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A new model of *in vitro* dentin intratubular contamination for *Fusobacterium nucleatum*: Validation by confocal laser scanning microscopy

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ABSTRACT

Objectives: To test and validate a new protocol for *in vitro* contamination of dentinal tubules using *Fusobacterium nucleatum* (*F. nucleatum*) by confocal laser scanning microscopy (CLSM), in addition to evaluating the effectiveness of conventional endodontic irrigants such as sodium hypochlorite (NaOCl) and chlorhexidine (CLX) on this biofilm.

Material and methods: Thirty lower premolars were contaminated with *F. nucleatum* (ATCC 51190) for 7 days under anaerobic conditions using the proposed new model. The specimens were divided into a control group and experimental groups, according to the irrigants: NaOCl 2.5% and CLX 2%. Then, the samples were submitted for analysis by CLSM and the LIVE/DEAD technique to quantify bacterial viability. Data normality was verified by the Shapiro-Wilk test. Intragroup and intergroup comparisons were performed using the Kruskal-Wallis test, followed by Dunn's post-test.

Results: The CLSM images obtained demonstrated the effectiveness of the proposed new contamination protocol, with a high percentage of viable bacteria in relation to the treated groups ($p < 0.05$). Lower viability values were observed for the 2.5% NaOCl group.

Conclusion: The new contamination protocol resulted in a high and homogeneous percentage of viable bacteria in the dentinal tubules in all specimens evaluated. Both irrigating solutions proved to be effective in reducing the intratubular microbiota, especially 2.5% NaOCl.

1. Introduction

Dentin contamination is a consequence of pulp necrosis and the establishment of an intraradicular infection, where bacteria invade and colonize the root canal system (RCS) [1]. Thus, to achieve long-term success, endodontic treatment must promote the reduction of the microbial load to levels compatible with the healing of periapical tissues [2]. Although biofilms should ideally be eliminated through instrumentation, the high complexity of this system limits this action, requiring the use of chemical compounds that act to

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remove organic tissues, bacteria, and especially their virulence factors [3,4].

Bacterial invasion of dentinal tubules is directly related to the persistence of endodontic infection and consequently to treatment failure in many cases [2], so the reduction of microbial load in dentinal tubules and strict areas of the root canal has been the subject of numerous studies.

In this sense, several antimicrobial solutions have been used, including sodium hypochlorite (NaOCl), used worldwide due to its high tissue dissolution capacity and known antimicrobial activity [5], and chlorhexidine (CLX) which, in addition to the antimicrobial action associated with substantivity, has a low degree of toxicity [6]. As irrigants, these solutions show excellent performance in reducing the microbial load of infected root canals [7–10]. The most used mechanism for distributing these solutions is conventional irrigation, performed using small-caliber syringes and needles, which has limitations, especially in areas of anatomical complexity, and may not be able to produce the necessary shear stress. Therefore, to improve the effectiveness of irrigation, the use of irrigants with activation, whether sonic, ultrasonic, or subsonic, seems essential, allowing a greater reach of the irrigation solution in these areas of complexity, cleaning of the dentin walls, and promoting decontamination [11,12].

Analysis of the microbiota of infected and necrotic pulp canals reveals the predominance of strict anaerobic bacteria, especially Gram-negative ones [13–15], which have in their outer cell wall lipopolysaccharides (LPS) responsible for a series of biological effects, including induction of clinical symptoms and periapical bone resorption [16–18]. Among the Gram-negative bacteria, *Fusobacterium nucleatum* (*F. nucleatum*) stands out, a strict anaerobe isolated in 100% of cases of primary endodontic infection [13,19] and extremely important in the formation of biofilms, as it acts as a bridge between primary and secondary colonizers that cannot interact [20]. Furthermore, it is present in the microbiota of both asymptomatic and symptomatic cases, being the most predominant species in symptomatic cases [21,22].

For analysis and validation of antimicrobial tests in Endodontics, homogeneous contamination of dentinal tubules is an essential factor [23,24]. Although 90% of the microbiota of an infected root canal consists of obligate anaerobes bacteria [25], to date, most *in vitro* studies using the dentin infection model make use of facultative microorganisms, which do not reflect the composition of the microbiota of primary infections. Furthermore, there are no comparative studies in the literature on the effects of NaOCl and CLX on intratubular bacterial reduction using a model of contamination with a Gram-negative strict anaerobe.

The aim of the present study was to test and validate a new protocol for *in vitro* contamination of dentinal tubules, enabling the use of a strict anaerobe, such as *F. nucleatum*, using confocal laser scanning microscopy (CLSM) as assessment tool. The effectiveness of conventional endodontic irrigants such as NaOCl and CLX on this pathogen was also evaluated.

2. Material and methods

2.1. Selection of specimens

The study protocol was approved by the Human Research Ethics Committee, Bauru School of Dentistry (#39330620.0.0000.5417). Permanent human teeth extracted for periodontal reasons were obtained from the Department of Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry. Thirty newly extracted single-rooted lower premolars were selected through mesiodistal and buccolingual radiographs. The teeth had fully formed roots and single canals, with no root resorption or calcifications in the root canals. All experiments were performed in accordance with approved guidelines and regulations. Informed consent was obtained from each patient for donation of the teeth.

2.1.1. Specimen preparation

The teeth were decoronated and the apical 3 mm of each root was removed with a water-cooled double-sided diamond disk 0.10 × 22 mm (KG Sorensen, Cotia, SP, Brazil), standardizing the root length (≅ 15 mm). Working length (WL) was determined by subtracting 1 mm from this measurement [26]. To standardize the diameter of the canals (i.e., the inner diameter of the dentin cylinders), they were enlarged with the X-1 blue 40.06 instrument (MK Life, Porto Alegre, RS, Brazil), opening space for the inoculation of microorganisms. The specimens were submitted to successive ultrasonic baths of 10 min each with 1% NaOCl (Asfer, São Caetano do Sul, São Paulo, Brazil), 17% EDTA (Biodinamica, Ibiporã, PR, Brazil) and distilled water, and covered with red nail polish. (Colorama, Rio de Janeiro, RJ, Brazil) The apical region was sealed with Natural Look composite resin (DFL Industria e Comércio S.A. Taquaral, RJ, Brazil). Then, they were sterilized in an autoclave (Cristofoli, Campo Mourão, PR, Brazil) at 121 °C for 24 min. All subsequent procedures were performed in laminar flow (Grupo Veco, Campinas, SP, Brazil).

2.1.2. Dentin infection with Fusobacterium nucleatum

For intradentinal infection, a protocol already described was used [26] with some modifications. The specimens were placed in pyrogenic microtubes (Eppendorf, Hamburg, Germany) with 1 mL of sterilized RCM (Reinforced Clostridial Medium, Probac do Brasil, São Paulo, SP, Brazil) broth and subjected to an ultrasonic bath for 15 min for maximum penetration of the broth into the dentinal tubules before the bacterial contamination step. Contamination was carried out in seven days, with centrifugation on alternate days.

The bacterial strain of *F. nucleatum* ATCC 51190 (American Type Culture Collection) was reactivated in RCM broth and incubated in anaerobic chamber (Whitley A35 anaerobic workstation, Dow Whitley, Victoria Works, Victoria, United Kingdom) for 48 h. After this period the bacterial suspension was adjusted to a concentration of 3×10^8 colony forming units (CFU/mL) using a spectrophotometer SF325NM (Bel Photonics do Brasil Ltda, Osasco, SP, Brazil) and taken back to the anaerobic chamber at 37 °C for 24 h to achieve exponential growth (first day). This amount of time (24 h) was defined based on the *F. nucleatum* growth curve defined in a pilot study (Fig. 1).

Colonial morphology and purity were evaluated several times throughout the experiment using Gram stain. The flowchart of the new stablished protocol is presented in Fig. 2.

On the second day, 1 mL of the previously standardized inoculum was added to each microtube containing the dentin sample. The microtubes were sequentially centrifuged at 1400 g, 2000g, 3600 g, and 5600 g (one time each for 5 min at 25 °C). Between each centrifugation, the inoculum was renewed. After the four cycles, the contaminated solution was replaced by 1 mL of sterilized RCM broth, and then the samples were incubated under anaerobic conditions at 37 °C. The following day (third day) the contaminated medium was discarded, 1 mL of sterilized RCM broth was inserted into microtubes, followed by a centrifuge cycle of 3600 g for 5 min at 25 °C and the microtubes were incubated again in anaerobic conditions at 37 °C for 24 h.

On the fourth day, a new inoculum of exponentially growing *F. nucleatum* was standardized. 24 h later (fifth day) it was inserted into the microtubes with the specimen and the centrifugation protocol (once at each speed, at 25 °C) was repeated. On the sixth day, the same procedures described for the third day were performed. On the seventh day, the specimens were removed from the microtubes, submitted to antimicrobial treatment, and analyzed in the CLSM. Ten specimens were randomly selected as a positive control (C+) to confirm intratubular contamination.

All RCM broth from this contamination process was pre-reduced inside the anaerobic chamber for 24 h before utilization, in the same manner as all culture incubation for the intratubular multiplication of *F. nucleatum.*

2.2. Antimicrobial tests

The specimens were randomly divided into two experimental groups (n = 10) according to the irrigant used: G1, 2.5% NaOCl (Farmácia Specifica, Bauru, SP, Brazil) (3 min) and G2, 2% CLX (Farmácia Specifica, Bauru, SP, Brazil) (3 min). The irrigant was delivered through a syringe and needle positioned 2 mm short of the working length. 5 mL of the solution were used at a flow rate of 0.0277 mL/s. NaOCl and CLX were inactivated by adding 5% sodium thiosulfate and tween 80 plus lectin, respectively, for 3 min. Finally, all specimens were irrigated with 5 mL of sterilized distilled water.

2.3. Antimicrobial assay by confocal scanning laser microscopy (CLSM)

After exposure to the irrigation protocols, the specimens were longitudinally sectioned in Isomet (Buehler, IL, USA), treated with 17% EDTA for 3 min, and washed with sterile saline to remove the smear layer resulting from the cutting procedure, as previously reported [27,28]. The specimens were then stained for 15 min in the dark with 20 μL of the LIVE/DEAD™ BacLight bacterial viability kit (Invitrogen Molecular Probes, Eugene, OR, USA). This kit contains SYTO 9™ Green Stain, which stains viable bacteria, and Propidium Iodide Red Stain™, which stains dead bacteria. The samples were examined with a Leica TCS-SPE confocal microscope (Leica Microsystems GmbH, Mannheim, Germany) at 40× magnification, 1 μm step depth, and a resolution of 1024 × 1024 pixels. Eight sequential images of the specimens were obtained: 4 in the superficial portion and 4 in the deep portion, totaling 80 images of each group, including the positive controls. This allowed visualization of all extensions of contaminated dentinal tubules from the main root canal to the cementum. The acquired images were fragmented using Leica Application Suite-Advanced Fluorescence software (LAS AF, Leica Microsystems GmbH, Mannheim, Germany) and converted to TIFF format. These images were exported to the bioImage_L v21 software to quantify the percentage of viable (green stained) and non-viable (red stained) bacteria.

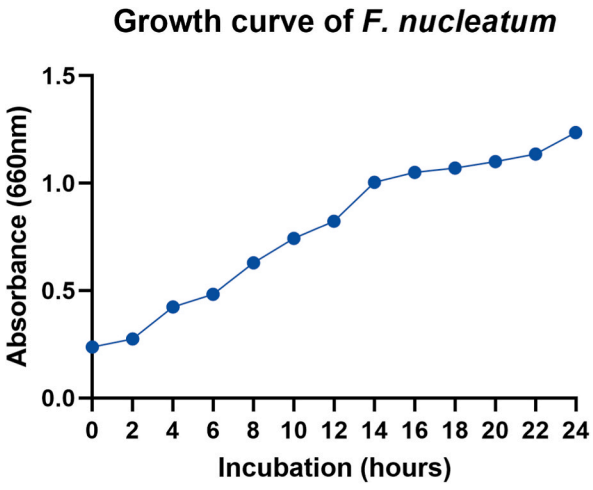

Fig. 1. Growth curve of *Fusobacterium nucleatum.*

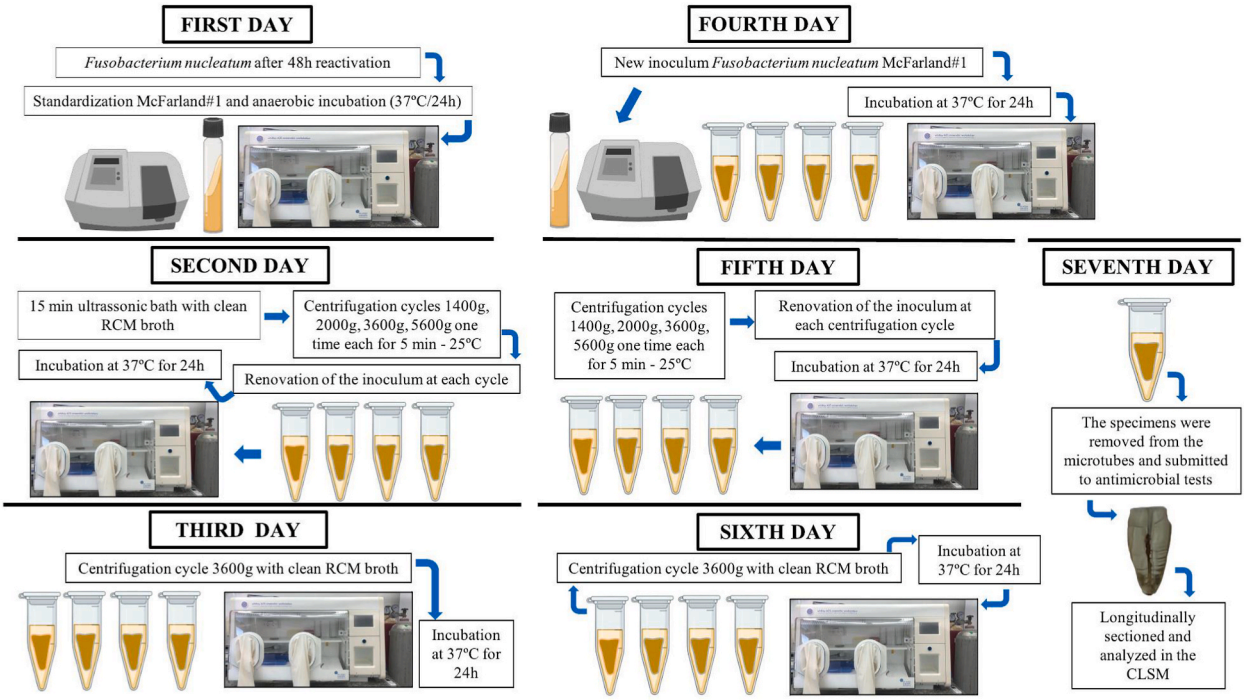

Fig. 2. Flowchart of the contamination protocol.

2.4. Statistical analysis

Data normality was evaluated using the Shapiro-Wilk test. Intragroup and intergroup comparisons were performed using the Kruskal-Wallis test, followed by Dunn's post-test. The GraphPad Prism 8.0 software (GraphPad San Diego, CA, USA) was used for the analysis, adopting a significance level of 5%.

3. Results

3.1. Intratubular contamination test (CLSM)

The CLSM images obtained from the positive control group (C+) demonstrated the effectiveness of the proposed new contamination protocol with a high percentage of viable bacteria, in addition to a dense and homogeneous contamination. The experimental groups showed lower viability values than the positive control group ($p < 0.05$). The comparison between the irrigants showed that in the total areas, superficial and deep, irrigation with 2.5% NaOCl produced a lower percentage of viable bacteria compared to the use of 2% CLX ($p < 0.05$). No significant differences were observed in intragroup comparisons regarding depths (superficial x deep) ($p > 0.05$) (Table 1). Representative confocal laser scanning microscopy images of the groups are shown in Fig. 3.

4. Discussion

This study showed a new method for intratubular contamination of dentin using a strict anaerobe, *Fusobacterium nucleatum*, in addition to evaluating the action of 2.5% NaOCl and 2% CLX on its biofilm. This was demonstrated by quantitatively comparing the percentage of live and dead bacteria within the dentinal tubules.

The selection criteria of this microorganism was based on its prevalence in primary and secondary endodontic infections, especially

Table 1
Median percentage (95% confidence interval) of viable bacteria in the dentinal tubules after the irrigation protocol for each group.

Groups	Total	Superficial	Deep
Control	75.50 (12.1–99.4)^{Aa}	73.66 (18.9–98.9)^{Aa}	70.00 (3.3–86.6)^{Aa}
2.5% NaOCl	9.72 (0.6–97.7)^{Ba}	6.62 (0.3–90.8)^{Ba}	8.95 (0.8–95.9)^{Ba}
2% CHX	35.01 (0.0–98.9)^{Ca}	28.66 (0.0–97.84)^{Aa}	40.24 (0.0–98.9)^{Ba}

Comparison by Kruskal-Wallis and Dunn tests ($p < 0.05$). Different superscript upper letters in a column represent significant differences between groups; different superscript lowercase letters on a line represent significant differences within groups.

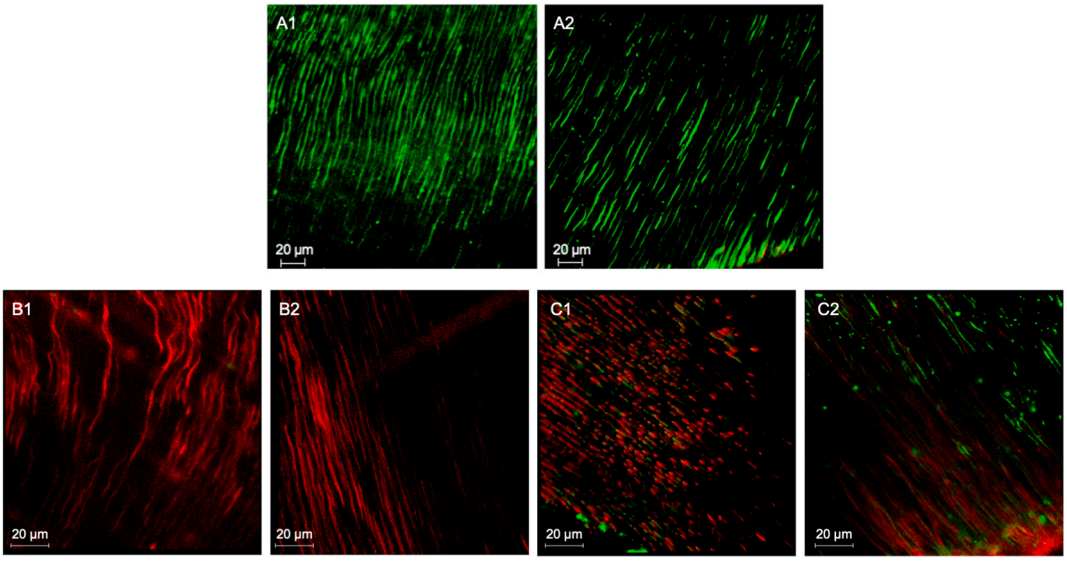

Fig. 3. Representative confocal laser scanning microscope images of (A) the control group, (B) 2.5% NaOCl–treated dentin and (C) 2% CHX–treated dentin. The image A1 corresponds to the superficial portion in relation to the main root canal of control group; A2 is the deep portion in relation to the main root canal of control group; B1 is the superficial portion of 2.5% NaOCl–treated group; B2 is the deep portion of 2.5% NaOCl–treated group; C1 is the superficial portion of 2% CHX–treated group and C2 is the deep portion of 2% CHX–treated group. Viable bacteria are indicated in green and non-viable bacteria are indicated in red. Magnification: 40×. Bars: 20.0 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in symptomatic cases [22], toxicity of its LPS [29,30], capacity of penetration in the entire thickness of dentin in the cervical, middle and apical regions [31], in addition to its role in reducing oxygen levels favoring the growth of anaerobic and more pathogenic bacteria [32] and acting as a bridge between several species [20]. Finally, although a monospecies biofilm was used in the present study, this method was designed as a way of evaluating the resistance and survival of this strict anaerobe, confirmed by the positive control, to the sequence of centrifugations proposed by the present protocol before associating it with other species associated with endodontic treatment failure.

Human lower premolars were used, due to their oval anatomy, with the same instrumentation technique in all canals to obtain comparable specimens. Another relevant aspect concerns the diameter and number of dentinal tubules per mm^2 in human permanent dentin, which vary considerably [33]. Therefore, to standardize the specimens collected, radiographs were taken in different directions. The CLSM has been used to identify and measure viability exclusively within the dentinal tubules through optical sections of the dentin mass, photographed at various depths up to 200 µm, which are stacked to produce a three-dimensional reconstructed image [23,27,28].

This new proposed experimental model is based on modifications to the protocol developed by Andrade et al. [26] with a reduction in the number of centrifugation cycles, allowing the use of a strict anaerobe in intratubular contamination. All the modifications introduced were gradually added after numerous pilot studies to obtain the best and most homogeneous contamination. For example, the reduction of centrifugation cycles was fundamental to obtain live bacteria inside the dentinal tubules, since the number of cycles present in the original protocol ended up generating a lethal amount of oxygen for *F. nucleatum*. Furthermore, it was necessary to add more days to the protocol, due to the fastidious growth of this microorganism. By obtaining its growth curve, it was possible to use this bacterium in the contamination of dentinal tubules in its optimal phase of proliferation.

Previous studies have reported high antibacterial activity of both NaOCl and CHX against this microorganism [8,34,35]. However, we emphasize that so far there are no *in vitro* studies on the effectiveness of endodontic disinfecting agents in intratubular decontamination on *F. nucleatum*, nor a model of dentin contamination with the same species, which makes the present study unprecedented. In the present study according to CLSM analysis, the two irrigating solutions promoted a reduced number of viable bacteria in infected dentin after their use, compared to the positive control.

NaOCl has a broad spectrum of action, removes biofilms from surfaces, leaves no toxic residues, is inexpensive and fast-acting [5]. CLX, in turn, is a cationic bisbiguanide that has excellent antibacterial activity, substantivity, and relatively good tissue compatibility [34]. In this study, teeth treated with 2.5% NaOCl showed the lowest percentages of bacterial viability, which confirms their antimicrobial and *anti*-biofilm activity. Rodrigues et al. [36] observed similar results with the use of 2.5% NaOCl in relation to intratubular antimicrobial activity when compared to 2% CLX. This same difference was verified in previous studies [7,37,38] that compared the decontamination capacity of the canals by the two solutions.

Rôças et al. [9] demonstrated similar antimicrobial action between these two solutions, a fact mainly attributed to the mechanical action of the instruments that act significantly in the decontamination of root canals. However, it should be noted that the teeth irrigated with 2% CLX showed a significant reduction in the percentage of viable bacteria compared to the positive control,

highlighting its role in this pathogen.

The selection of the microorganism depends on the focus of the study and most of the time an intratubular biofilm of *Enterococcus faecalis* is used [23,26,28,36,39–41], mainly due to its characteristic of being commonly isolated from persistent endodontic infections [42], although the microbiota of infected root canals is mainly composed of strict anaerobes [14,15,25]. There are no reproducible *in vitro* intratubular models for strict anaerobes, such as *F. nucleatum*, predominant in primary infections. These models are useful for evaluating new antimicrobial treatment options, mainly if a new therapy has not yet been proven in randomized controlled clinical trials, in addition to considering the anatomic complexity of RCS.

According to a recent study [43], the susceptibility of the cultured biofilm to evaluated treatment depends on parameters such as biofilm composition, maturation time, and incubation atmosphere. In this way, the observed antimicrobial results can be explained not only by the effectiveness of the substances but also by the use of a monospecies biofilm. Although the literature presents intradentinal contamination protocols that use centrifugations, these models use facultative anaerobic or aerobic microorganisms and are lethal for strict anaerobes, which justifies the initial choice of a monospecies biofilm in this protocol. Our results showed high bacterial viability, which will allow the use of other species together with *F. nucleatum*. Furthermore, this type of contamination is closer to the clinical reality compared to the growth of biofilms on a substrate, since the endodontic infection is not contained only in the main canal, but throughout the entire root canal system.

As limitations of the study, we highlight the difficulty of cultivating this strict anaerobic microorganism, requiring that all procedures be performed in an anaerobic chamber with a specific culture medium, in addition to the longer time required for growth. Furthermore, centrifugations were carried out cautiously using speeds that could reconcile viability and extensive contamination of the dentinal tubules, from the main canal to the dentinal cementum. From a clinical point of view, this contamination model using *F. nucleatum*, predominant in primary endodontic infections, allows the evaluation of new antimicrobial treatment options that are complementary to the methods already employed, increasing the success rates of endodontic treatment in these cases, and whose main target is the elimination of this pathogen responsible for the development of clinical symptoms, periapical bone rarefaction and which has extremely cytotoxic endotoxins in its cell wall, capable of promoting systemic repercussions.

In conclusion, this study demonstrates a new protocol for the contamination of dentinal tubules by a strict anaerobe, promoting a homogeneous proliferation of viable bacteria. In addition, it was observed that both irrigating solutions studied proved to be effective in reducing the intratubular *F. nucleatum* with the ability to penetrate deep into the dentinal tubules, with emphasis to 2.5% NaOCl, which showed superiority in the results.

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Author contributions

Mirela Cesar de Barros & Victor Feliz Pedrinha: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Marcia Sirlene Zardin Graeff: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Clovis Monteiro Bramante & Marco Antonio Hungaro Duarte: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Flaviana Bombarda de Andrade: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be available on request.

Declaration of competing interest

The authors have no competing of interest to declare.

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