in dental root canal infections. Oral Microbiol Immunol. 1992 Oct;7(5):257-62.

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