Biomedicine & Pharmacotherapy 183 (2025) 117833

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Antimicrobial efficacy of alternative root canal disinfection strategies: An evaluation on multiple working models

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ARTICLE INFO

Keywords:
Dental pulp diseases
Endodontics
Root canal irrigants
Root canal medications
Root canal preparation

ABSTRACT

Researching disinfection strategies is pivotal because effectively eliminating bacteria and their byproducts during root canal treatment (RCT) remains a challenge. This study investigated the antimicrobial efficacy of natural antimicrobial compounds, propolis (PRO) and copaiba oil-resin (COR), compared to conventional agents in Endodontics. Antimicrobials were tested against endodontic pathogens via macrodilution with standardized inoculums to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). Biofilm killing efficacy were performed using two dual-species biofilms: *Enterococcus faecalis* (ATCC 29212) and *Streptococcus mutans* (ATCC 20523) and *Streptococcus oralis* (J22) and *Actinomyces naeslundii* (T14V-J1) grown on dentine discs. At the intratubular level (dentine cylinders), dentine tubule contamination was performed with *E. faecalis* and *S. mutans*. The specimens were exposed to antimicrobials to simulate their use at different sets of RCT and bacterial viability was quantified using Live/Dead staining via confocal laser scanning microscopy (CLSM). Biofilm characteristics and immediate removal of *S. oralis* and *A. naeslundii* biofilm model were evaluated employing optical coherence tomography (OCT) and CFU/mL counting. Statistical tests were applied according to data distribution for each analysis (α=0.05). Macrodilutions showed different effects against endodontic pathogens. Direct contact and intratubular analysis showed that PRO and COR promoted disinfection like conventional agents ($p > 0.05$). According to OCT analysis, PRO and COR showed similar biofilm reduction after immediate contact ($p < 0.05$). CFU/mL counting showed decontamination ($p < 0.05$) after

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https://doi.org/10.1016/j.biopha.2025.117833
Received 17 September 2024; Received in revised form 26 December 2024; Accepted 9 January 2025
Available online 18 January 2025

using natural and conventional agents. PRO and COR showed antimicrobial effects, indicating their suitability as complementary approaches in RCT to eliminate as much microbial load as possible.

1. Introduction

Bacterial infection through the root canal system is responsible for the development of apical periodontitis, which is one of the most common inflammatory diseases in humans [1–3]. Despite the high success rate of root canal treatment (RCT), persistent infections may occur even in well-treated teeth, resulting in the presence of periapical tissue inflammation (5–15 % of cases) [2,4]. To understand this, it is necessary to consider the complex structure of the root canal system itself. Since bacteria tend to grow in biofilms, adhering to a surface or liquid interface and co-adhering to each other [5], the root canal walls serve as a surface where biofilms can be developed [6–8].

In this context, a satisfactory performance of antimicrobial strategies is pivotal since successful treatment outcome depends on effective infection control during RCT [7,9]. The chemomechanical preparation is considered the main mechanism responsible for killing microorganisms (planktonic or biofilm), eliminating infection, or at least reducing the bacterial load to levels that could favor tissue repair. In other words, it promotes a bacterial level insufficient to cause or maintain periapical disease [10,11], providing cleaning, shaping, and debridement of root canal walls [11,12]. However, it is well known that irregular canals and difficult-to-reach areas are not thoroughly cleaned and disinfected under different chemomechanical preparation techniques, often retaining residual bacteria and/or pulp tissue remnants [7,10–12]. This situation represents a potential cause of persistent infection and poor treatment outcome [13].

Chemical substances are used in combination with the mechanical action of endodontic instruments [7,8]. Sodium hypochlorite (NaOCl) is the most widely used irrigation solution in Endodontics, as it is the only one capable of dissolving pulp tissue and other organic components within the root canal system, in addition to its strong antimicrobial effect [14,15]. However, NaOCl presents some disadvantages. It cannot dissolve inorganic components [16] and affects the integrity of dentin tissue through collagen alteration, known as proteolytic action, contributing to teeth weakness, and consequently predisposing to teeth fracture [17–19], beyond its toxicity to periapical tissues [20]. Indeed, the adverse effects resulting from the use of NaOCl underscore the pressing need for alternative endodontic solutions to provide complementary disinfection in RCT with non-toxic substances. Moreover, for teeth with incomplete rhizogenesis, meaning incomplete root development, the use of non-aggressive and less toxic irrigation solutions is recommended [21,22]

Also, considering the treatment of an infected tooth, the use of intracanal medications is recommended to eliminate residual bacteria and their byproducts within the root canal system, despite chemomechanical preparation, aiming to avoid the possibility of reinfection [23,24]. For that reason, calcium hydroxide (CH) paste is the most frequently used as intracanal medication, being delivered into the root canal for a period of at least seven days between treatment sessions [25,26]. The antibacterial property of CH paste occurs through alkalinization of the internal surfaces of the canal walls and neutralization of acidic products from the inflammatory process [27], facilitating periapical tissue repair due to the activation of alkaline phosphatase, related to hard tissue formation [28].

Despite that, CH also shows some limitations regarding the elimination of bacteria placed in the deep dentine tubules and all ramifications due to the lack of direct contact with the microorganisms and the limited penetration into difficult-to-reach areas of the root canal system [29,30]. In addition, the properties of CH pastes vary according to the type of vehicle which they are formulated [28,31]. Alkalinization, antimicrobial action, and volumetric alteration are some of the properties that could present different behaviors [31–33]. In a clinical scope, is necessary to well know the performance of each CH paste to make an adequate choice according to the case treated. Beyond that, CH pastes present the potential for teeth weakening [24], reducing root fracture resistance when used for long periods [34], and can cause injury to the surrounding periapical tissues if medication is accidentally extruded [35]. These aspects support the need to investigate biocompatible alternatives for intracanal medications.

Some natural antimicrobial compounds have been explored representing promising alternatives. Propolis (PRO), also known as bee glue, is a resinous substance collected by honeybees from various plants and used within their hives [36,37]. It possesses anti-inflammatory and antioxidant properties due to the presence of flavonoids and other phenolic compounds in its composition [36,37]. In Endodontics, PRO has already demonstrated significant antibacterial, antifungal, and anti-inflammatory properties [32,36,38,39]. PRO showed antibacterial efficacy comparable to CH and triple antibiotic paste [39], as well as similar intratubular penetrability to CH [32] and bacterial viability [40]. Furthermore, in regenerative endodontic therapy, PRO has been found to elicit less inflammatory reaction and increased bioactivity [41].

Copaiba oil-resin (COR) has demonstrated potential therapeutic properties for dental applications [42,43]. Sourced from the Brazilian Amazon and extracted from trees of the genus *Copaifera*, also known as *copaibeiras* [44], this resin exhibits anti-inflammatory and healing properties [43]. Additionally, it possesses antimicrobial effects attributed to its constituents, including diterpenes and sesquiterpenes [43–45]. COR has already been mixed into a root canal sealer, showing promising results regarding the physicochemical properties of the sealer, suggesting its potential suitability for RCT applications [46].

Strategies could involve the use of PRO or COR to provide complementary disinfection for RCT, embracing the irrigation solution and intracanal medication scopes. A previous finding suggested that PRO and COR could induce proliferative activity in human periodontal ligament fibroblasts (PDLFs), without increasing cytokine liberation [47]. These findings provide background for determining new studies aiming at the development of products based on natural antimicrobial compounds, since PDLFs are the cells present around the apical area and are involved in the tissue response generated by endodontic materials [48]. However, when addressing an endodontic infection, it is crucial to investigate not only the biocompatibility properties of substances but also to conduct antimicrobial evaluations using different working models. This is necessary due to the diversity of microbiota and bacterial states, such as planktonic and biofilm forms, present in the root canal system.

To our knowledge, no studies have explored the effectiveness of PRO and COR against different endodontic pathogens compared to conventional antimicrobial agents through various experimental setups simulating irrigation solutions and intracanal medications. To fill this gap in knowledge, our study conducted experiments encompassing different planktonic bacteria via macrodilution test, beyond direct contact, and intratubular analyses on a dual-species biofilm confected with traced bacteria in root canal infections. Additionally, immediate disinfection and biofilm reduction were analyzed on a second type of dual-species biofilm model, which presents properties comparable to *in vivo* oral biofilms. The objective of this work was to explore the potential of natural antimicrobial compounds, PRO and COR, as viable endodontic products, searching for alternatives to complement disinfection in RCT.

2. Materials and methods

2.1. Characterization of PRO and COR

The solid green propolis was obtained from the state of Minas Gerais (Brazil) and typified as Brazilian Green Propolis (BRPX). The crude resin was extracted with ethanol, dried, to obtain the "dry extract of propolis" (PRO) and analyzed by high-performance liquid chromatography (HPLC) according to previous studies with L-7100 pumps and an L-7200 auto-sampler (Merck-Hitachi, Darmstadt, Germany) [32,47,49]. The quantification of polyphenols in PRO was performed using standards and the dried soft extract presented a high content of phenolic compounds as described previously [47]. The p-coumaric acid, 3,5-di-*O*--caffeoylquinic acid, 3-prenyl-4-hydroxycinnamic acid (Drupanin), and 2,2-dimethyl-2-H-1-benzopyranpropenoic acid (Culifolin) are some of the constituents, being the 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin-C) the main compound in Brazilian green propolis BRPX type [47].

COR was collected as previous described, in the experimental field of Brazilian Agricultural Research Corporation (EMBRAPA) Eastern Amazon, in the city of Belterra, State of Pará, Brazil and *Copaifera reticulata* Ducke was the tree species used for the oil-resin extraction [47]. The oil-resin presented the following characteristics: light yellow color (gold), dense, clear, and translucent.

COR components were identified through gas chromatographic mass spectrometry (GC-MS) analysis on an Agilent 6890 gas chromatograph (Agilent Technologies model 6890 N, Santa Clara, USA) coupled to a mass spectrometer detector 5975 model and fitted with a fused silica HP5-MS capillary column (30 m x 0.25 mm i.d.; 0.25 μm film thickness). Injector and detector temperatures were set at 220 °C and 290 °C, respectively. The column temperature program was set to increase from 120 °C to 160 °C at a rate of 2 °C/min, and then to 300 °C at a rate of 10 °C/min (held for 13 min). Mass spectrometer detection was conducted at 70 eV. Retention indices (RI) were determined in relation to a homologous series of n-alkanes (C8-C30) under the same operating conditions.

The components were identified by direct comparison of mass fragmentation patterns with those obtained from the NIST-11 library database and by comparison of their mass spectra with those reported in the literature from Adams, 1995 [50]. Component percentages were expressed as peak area normalization. Diterpenic acids from COR were analyzed in the methyl ester form according to the methodology described by Pasquel-Reátegui *et al.* [51]. COR constituents are described in Table 1.

Table 1
Chemical profile of *Copaifera reticulata* oil-resin (COR) by GC-MS.

Constituents	RI $_{calculated}$	RI $_{from\ literature}$	%
Cyclosativene	1359	1368	0.41
α-Copaene	1379	1376	0.99
β-Elemene	1388	1391	1.01
Caryophyllene	1420	1418	0.46
α-Bergamotene	1432	1434	18.20
α-Guaiene	1436	1439	0.98
(*E*)-β-Farnesene	1456	1456	1.33
(E.E)-α-Farnesene	1489	1505	2.12
β-selinene	1487	1485	1.41
α-Selinene	1495	1494	0.69
α-Bisabolene	1498	1504	2.71
β-Bisabolene	1506	1509	22.56
γ-Cadinene	1520	1513	0.58
β-Sesquiphellandrene	1527	1524	1.50
Kauren−16-ene	2050		1.93
Epeuric acid *	2239		2.54
Cativic acid *	2274		3.06
Kaur−16-en−18-oic acid *	2316		9.74
Copalic acid *	2325		3.92
Kauran−19-oic acid *	2335		8.31
Polyalthic acid *	2381		4.98
Pinifolic acid *	2513		2.50
Hydrocarbon sesquiterpenes			57.36
Hydrocarbon diterpene			1.93
Oxygenated diterpenes			39.43
Total			98.73
* in the methyl ester form			
ni = not identified			8.08

2.2. Macrodilution assay

2.2.1. Bacterial strains

Four bacterial reference strains were used: *Enterococcus faecalis* (ATCC 29212), *Streptococcus mutans* (ATCC 20523), *Streptococcus oralis* (J22), and *Actinomyces naeslundii* (T14V-J1). Briefly, the bacteria were collected from stocks at −80 ºC and streaked on blood agar plates and incubated at 37 ºC. For *A. naeslundii*, the plate was incubated in anaerobic chamber (Baker Ruskinn, Bridged, United Kingdom). After 48 hours, single colonies of *E. faecalis* and *S. mutans* were used to inoculate 10 mL of brain-heart infusion broth (BHI, Oxoid, Basingstoke, UK). For single colonies of *S. oralis* and *A. naeslundii*, the inoculation was performed in 10 mL modified brain–heart infusion broth (BHI^{+}; 37.0 g L^{-1} BHI, 1.0 g L^{-1} yeast extract, 0.02 g L^{-1} NaOH, 0.001 g L^{-1} vitamin K1, 5 mg L^{-1} L-cysteine-HCl, pH 7.3; BHI; Oxoid Ltd., Basingstoke, UK). Subsequently, facultatively anaerobic bacteria *E. faecalis*, *S. mutans*, and *S. oralis* were cultured at 37 °C for 24 h in enviroment air and *A. naeslundii* at 37 °C for 24 h in an anaerobic chamber. The bacterial purity was checked by means of agar plate cultures. Beyond that, 10 μL from subcultures were visualized on slides through a microscope (CH series, Olympus, Hachioji, Tokyo, Japan) under 40 × magnification before experiments.

The inoculum was obtained after two successive 24-hour cultures in both BHI (*E. faecalis* and *S. mutans*) and BHI^{+} (*S. oralis* and *A. naeslundii*) media incubated at 37°C under aerobic or anaerobic (*A. naeslundii*) conditions. The tube's turbidity readings were performed in a spectrophotometer (GENESYS™ 30 Visible Spectrophotometer; Thermo Fisher Scientific, Groningen, The Netherlands) at 540 nm. The cultures were diluted to the 0.5 MacFarland standard and further diluted to the concentration of 5×10^5 colony forming units (CFU/mL) and distributed in 3 mL volumes to each tube containing broth-diluted substances.

2.2.2. Tested substances

Firstly, the tested substances were pure Hydrocortisone (H) (Bauru Fórmulas, Bauru, SP, Brazil) and Otosporin® (FQM, Rio de Janeiro, RJ, Brazil), which consists of two antibiotics, polymyxin B sulfate (10.000 UI or 1.19 mg/mL) and neomycin sulfate (5 mg/mL), associated with H (10 mg/mL) in an aqueous vehicle. Additionally, 10 % PRO and 10 % COR were tested, diluted in two different vehicles: pure ethanol (Merck, Darmstadt, Germany) and propylene glycol (De Hekserij, IJsselmuiden, The Netherlands). Pure ethanol and propylene glycol were used as controls.

To obtain a 10 % concentration of PRO, either ethanol or propylene glycol was added to the dried extract of propolis, BRPX, in a volumetric balloon. Similarly, 2 mL of COR was added to 18 mL of ethanol or propylene glycol to obtain a 10 % concentrated stock solution. The H in powder was used to prepare a 10 % (100 mg/mL) stock solution diluted in propylene glycol. Since the Otosporin® is already in an aqueous vehicle, the medication per se was considered a stock solution. For the macrodilution test, screw-capped tubes were used to transfer accurate volumes of substances taken from the stock solutions, producing simple dilutions. BHI or modified BHI^{+} media were added to complete a volume of 3 mL in each tube. In a second stage, based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the natural antimicrobial compounds, the PRO + COR combination was tested against each bacterial strain.

2.2.3. MIC and MBC determination

Since some substances can alter the media, it was necessary to

compare the turbidity readings in the spectrophotometer before and after the incubation period to detect tubes with bacterial growth. The readings of the tubes determined the minimum inhibitory concentration (MIC) for each substance. Before reading the substances, each tube was vortexed for 10 seconds (Reax top, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany).

The time required to promote growth was 24 hours in BHI for *E. faecalis* and *S. mutans*, 24 hours in BHI^+ for *S. oralis*, and 48 hours in BHI^+ for *A. naeslundii*, considering the fastidious nature of this strict anaerobic bacteria. These periods were determined based on the bacterial growth curve established prior to the experiments (Fig. 1). Negative and positive controls for bacterial growth were included.

After reading the final absorbances, 100 μL of each tube was transferred to BHI-agar or BHI^+-agar plates. Subsequently, these plates were incubated aerobically or anaerobically at 37°C for 48 hours to determine the minimum bactericidal concentration (MBC). The MBC was defined as the lowest substance concentration that inhibited bacterial growth on the plates. These tests were performed in triplicate.

2.2.4. Fractional bactericidal concentration

The antimicrobial activity of the PRO + COR combination was interpreted into one of the following categories: synergy, additive effect, indifference, or antagonism. The fractional bactericidal concentration (FBC) of them was calculated as the MBC of each one in combination, divided by the MBC of each one alone, applying the following formulas [52]:

FBC (a) = MBC (a) in combination with (b) / MBC (a) alone

FBC (b) = MBC (b) in combination with (a) / MBC (b) alone

The sum of the FBC or FBC index (FBCI) was therefore analyzed as:

$\Sigma FBC = FBC\ (a) + FBC\ (b)$

The index values were analyzed as follows: FBC index values ≤ 0.5, synergism; FBC index values > 0.5 to < 1.0, additive; FBC index values ≥ 1.0 to < 4.0, indifference; and FBC index values ≥ 4 antagonism [53].

2.3. Antibiofilm direct contact assay

2.3.1. Preparation of the specimen

Bovine incisor teeth, recently extracted and acquired through donation from a slaughterhouse, were preserved in a 0.1 % thymol solution at 4 °C. Using a 5.0 mm trephine bur (Härte Surgical Instruments, Ribeirão Preto, SP, Brazil) attached to a handpiece (Kavo Kerr, Joinville, SC, Brazil), the dentine discs were obtained. The dentine surfaces underwent sequential preparation to ensure standardized specimens, following protocols outlined previously [31,54]. Subsequently, the discs underwent treatment in an ultrasonic tub containing 17 % ethylenediaminetetraacetic acid (EDTA) and 5 % sodium thiosulfate ($Na_2S_2O_3$, Sigma-Aldrich, St Louis, MO, USA) for 5 minutes each, aimed at removing the smear layer. Following this, the discs were washed with distilled water and subjected to sterilization at 121 °C for 20 minutes.

2.3.2. Biofilm growth

All microbiological procedures were performed in a laminar flow chamber under aseptic conditions (VecoFlow Ltd., Campinas, SP, Brazil). Purity was confirmed through colony morphology and Gram staining (Oxoid, Basingstoke, UK). To construct a biofilm consisting of *E. faecalis* + *S. mutans*, the *S. mutans* strain stored by freezing (ATCC 20523) was reactivated in 3 mL of sterilized BHI and incubated at 37 °C until it reached exponential bacterial growth (Fig. 1). Then, the inoculum was transferred to a flask containing 50 mL of BHI and incubated at 37 °C. Meanwhile, the *E. faecalis* strain (ATCC 29212) was reactivated.

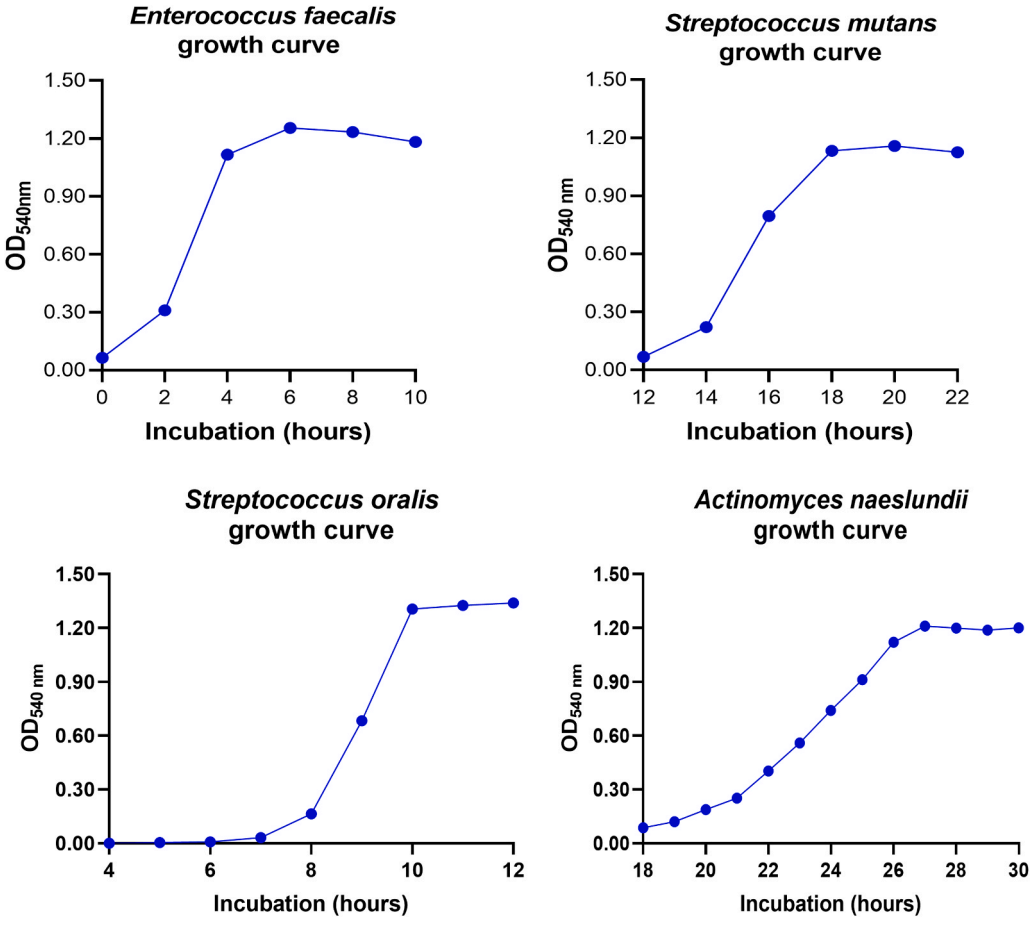

Fig. 1. Growth curve for each bacteria investigated. OD: optical density.

After 24 hours, the *S. mutans* main culture was adjusted according to McFarland standard #1 (3×10^8 CFU/mL).

S. mutans inoculum (100 μL), 1 dentine disc, and 900 μL BHI were individually inserted into each well of the 24-well plate. The plates were then incubated aerobically at 37 ºC. The following day, the *E. faecalis* main culture was adjusted to McFarland standard #1 (3×10^8 CFU/mL), and the BHI from each well of the plate was replaced with 900 μL BHI and 100 μL of *E. faecalis* inoculum. This media renewal process was conducted daily for a period of 10 days. Following the incubation period, the discs were randomly assigned according to the experimental conditions, simulating either an irrigation solution or an intracanal medication ($n = 8$/group). Additionally, eight specimens were used as positive controls (no treatment) to assess bacterial viability before administering antimicrobial agents, while two specimens served as negative controls.

2.3.3. Application of irrigation solutions

For the irrigation solution assay, four groups were investigated: 2.5 % NaOCl (Sigma-Aldrich, St Louis, MO, USA); 10 % PRO; 10 % COR; and the mixture of 10 % PRO and 10 % COR (PRO + COR) in a ratio of 1:1. To ensure the proper concentration of the NaOCl, a thiosulfate titration assay was performed previously to the experiment. The natural antimicrobial solutions were prepared in propylene glycol as a vehicle. Before the treatments, the infected samples were submerged in 1 mL of distilled water to remove loosely adhered planktonic bacteria. Subsequently, the dentine discs were submerged in 600 μL of the irrigating solutions for 3 minutes. The 2.5 % NaOCl solution was inactivated by adding 1 mL of 1 % sodium thiosulfate for 1 minute, followed by distilled water. For PRO, COR, and PRO + COR treatments, a solution consisting of 0.5 % Tween 80 + 0.07 % lecithin (w/v) was used, followed by distilled water.

2.3.4. Application of intracanal medications

In the intracanal medication assay, five groups were investigated: Otosporin®, CH paste, PRO, COR, and PRO + COR. Unlike the irrigation solution test, the natural antimicrobial compound groups (PRO, COR, and PRO + COR) were diluted in propylene glycol based on the CBM values obtained in the macrodilutions. For conventional agents like CH paste, the powdered CH (Merck & Co, Kenilworth, NJ) was mixed with propylene glycol in a ratio of 3:1 (powder weight/vehicle weight) and delivered to the dentine disc surfaces. For Otosporin® as well as the natural antimicrobial groups, 100 μL of medication was used since it was an amount capable of covering all the dentine disc surfaces, warranting maximum contact between biofilms and medications. The dentine discs with biofilms were immersed in experimental medications and incubated at 37 ºC for 7 days for the direct contact test. After the incubation period, the medications were removed using 10 % citric acid (Específica Pharmacy, Bauru, SP, Brazil), 0.5 % Tween 80 + 0.07 % lecithin (w/v), phosphate buffered solution (PBS) for CH, natural antimicrobial groups, and Otosporin® respectively, to neutralize any residual medication action. A final rinse with distilled water was performed to remove loosely adherent planktonic bacteria.

2.3.5. CLSM image acquisition and analysis for dentine disc model

After exposure to the treatments, the biofilms were prepared for analysis with the confocal laser scanning microscope (CLSM). They were stained with 10 μL of the LIVE/DEAD® BacLight viability kit (Molecular Probes, Eugene, OR, USA) for 10 minutes in a dark environment. The LIVE/DEAD® kit contains SYTO 9 dye, which stains live cells with a green pigment, and propidium iodide dye, which stains dead cells with a red pigment. The specimens were placed on a glass slide with immersion oil and observed under a Leica TCS-SPE confocal microscope (Leica Microsystems GmbH, Mannheim, Germany) at 40 × magnification. The settings for image acquisition were based on previous studies [31,33], and images were exported and analyzed using Leica LAS X Life Science software (Leica Microsystems GmbH, Mannheim, Germany). Four sequential images were obtained from different parts of each dentine disc. The images were acquired using 23 deep sections with a step size of 1 μm and a resolution of 1024 × 1024 pixels. A formula described in previous studies was applied to calculate the percentage of bacterial viability [31,55].

2.4. Antibiofilm intratubular assay

2.4.1. Preparation of the specimen

Bovine incisors were decoronated, and the 5 mm apical end of the roots was excised using an Isomet saw (Isomet® 1000, Buehler Ltd., Lake Bluff, IL, USA) with a diamond disc at 250 rpm under continuous irrigation. Standardized 8 mm length dentine cylinders were then obtained. The inside diameters of the dentine cylinders were shaped using a Gates Glidden drill size 4 (Dentsply Maillefer, Ballaigues, Switzerland) to achieve a standardized diameter of 1.1 mm, following established protocols [31,40,56,57]. Subsequently, the dentine cylinders underwent sequential ultrasonic baths with 1 % NaOCl (Fórmula e Ação, São Paulo, SP, Brazil), 17 % EDTA, 5 % sodium thiosulfate (Fórmula e Ação, São Paulo, SP, Brazil), and distilled water for 10 minutes each. Finally, the specimens were coated with red nail polish (Colorama, Rio de Janeiro, RJ, Brazil) and sterilized in an autoclave (Cristófoli, Campo Mourão, PR, Brazil) at 121°C for 24 minutes.

2.4.2. Dentinal tubule contamination

The specimens were placed in microtubes (Eppendorf, Hamburg, Germany) containing sterile BHI media and subjected to an ultrasonic bath for 10 minutes to enhance the penetration of the culture broth into the dentinal tubules [8,31,32,40,55,56,58]. On the first day of the contamination protocol, a main culture of *S. mutans* (ATCC 20523) was utilized for contamination, and the bacterial suspension was adjusted to McFarland standard #1 (3×10^8 CFU/mL). Each dentine cylinder was then placed in a 1.5 mL microtube, filled with 1 mL of the *S. mutans* suspension, and subjected to the sequence of centrifugation steps described by Ma et al. [59] and Andrade et al. [58] with some modifications. Subsequently, the specimens were incubated at 37 ºC.

On the second day, the BHI media for each specimen was refreshed, and a centrifugation step of 3.600 g for 5 minutes at 25 ºC was carried out. On the third day, a main culture of *E. faecalis* (ATCC 29212) adjusted to McFarland standard #1 (3×10^8 CFU/mL) was employed for contamination. The media for each specimen was replaced with the *E. faecalis* inoculum, and the centrifugation steps were repeated. On the fourth day, the procedures mirrored those of the second day. Moving to the fifth day, a previously mixed inoculum containing main cultures of *S. mutans* and *E. faecalis* was adjusted to McFarland standard #1 (3×10^8 CFU/mL) and utilized for the centrifugation steps. On the sixth day, the procedures were repeated as described for the second and fourth days.

After an overnight incubation, the specimens were randomly assigned to experimental groups based on the type of irrigation solution or intracanal medication used ($n = 8$), like the dentine disc tests. Additionally, eight specimens were randomly chosen as positive controls to verify intratubular contamination, and two additional specimens were selected as negative controls to confirm sterility.

2.4.3. Application of irrigation solutions

For the irrigation solution intratubular assay, four groups were investigated: 2.5 % NaOCl; 10 % PRO; 10 % COR; and the mixture of PRO + COR in the ratio of 1:1. The specimens were placed in a sterilized metal device inside the laminar flow chamber. The apical opening of the root was sealed with composite resin (DFL Industria e Comércio S.A., Taquaral, RJ, Brazil) and the root canals were irrigated using sterilized 30-gauge NaviTip needles (Ultradent Products Inc., South Jordan, UT, USA). The irrigation involved inward and outward movements with a 5 mm amplitude, along with simultaneous aspiration. The groups were irrigated with 10 mL for 90 seconds in a flow rate of 0.11 mL/s. At

120 seconds, the aspiration was suspended, leaving the solution inside the canals for 60 seconds, making a total of 180 seconds (3 minutes) of contact between the irrigation solution and the root canal walls. To neutralize the effects of the 2.5 % NaOCl, and natural antimicrobial solutions, 5 mL of 1 % sodium thiosulfate, and 0.5 % Tween 80 + 0.07 % lecithin (w/v) were used respectively. The inactivating agents were removed using 5 mL of distilled water.

2.4.4. Application of intracanal medications

For intracanal medications, new solutions diluted in propylene glycol were prepared based on the CBM concentrations of PRO, COR, and PRO + COR. The CH paste was applied to the root canals using a K-file #40 (Dentsply Maillefer, Ballaigues, Switzerland) until complete filling was achieved. Otosporin® and the natural antimicrobial groups were introduced into the root canals using sterilized 30-gauge NaviTip needles until complete filling, ensuring visualization of medications at the canal openings. After insertion of the medication, a temporary sealant (Coltosol®, Coltene, Rio de Janeiro, RJ, Brazil) was applied to the opening of the dentine cylinders. The medication remained in the specimens, which were kept in sterilized microtubes with small pieces of gauze soaked in sterilized distilled water at 37 ºC for 7 days. After the incubation period, the medications were removed, and neutralization steps were performed, followed by a final rinse with distilled water, like the process performed for medications applied over to dentine discs.

2.4.5. CLSM image acquisition and analysis for intratubular model

After exposure to the treatments, the specimens underwent longitudinal sectioning using an Isomet machine with a diamond disk under continuous irrigation with sterile saline solution. The smear layer resulting from the cut was eliminated by immersion in 17 % EDTA for 3 minutes, followed by rinsing with distilled water and drying with sterilized absorbent paper [8,31,32,40,55,56,58]. Subsequently, the dentine cylinders were stained with 30 μL of the LIVE/DEAD® BacLight viability kit for 20 minutes in darkness. CLSM analysis was conducted following the same specifications as the direct contact test for image acquisition. However, for intratubular analysis, eight sequential images were captured from each sample: four near the main root canal (superficial area) and four further away from the main canal (deep area) [8,31,40,58]. The percentages of bacterial viability were then quantified.

2.5. Immediate antibiofilm effect

2.5.1. Preparation of the specimen

Sterilized dentine discs were saliva-coated to confect a biofilm model consisting in clinical isolates of *S. oralis* (J22) and *A. naeslundii* (T14V-J1) according to previous studies [8,60,61] with some modifications. For that, a stock lyophilized saliva was dissolved in 30 mL adhesion buffer (1.5 g/L), stirred for 30 minutes, and centrifuged at 15.000 g, 10 °C for 5 minutes. The reconstituted saliva was filtered using a 0.22 μm pore size membrane filter (Merck Millipore, Burlington, Massachusetts, USA). The dentine discs were exposed to the reconstituted saliva for 14 h at 4 °C under static conditions.

2.5.2. Biofilm growth

The bacteria were grown as previously described [60,61]. Pre-cultures were utilized to inoculate 200 mL of BHI^+ and incubated for 16 hours to obtain main cultures. Bacteria were harvested via centrifugation (at 6350 g) and washed twice in sterilized adhesion buffer (containing 3.728 g/L KCl, 0.147 g/L $CaCl_2$, 0.174 g/L K_2HPO_4, and 0.136 g/L KH_2PO_4 in sterilized demineralized water, pH 6.8). The bacterial pellets were resuspended in 10 mL of sterilized adhesion buffer and sonicated intermittently in ice water for 3 cycles of 10 seconds at 30 W using a Vibra cell model 375 (Sonics and Materials Inc., Newtown, CT, USA) to disrupt bacterial chains. Bacterial counts were performed using a Bürker-Türk counting chamber (Marienfeld-Superior, Lauda-Königshofen, Germany) to determine the concentration. The mono-suspensions were diluted in sterilized adhesion buffer to prepare a dual-species bacterial suspension with a concentration of 6×10^8 bacteria mL^{-1} for *S. oralis* J22 and 2×10^8 bacteria mL^{-1} for *A. naeslundii* T14V-J1.

The dentine discs with adhered pellicle were placed in a disposable sterile 60 mL screw cap container along with a mixture of *S. oralis* J22 and *A. naeslundii* T14V-J1 bacteria. They were then left for 2.5 hours at room temperature for bacterial adhesion. Subsequently, the samples were transferred individually to wells in 24-well plates containing 1 mL of fresh BHI^+ media and incubated at 37 °C to promote biofilm formation. The media was refreshed every 24 hours for a total of 7 days. This duration was chosen based on established protocols for *S. oralis* J22 and *A. naeslundii* biofilm formation in previous studies, which compared 4-day and 10-day biofilms [60,61]. Additionally, after conducting several pilot studies, it was demonstrated that a 7-day biofilm reaches a standardized thickness and similar form visualized on CLSM and characterized by optical coherence tomography (OCT).

2.5.3. Application of irrigation solutions

For this analysis, irrigation solution treatments were selected to evaluate their immediate effect on biofilm reduction. To achieve this, 10 % concentrations of natural antimicrobial compounds were utilized and compared to 2 % NaOCl (Sigma-Aldrich, St Louis, MO, USA), known for its biofilm dissolution properties and already tested against this biofilm model [8,59,60]. The samples were divided into six groups according to the irrigation solution applied ($n = 9$/group): adhesion buffer (control), 2 % NaOCl, 10 % PRO, 10 % COR, PRO + COR in a 1:1 ratio, and propylene glycol, as natural antimicrobials were diluted in this vehicle. Three independent experiments were performed, during which 3 independent biofilm-carrying dentine discs from each irrigant - group were treated ($n = 54$/total). The sample size was determined based on findings from preliminary investigations and previously published data [8,60–62].

The solutions were applied statically (without flow). 40 μL of irrigation solution were gently pipetted onto the surface angles of the dentine discs, creating a thin layer over the biofilms. The samples were left undisturbed for 30 seconds. Subsequently, the biofilms were gently washed in 3 mL of adhesion buffer for 1 minute to neutralize the natural antimicrobial compounds and propylene glycol, while 2 % NaOCl solution was neutralized using 1 % sodium thiosulfate followed by adhesion buffer.

2.5.4. Optical coherence tomography (OCT) and biofilm assessment

Biofilm removal quantification was conducted using OCT, comparing images acquired before and after irrigation solution contacts [8,60,61]. For OCT imaging, the dentine discs with biofilms were placed individually in wells of a 12-well plate containing adhesion buffer. Real-time 2D cross-sections representing the biofilm were obtained using an OCT scanner (Thorlabs, Newton, NJ, USA). The field of view was set to 45 mm, the refractive index to 1.33, equal to the one of water, and images were processed using the ThorImage OCT software (Thorlabs) [63].

For image analysis, the base of the biofilm was defined by fitting a parabola to the 2D images, connecting the white pixels generated by light reflection on the dentine substrate surface. A gray-value threshold, which distinguishes the biofilm from the background, was determined using the gray-value histogram of the entire selected region of interest (ROI) [64,65]. The upper boundary of the biofilm was determined by identifying pixels with the greatest distance from the bottom, having a gray value exceeding the predetermined threshold, and being connected to the biofilm's base. Any objects not connected to the base were excluded from the biofilm structure. The mean thickness of the biofilm was then calculated by measuring the number of pixels between the biofilm's base and the upper boundary along each vertical line in the image as described previously for OCT biofilm analyzes [66]. For biofilm reduction, the percentage reduction was calculated by comparing the

OCT measurements of the biofilms before and after irrigation solutions were applied.

In terms of biofilm roughness, the analysis determined the roughness coefficient (Ra*) using the parameters established by Murga *et al.* [67]. This coefficient enables the comparison of biofilm structures across various studies and scales, as it is normalized to the mean biofilm thickness [66,68]. Additionally, the percentage of biofilm porosity is quantified based on the volume of non-connected pores per unit area of the ROI. This value is calculated by multiplying the number of non-connected voxels in an image by the voxel size and then normalizing it by the area [66].

2.5.5. Determination of CFU/cm^2 counting

Biofilms on dentine discs were exposed to irrigation solutions in an immediate action setting of 30 seconds ($n = 5$/group), following the procedure outlined above. Subsequently, the biofilms were transferred to 15 mL Greiner tubes (Fisher Scientific, Landsmeer, The Netherlands) containing 2 mL of PBS. The biofilms underwent sonication in ice water for 3 cycles of 10 seconds at 30 W, followed by homogenization using a vortex for 10 seconds. Then, 200 µL of the content was transferred to 1800 µL of PBS until a concentration of 10^{-4} was reached. Finally, 10 µL of the dilutions were plated on BHI^{+}-agar plates and incubated at 37 °C for 48 hours for subsequent colony forming units per cm^2 (CFU/cm^2) counts, which were log-transformed before statistical analysis.

2.6. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk normality test was employed to assess data distribution. Upon evaluation, OCT and CFU/mL counting showed a parametric distribution, while direct contact and intratubular data exhibited a non-parametric distribution. Consequently, for OCT and CFU/mL analysis, one-way ANOVA followed by Tukey's test was conducted and the Kruskal–Wallis test followed by Dunn's test was performed for direct contact and intratubular tests. A significance level of 5 % was adopted.

3. Results

3.1. Macrodilutions

The tested substances demonstrated the ability to inhibit and eliminate the strains, except for H, which did not promote bacterial growth inhibition. In addition, the ethanol and propylene glycol (vehicles) did not provide an additional antimicrobial effect to the substances. Tables 2 and 3 display the MIC and MBC concentrations for PRO and COR diluted in ethanol and propylene glycol, respectively. Higher MIC and MBC concentrations were required for bacterial inhibition and elimination of *E. faecalis* and *S. mutans* strains. Due to interactions between the media, vehicle, and substances, some MIC values for PRO and COR diluted in propylene glycol could not be determined.

Table 4 shows the MIC and MBC concentrations for the Otosporin® antibiotics, composed of neomycin sulfate and polymyxin B, wherein for the anaerobic strain of *A. naeslundii*, smaller concentrations were observed, while for facultative bacteria higher concentrations were found. However, it is necessary to note that both antibiotics act in combination in this drug. In relation to H, as it is an anti-inflammatory substance (steroid), no antibacterial action was observed in all concentrations tested.

Table 5 presents the MIC and MBC concentrations for PRO + COR diluted in ethanol and propylene glycol, along with the determination of FBC indexes and their interpretation of effect. Higher values for MIC and MBC were obtained against *E. faecalis* and *S. mutans* strains. Regarding the FBC index interpretations, an additive effect was observed for the *E. faecalis* strain, a synergistic effect for the *A. naeslundii* strain, an indifferent effect for *S. oralis*, and a synergistic or indifferent effect for the *S. mutans* strain between the dilutions in ethanol and propylene glycol, respectively.

Table 2
MIC and MBC in µg/mL of each substance diluted in ethanol using the macrodilution method against different bacteria.

Microorganism	Propolis		Copaiba		Ethanol	
	MIC	MBC	MIC	MBC	MIC	MBC
E. faecalis	1560	6250	1560	3120	278,200	556,425
S. mutans	6250	6250	6250	12500	278,200	556,425
S. oralis	195.0	195.0	97.50	97.50	278,200	278,200
A. naeslundii	390.00	780.0	780.0	1560	278,200	278,200

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.

Table 3
MIC and MBC in µg/mL of each substance diluted in propylene glycol using the macrodilution method against different bacteria.

Microorganism	Propolis		Copaiba		Propylene glycol	
	MIC	MBC	MIC	MBC	MIC	MBC
E. faecalis	1560	6250	780.0	780.0	187,500	375,000
S. mutans	-	1560	780.0	1560	187,500	375,000
S. oralis	-	195.0	-	195.0	375,000	750,000
A. naeslundii	-	390.0	390.0	390.0	187,500	187,500

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; (-): undetermined.

Table 4
MIC and MBC in µg/mL of antibiotics contained in the Otosporin® and hydrocortisone using the macrodilution method against different bacteria.

Microorganism	Neomicin sulfate in Otosporin®		Polimixin B in Otosporin®		Hydrocortisone	
	MIC	MBC	MIC	MBC	MIC	MBC
E. faecalis	78.125	312.50	7.812	31.25	-	-
S. mutans	78.125	312.50	7.812	31.25	-	-
S. oralis	625.0	625.0	62.50	62.50	-	-
A. naeslundii	9.760	9.760	0.976	0.976	-	-

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; (-): undetermined.

3.2. Direct contact tests

For irrigation solution settings against *E. faecalis* with *S. mutans* biofilms, the natural antimicrobial compounds, PRO, COR, as well as PRO + COR showed lower values of bacterial viability compared to the control ($p < 0.05$) and they did not differ from each other and were similar to 2.5 % NaOCl ($p > 0.05$). The graph of the percentage of bacterial viability and representative images of biofilms after direct contact with irrigation solutions analyzed in CLSM are shown in Fig. 2.

All the intracanal medication substances showed lower values of bacterial viability compared to the control ($p < 0.05$) with the exception of Otosporin® which presented intermediary values between the control and other medications ($p > 0.05$). CH paste, PRO, COR, and PRO + COR presented decreasing values for bacterial viability with no significant difference ($p > 0.05$). The graph of the percentage of bacterial viability and representative images of biofilms after direct contact with intracanal medications are in Fig. 3.

3.3. Intratubular model

According to CLSM image analysis of irrigation solutions against *E. faecalis* with *S. mutans* intratubular biofilms, the total percentage of bacterial viability for 2.5 % NaOCl and natural antimicrobial

Table 5
MIC and MBC in μg/mL related to the combination of propolis and copaiba oil-resin diluted in ethanol and propylene glycol using the macrodilution method and determination of Fractional Bactericidal Concentration (FBC) and its effect against different bacteria.

Microorganism	Propolis + Copaiba in ethanol		Propolis + Copaiba in propylene glycol		FBC Index in ethanol	FBC Index in propylene glycol
	MIC	MBC	MIC	MBC	Interpretation	Interpretation
E. faecalis	780 + 90	3120 + 390	780 + 90	3120 + 390	0.99 - Additive	0.99 - Additive
S. mutans	780 + 780	1560 + 1560	780 + 780	1560 + 1560	0.37 - Synergy	2.0 - Indifference
S. oralis	54 + 109	54 + 109	195 + 195	195 + 195	1.39 - Indifference	2.0 - Indifference
A. naeslundii	54 + 109	109 + 219	97.5 + 97.5	97.5 + 97.5	0.28 - Synergy	0.5 - Synergy

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.

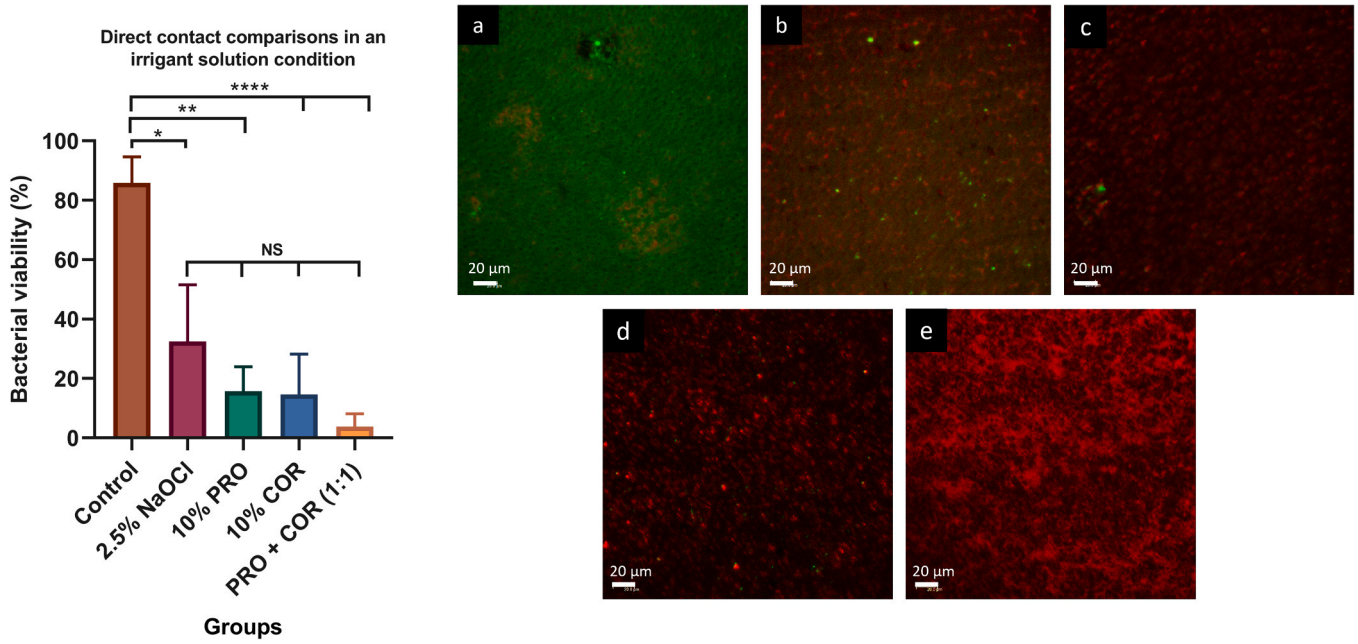

Fig. 2. The graph illustrates significant differences for bacterial viability between groups as determined by Kruskal-Wallis and Dunn tests, denoted by * for $p < 0.05$; * * for $p < 0.005$; * ** * for $p < 0.0005$. Non-significant differences are marked as NS. The bars represent the median, and the vertical dashes above the bars indicate the confidence interval. PRO: propolis; COR: copaiba oil-resin; NaOCl: sodium hypochlorite. Representative confocal laser scanning microscope images of biofilms after direct contact with irrigation solutions. (a) Control group; (b) 2.5 % NaOCl; (c) 10 % PRO; (d) 10 % COR; (e) PRO + COR (1:1). Viable bacteria are shown in green, and non-viable bacteria are shown in red. Magnification: 40 ×. Bars: 20.0 μm.

compounds (10 % PRO, 10 % COR, and PRO + COR in a 1:1 ratio) did not differ significantly from each other ($p > 0.05$), demonstrating antimicrobial effectiveness compared to the control group (no irrigation treatment) ($p < 0.05$). Similar results were observed when evaluating the superficial and deep areas of dentine tubules (Fig. 4).

Regarding intracanal medications, all tested groups exhibited lower values for total bacterial viability compared to the control group (no medication treatment) ($p < 0.05$). The natural antimicrobial compounds, prepared based on MBC values (PRO, COR, and PRO + COR groups), did not show significant differences from CH paste and Otosporin® ($p > 0.05$). CH, PRO, and PRO + COR showed lower bacterial viabilities in the superficial areas ($p < 0.05$), while CH and COR exhibited lower bacterial viabilities in the deep area ($p > 0.05$) (Fig. 5).

3.4. Immediate biofilm reduction

Fig. 6 presents the data regarding *S. oralis* J22 with *A. naeslundii* T14V-J1 biofilm thickness (height) in μm^3 after the application of irrigation solutions for 30 seconds, along with the percentage of biofilm reduction for the treated groups. Significant reductions in biofilm mean heights were observed for 2 % NaOCl, 10 % PRO, 10 % COR, and PRO + COR (1:1) compared to the control (adhesion buffer) and the vehicle (propylene glycol) ($p < 0.05$). The natural antimicrobial compounds (PRO, COR, and PRO + COR) could promote immediate biofilm reduction without significant differences between them ($p > 0.05$). The 2 % NaOCl group exhibited lower biofilm thickness followed by 10 % COR. Additionally, 2 % NaOCl showed the greatest biofilm reduction compared to PRO, COR, and PRO + COR ($p < 0.05$). Fig. 7 shows representative OCT images of biofilms after contact with the irrigation solutions employed, along with the values representing the mean and standard deviation (± SD) for each group.

Fig. 8 presents the data regarding biofilm roughness coefficients and porosity by means of OCT analyzes after irrigation solutions. The 2 % NaOCl and PRO + COR (1:1) groups produced biofilms with higher roughness coefficients ($p < 0.05$). The 10 % PRO group showed the highest mean value for porosity percentages, statistically differing from the other irrigation solutions ($p < 0.05$).

3.5. Microbiological culture evaluation

The results of CFU/cm^2 counting are displayed in Fig. 9. The irrigation solutions resulted in fewer colonies of the *S. oralis* J22 with *A. naeslundii* T14V-J1 biofilms, with 2 % NaOCl demonstrating superior performance ($p < 0.05$). 10 % PRO, 10 % COR, and PRO + COR (1:1) exhibited reductions that were statistically similar between them ($p > 0.05$). The control and vehicle groups exhibited the highest number of colonies, with no statistical differences observed between them ($p > 0.05$).

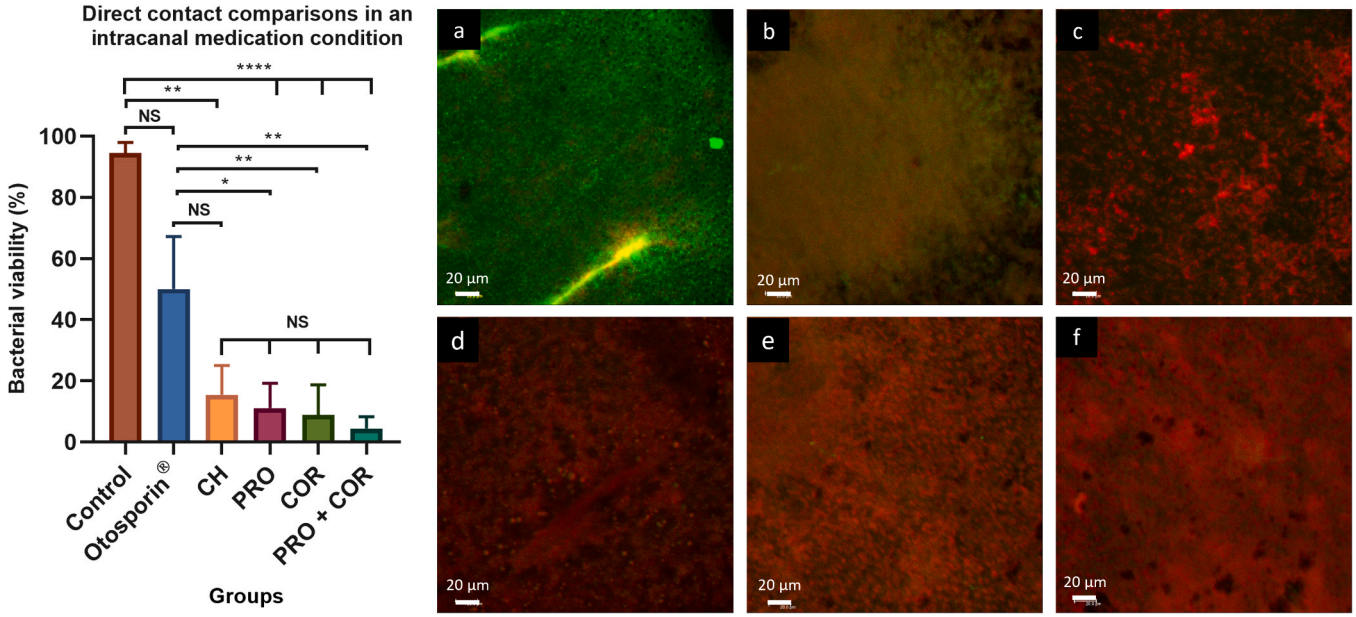

Fig. 3. The graph illustrates significant differences for bacterial viability between groups as determined by Kruskal-Wallis and Dunn tests, denoted by * for $p < 0.05$; * * for $p < 0.005$; * ** * for $p < 0.0005$. Non-significant differences are marked as NS. The bars represent the median, and the vertical dashes above the bars indicate the confidence interval. PRO: propolis; COR: copaiba oil-resin; CH: calcium hydroxide. Representative confocal laser scanning microscope images of biofilms after direct contact with intracanal medications. (a) Control group; (b) Otosporin®; (c) CH; (d) PRO; (e) COR; (f) PRO + COR. The concentration of natural antimicrobial compounds (PRO, COR, and PRO + COR) was determined based on the MBC values. Viable bacteria are shown in green, and non-viable bacteria are shown in red. Magnification: 40 × . Bars: 20.0 µm.

4. Discussion

Given the challenges in eradicating bacteria and their byproducts during RCT, the quest for alternative/complementary agents becomes paramount. As part of this endeavor, natural substances were tested in various antimicrobial assays. The effects of natural antimicrobial compounds PRO, COR, and the combination PRO + COR were investigated against planktonic bacteria, as well as different dual-species biofilms, considering their effects on direct contact and intratubular scopes using traced strains of *E. faecalis* 29212 and *S. mutans* 20523. Additionally, an immediate biofilm reduction and antimicrobial action against *S. oralis* J22 and *A. naeslundii* T14V-J1 biofilms were investigated. The results of these experiments suggest that PRO and COR hold promise as viable alternative antimicrobial substances.

4.1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC were ascertained via the macrodilution assay, exposing different concentrations of the substances to standardized bacterial suspensions by direct contact and subsequent streaking in agar plates for CFU/mL counting. The macrodilution is a standardized method which enables the simultaneous testing of multiple samples and is characterized by its accuracy [69]. By allowing samples to be diluted in larger volumes compared to the microdilution test, it reduces the risk of errors during concentration determination [38,52,70].

In this study, various pathogens were exposed to both natural antimicrobial compounds and conventional agents, recognizing the polymicrobial nature of endodontic infections [71,72]. PRO, COR, and PRO + COR demonstrated higher MIC and MBC concentrations against *E. faecalis* and *S. mutans*. The performance of these natural compounds against these bacterial strains may be attributed to their ability to survive. Both bacteria are Gram-positive and facultative. *E. faecalis* exhibits virulence characteristics, including resistance to intracanal medications and the ability to survive in adverse environments [73–75] and was reported as the less susceptible strain in contact with PRO diluted in ethanol via a macrodilution test [38]. *S. mutans* is a major contributor to the formation of cariogenic biofilms [76] and is commonly found in infected root canals during necrosis [77,78].

Despite *S. oralis* and *A. naeslundii*, also Gram-positive bacteria, being associated with persistent endodontic infections [79,80] they were found to be more susceptible to the action of PRO, COR, and PRO + COR, as low concentrations of these substances were sufficient to achieve bactericidal effects. The planktonic status of the bacteria tested may renders them more susceptible to the action of antimicrobial substances compared to biofilm-associated bacteria. When structured in biofilms, *S. oralis* and *A. naeslundii* demonstrate the capacity for coaggregation and the formation of robust biofilms with viscoelastic properties comparable to those of *in vivo* oral biofilms [61,62,81].

It's worth mentioning that the media used for *S. oralis* and *A. naeslundii* was BHI^{+}, which contains more nutrients compared to the BHI media used for *E. faecalis* and *S. mutans*. This approach was adopted to determine the MBC concentrations for the substances, which would be subsequently applied to the *S. oralis* and *A. naeslundii* biofilm model due to the coaggregation of a facultative (*S. oralis*) and a strict and fastidious bacterium (*A. naeslundii*) [8,60,61]. Despite the nutrient-rich conditions provided by the BHI^{+} medium, strains exposed to PRO, COR, and PRO + COR did not exhibit enhanced survival under the current experimental conditions. In closed root canals, strict anaerobes such as *A. naeslundii* frequently establish themselves as secondary and opportunistic invaders. The interaction and synergism between these anaerobes and other bacteria may result in a reduction in the number of facultative bacterial species [82].

The ethanol and propylene glycol were investigated as vehicles to check the possibility of influence of them on the antimicrobial action of the substances. The results suggest that the vehicles were not able to exert an extra antimicrobial activity to natural compounds, since they presented higher MIC and MBC concentrations (more than 10 times) and acting differently from natural compounds to the bacteria. A non-influence of ethanol regarding to antimicrobial properties of PRO was already showed when used as a vehicle [38]. Propylene glycol is commonly used in pharmaceutical being a viscous vehicle particularly

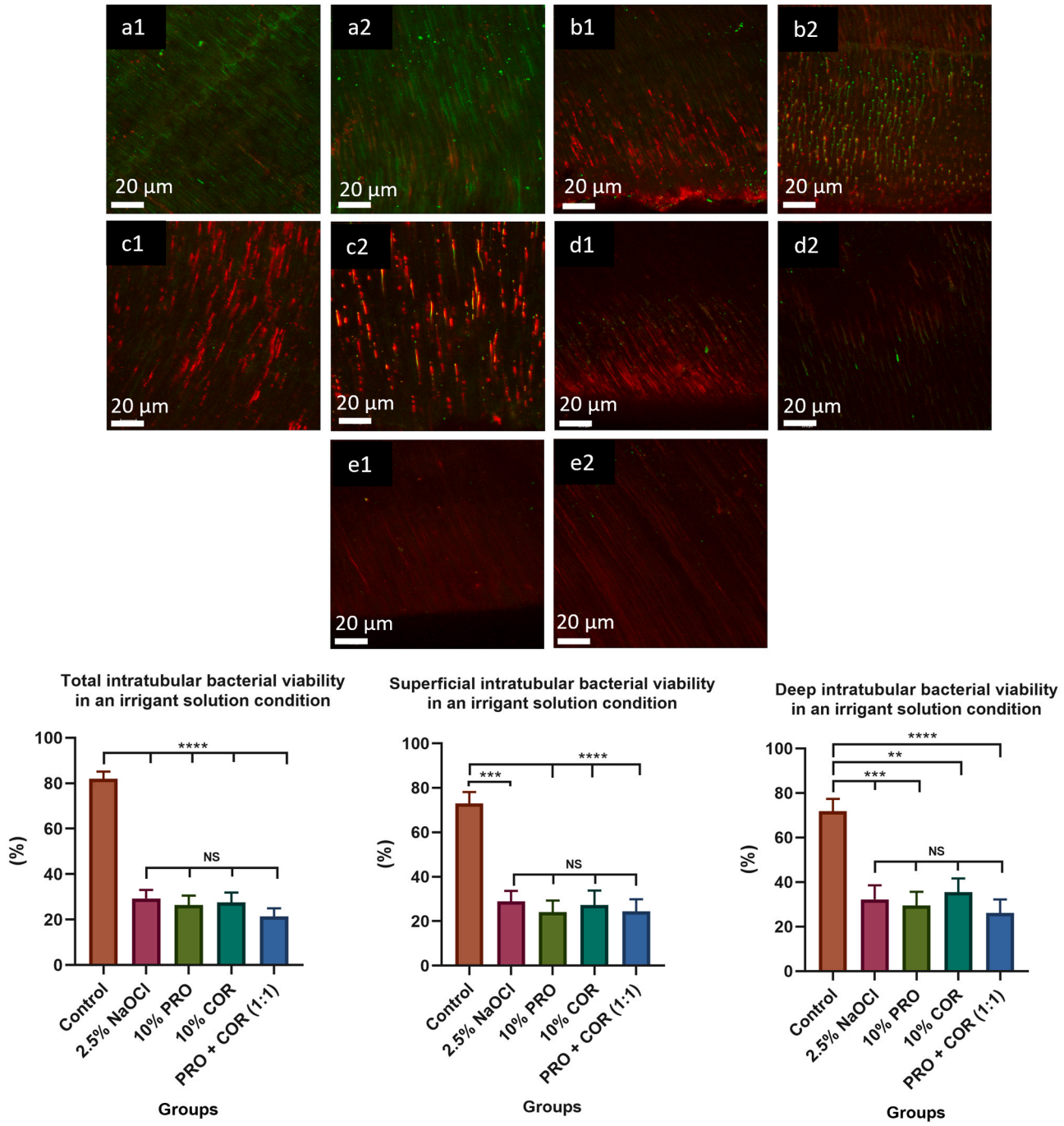

Fig. 4. Representative confocal laser scanning microscope images of intratubular biofilms after irrigation solutions. Number 1 corresponds to the superficial area and number 2 is the deep area. (a) Control group; (b) 2.5 % NaOCl; (c) 10 % PRO; (d) 10 % COR; (e) PRO + COR (1:1). Viable bacteria are shown in green, and non-viable bacteria are shown in red. Magnification: 40 × . Bars: 20.0 µm. The graphics illustrate significant differences for bacterial viability between groups as determined by Kruskal-Wallis and Dunn tests, denoted by * for $p < 0.05$; * * for $p < 0.005$; * ** for $p < 0.0005$; and * ** * for $p < 0.0001$. Non-significant differences are marked as NS. The bars represent the median, and the vertical dashes above the bars indicate the confidence interval. NaOCl: sodium hypochlorite; PRO: propolis; COR: copaiba oil-resin.

present in CH pastes and root canal sealers, and it is associated with inducing a more favorable release of calcium and hydroxyl ions compared to aqueous vehicles like distilled water and saline solution [83–85] and it did not exert antimicrobial effect [32]. Due to the hydrophobic nature of PRO and COR, most of their active components are scarcely soluble in water [86,87].

Based on the results, the natural compounds tested in both ethanol and propylene glycol showed variations in MIC and MBC concentrations against the strains, generally with lower concentrations observed for substances in propylene glycol. These variations are anticipated, as the polarity of each solvent influences their solubility characteristics [88–90]. While both propylene glycol and ethanol possess two hydroxyl (-OH) groups, the longer carbon chain in propylene glycol reduces its overall polarity compared to ethanol. Nonetheless, propylene glycol still maintains significant polarity due to the presence of hydroxyl groups [91].

Some substances have the potential to alter the media due to their miscibility, beyond this, absorbance values are also influenced by

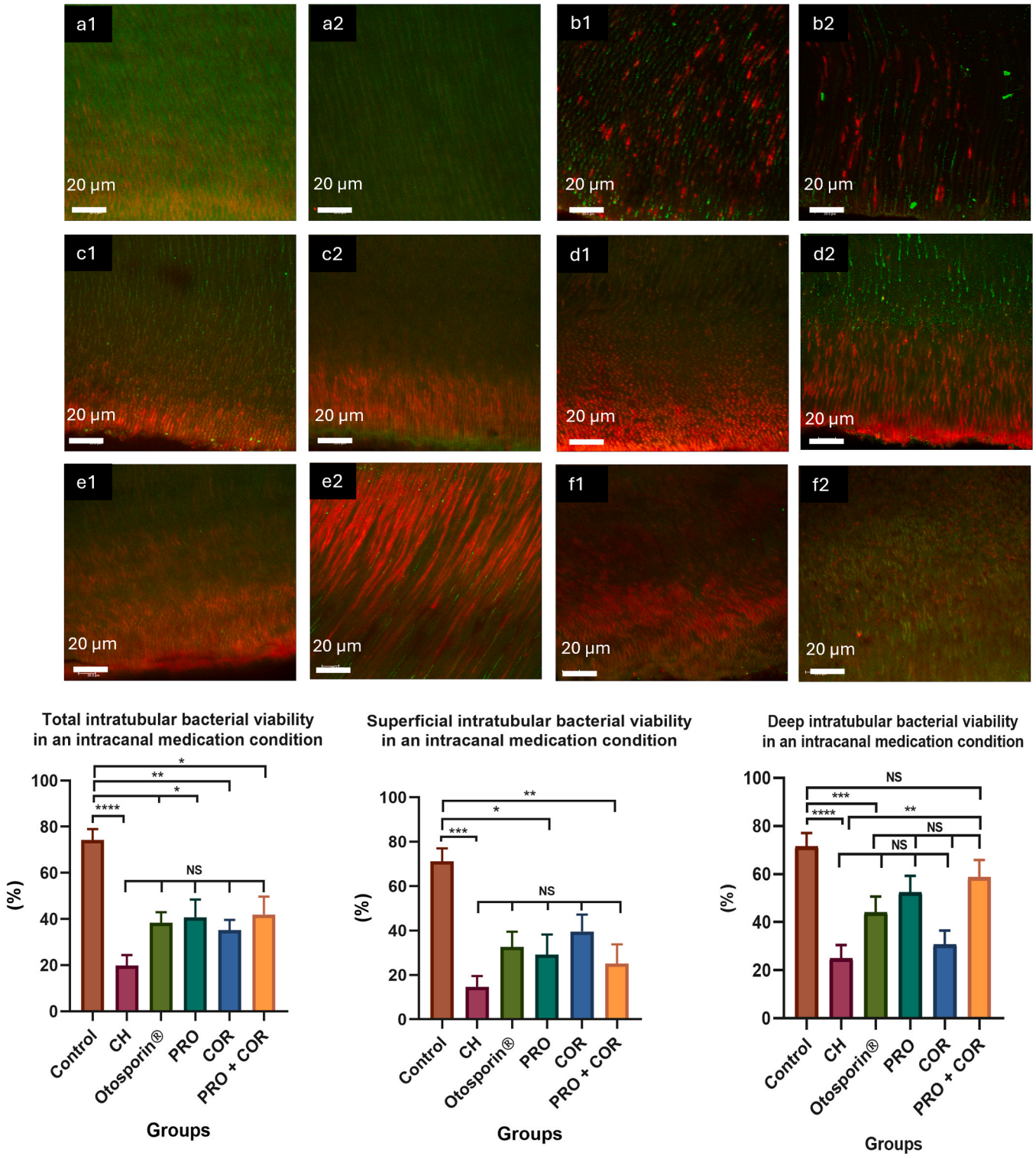

Fig. 5. Representative confocal laser scanning microscope images of intratubular biofilms after intracanal medications. Number 1 corresponds to the superficial area and number 2 is the deep area. (a) Control group; (b) Otosporin®; (c) CH; (d) PRO; (e) COR; (f) PRO + COR. The concentration of natural antimicrobial compounds (PRO, COR, and PRO + COR) was determined based on the MBC values. Viable bacteria are shown in green, and non-viable bacteria are shown in red. Magnification: 40 × . Bars: 20.0 µm. The graphics illustrate significant differences for bacterial viability between groups as determined by Kruskal-Wallis and Dunn tests, denoted by * for $p < 0.05$; * * for $p < 0.005$; * ** for $p < 0.0005$; and * ** * for $p < 0.0001$. Non-significant differences are marked as NS. The bars represent the median, and the vertical dashes above the bars indicate the confidence interval. CH: calcium hydroxide; PRO: propolis; COR: copaiba oil-resin.

bacterial growth [40], making it difficult to ascertain MIC concentrations for natural compounds in propylene glycol in the present study. Given that natural compounds often comprise organic components, it can be inferred that when diluted in propylene glycol, they may achieve a more uniform distribution within the solvent. Furthermore, the decision to dilute PRO and COR in propylene glycol was aimed at replicating endodontic conditions, where medications are typically delivered within the root canal using a solvent-based dilution. The lower surface tension

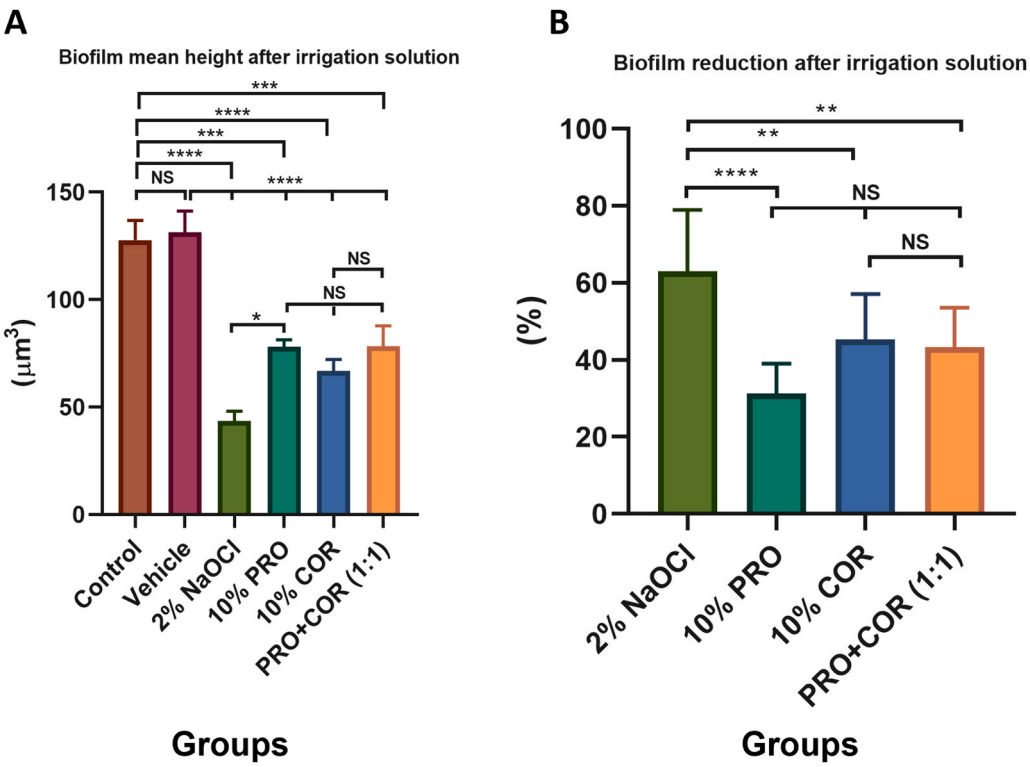

Fig. 6. (A) Biofilm mean thickness (height) after exposition to irrigation solutions by 30 seconds; (B) Biofilm reduction percentage promoted by irrigation solutions. No reductions were observed for the control (adhesion buffer) and the vehicle (propylene glycol). Significant differences between groups were determined using one-way ANOVA and Tukey tests and indicated by * for $p < 0.05$, * * for $p < 0.01$, * ** for $p < 0.001$ and * ** * for $p < 0.0001$. Non-significant differences are indicated by NS. The bars indicate the mean, and the vertical dashes above the bars indicate the standard deviation. PRO: propolis; COR: copaiba oil-resin; NaOCl: sodium hypochlorite.

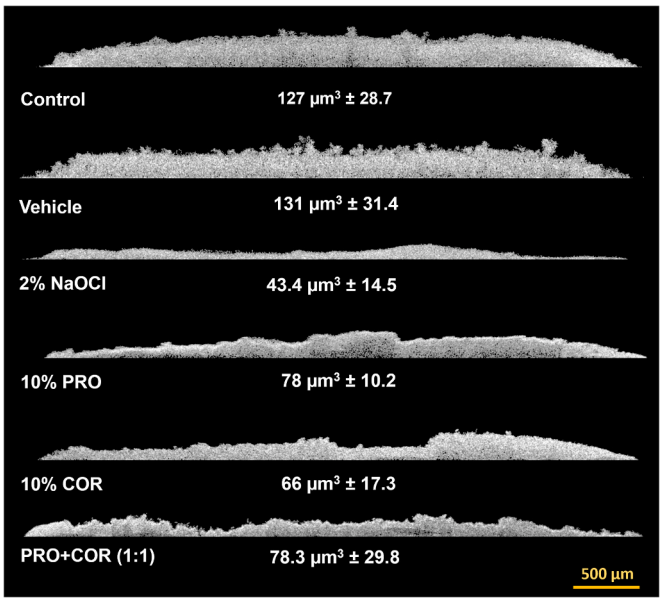

Fig. 7. Representative optical coherence tomographic images of S. oralis J22 with A. naeslundii T14V-J1 biofilms after irrigation solutions applied by 30 seconds. The values represent the mean ± standard deviations. No reductions were observed for the control (adhesion buffer) and the vehicle (propylene glycol). PRO: propolis; COR: copaiba oil-resin; NaOCl: sodium hypochlorite. Scale: 500 µm.

of this vehicle also contributes to greater penetration of the medication into anatomical complexities and dentinal tubules [33,34,92–94].

Comparing the present findings with prior studies poses challenges due to variations in methodologies and types of natural compounds used. Standardizing techniques is crucial to accurately assess the antimicrobial efficacy of substances. When applied clinically, it's imperative to use these substances to eradicate microorganisms within root canals, while ensuring minimal concentrations to prevent damage to periapical tissues [20,38,47,49].

Previous studies have assessed the activity of PRO against anaerobic bacteria, including various strains of *A. naeslundii*, using the agar diffusion test. However, this method may not be recommended for strict microorganisms due to their complex and slow growth, despite its occasional use by researchers [95–97]. In a separate study, a different form of COR extracted from *Copaifera pubiflora* was evaluated, both in its pure form and as isolated compounds, against several oral pathogens [53]. Interestingly, when combined with chlorhexidine, the oil-resin did not show variable interaction responses, with no sign of synergistic or additive effects. However, antibacterial activity against cariogenic and endodontic anaerobic bacteria, and a biofilm formation inhibition consisted in promising results [53].

To provide a comparison between natural antimicrobial compounds and a conventional agent in Endodontics, Otosporin® was selected due to its antibiotic composition, being typically used in otology, and often used in cases of vital pulp, between endodontic treatment sessions. It contains two antibiotics, polymyxin B sulfate and neomycin sulfate, along with the corticosteroid H, being this last one an anti-inflammatory [47,98]. However, the use of this medication raises questions since it is commonly employed in cases where the infectious condition is confined to a limited superficial area, such as vital pulp inflammation, where the root section of the pulp is inflamed without microbial involvement and maintains its vitality. Moreover, given the increasing prevalence of

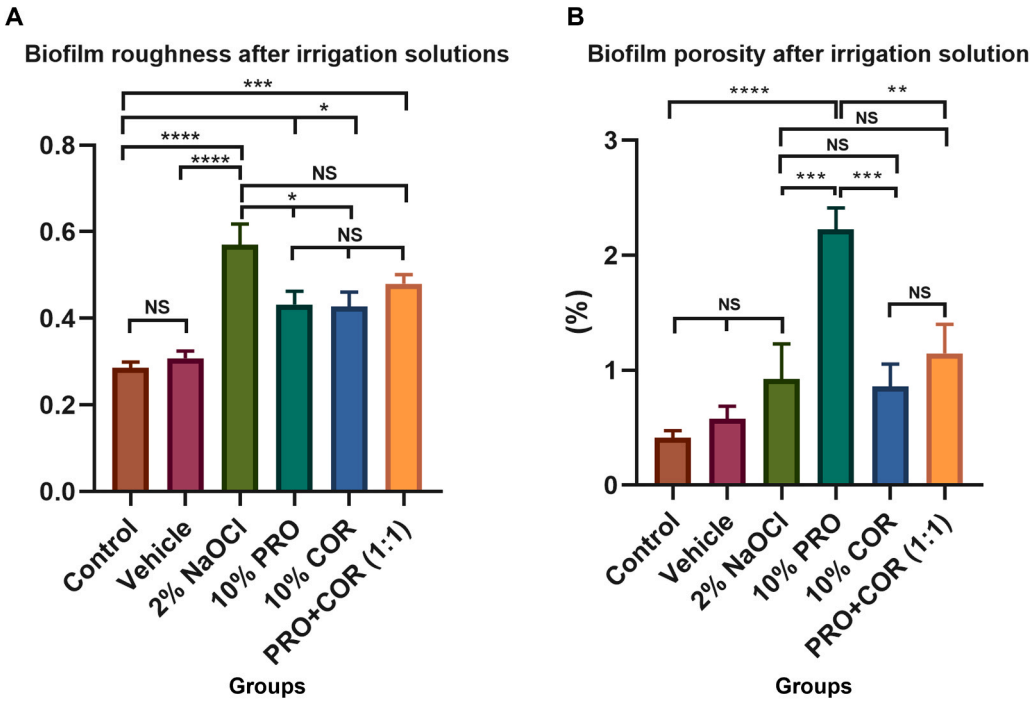

Fig. 8. (A) Biofilm roughness coefficient after exposition to irrigation solutions by 30 seconds; (B) Biofilm porosity percentage promoted by irrigation solutions. Significant differences between groups were determined using one-way ANOVA and Tukey tests and indicated by * for $p < 0.05$, * * for $p < 0.01$, * ** for $p < 0.001$ and * ** * for $p < 0.0001$. Non-significant differences are indicated by NS. The bars indicate the mean, and the vertical dashes above the bars indicate the standard deviation. PRO: propolis; COR: copaiba oil-resin; NaOCl: sodium hypochlorite.

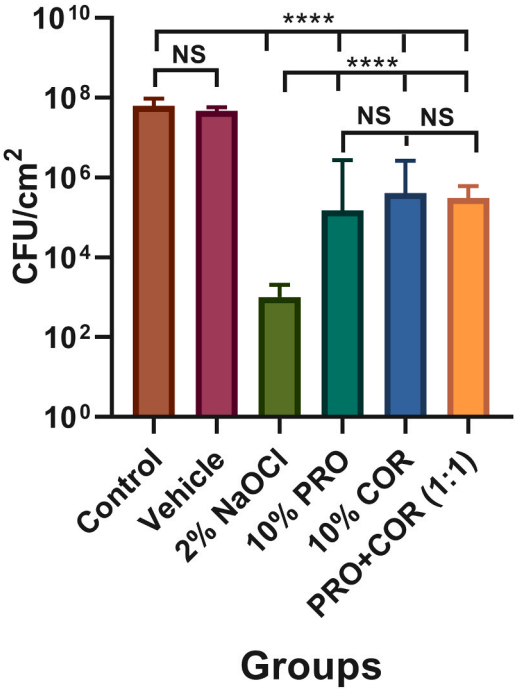

Fig. 9. Colony forming units (Log 10 CFU/cm2) counting after exposure of S. oralis J22 with A. naeslundii T14V-J1 biofilms to irrigation solutions for 30 seconds. Significant differences between groups were determined using one-way ANOVA and Tukey tests, with significance denoted by * ** * for $p < 0.0001$. Non-significant differences are indicated by NS. The bars represent the mean, and the vertical dashes above the bars indicate the standard deviation. Control: adhesion buffer; Vehicle: Propylene glycol; PRO: propolis; COR: copaiba oil-resin; NaOCl: sodium hypochlorite.

antibiotic resistance among pathogenic bacteria, the use of antibiotics in a superficial infection seems not completely reasonable. In this way, natural compounds may offer novel mechanisms of action capable of combating antibiotic-resistant strains being important to compare their antimicrobial capacity with conventional antibiotics [47,99,100].

In the present study, the antibiotics polymyxin B sulfate and neomycin sulfate, found in Otosporin®, exhibited lower MIC and MBC values for the tested strains compared to PRO, COR, and PRO + COR, except for *S. oralis*. This difference in susceptibility may be attributed to resistance mechanisms specific to *S. oralis*. Additionally, in the oral environment, *S. oralis* can resist the antimicrobial components of saliva and effectively compete with other oral microorganisms, potentially enhancing its overall resistance in planktonic status [92,93].

It is noteworthy that the MIC and MBC concentrations were determined considering the simultaneous action of these antibiotics in Otosporin®. Higher concentrations are expected if they were used independently. Nonetheless, polymyxin B sulfate and neomycin sulfate are frequently utilized topically to treat skin and mucous membrane infections, often in formulations such as ointments or suspensions. To optimize their efficacy while minimizing toxicity risks, these antibiotics are commonly combined [94]. Regarding to H, MIC and MBC were not possible to be determined in the concentrations tested since bacterial grow was detected in all the samples. The anti-inflammatory action of steroids is well established, however, their influence on bacteria still unclear [101].

Investigating potential synergistic interactions between PRO and COR, FBC indexes were assessed for their combination in propylene glycol and ethanol. A synergistic effect was observed against the *A. naeslundii* strain for PRO + COR in both vehicles, likely due to the reduced survival when exposed to antimicrobial agents in a planktonic state and not associated with other bacterial species. Different interpretations of FBC indexes were observed for *S. mutans* between the vehicles, possibly due to variations in compound distribution within the solvents and the acid tolerance of the bacteria [76–78].

Similar behaviors between vehicles were showed for *E. faecalis* and

S. oralis strains, with addictive and indifference FBC indexes respectively. The detection of indifferent and antagonistic effects has led researchers to explore other combinations with different classes of antimicrobials or even with other compounds isolated from nature that have shown promising antibacterial activity [53]. In our study, typified samples of PRO and COR as well as the combination of them was chosen based on the presence of hydrophobics acids in their composition, which may promote some interactions. The PRO type utilized, BRPX, is known for its high concentration of bioactive compounds such as Artepillin C and Culifolin [47,50], while COR from *Copaifera reticulata Ducke* contains essential oils, predominantly composed of sesquiterpenoids such as caryophyllenes, in addition to diterpene acids [44,45,47]. Further comparisons are necessary to determine the optimal delivery system for natural compounds containing hydrophobic moieties and oil components, such as oil-in-water emulsions with surfactants, and their performance in an antimicrobial context [91].

4.2. Direct contact and intratubular antibiofilm tests

The antibiofilm properties of natural compounds were compared to conventional endodontic agents through a direct contact test, followed by an intratubular disinfection test. In dental infections, most bacteria are not in suspension but rather associated in biofilm communities [4–7,102]. In this context, root dentine tissue represents a particular substrate where microorganisms can adhere and co-aggregate, as seen in dental caries and endodontic infections. Root dentine tissue is a composite consisting of an organic fraction, mainly collagen, and an interpenetrated inorganic fraction, primarily composed of hydroxyapatite [103].

In these experiments, *E. faecalis* and *S. mutans* were selected based on their tracer characteristics. Both bacteria are known for their strong biofilm-forming abilities in oral infections. *E. faecalis* has been detected more frequently in secondary and persistent endodontic infections compared to primary infections. This is attributed to its ability to penetrate dentinal tubules, adhere to dentin collagen, survive with little nutrition and resist common intracanal disinfection procedures. Additionally, its tendency to form biofilms and withstand high pH conditions contributes to its persistence [31,74,75]. Meanwhile, *S. mutans* plays a central role in the formation of cariogenic biofilms [76,78] but it is also found in infected root canals, especially as caries progresses to pulpal inflammation and necrosis [77,78]. *S. mutans* adheres to and forms biofilms on tooth surfaces, producing organic acids as an acidogenic microorganism and exhibiting tolerance to low pH (aciduric) and oxidative stresses [76–78]. As the biofilm matures, *S. mutans* within mixed-species biofilms upregulates the expression of genes involved in matrix exopolysaccharides (EPS) synthesis, which protect the biofilms against antimicrobial agents [104].

Unlike studies that conducted a direct contact test using substances in the absence of a dentine substrate, but rather on polystyrene pegs [105,106], our study submerged dentine discs with biofilms in the evaluated substances. Given that conventional irrigation solutions were evaluated, a test not utilizing a dentine substrate should not be employed to compare their antimicrobial activity [107], particularly concerning NaOCl, which exhibits sanitizing and dissolution properties.

Regarding the application of the intratubular method, it facilitates the investigation of the action of antimicrobial agents on bacteria situated deeper within the dentinal tubules [8,31,32,55]. This differs from the direct contact method as it involves antimicrobial action at distance. These bacteria are likely not associated with large amounts of extracellular matrix, as dentine itself may serve as their primary protection and source of nutrition [31]. That is another reason why the use of dentine substrate has been pivotal.

None of the antimicrobials tested in the present study were able to completely eradicate bacterial viability. Regarding irrigation solutions, all substances demonstrated similar disinfection efficacy in both the direct contact test and in terms of total, superficial, and deep bacterial viability in the intratubular test. PRO and COR when applied at a concentration of 10 % exhibit disinfection comparable to 2.5 % NaOCl. Interestingly, the combination of PRO + COR (in a 1:1 ratio) showed the lowest quantitative values for bacterial viability (direct contact and total intratubular) in relation to 2.5 % NaOCl (not significant). It can suggest that the antimicrobial effects of natural compounds and their combination are influenced by the concentration used as well as the interactions between their components. The effects for NaOCl can also varies according to its concentration [60–62,108]. However, is important to highlight differences between working mechanisms regarding to PRO, COR, and NaOCl.

The antimicrobial mechanism of action of NaOCl can be observed by examining its physicochemical characteristics being a non-specific oxidizing and proteolytic agent that is capable of degrading organic components [19,109,110]. As part of its mechanism, its activity depends on the free available chlorine, which makes part of hypochlorite ions and hypochlorous acid [110], inhibiting bacterial enzymes, including those present in the membrane. Additionally, it is important to note that a high pH of NaOCl (> 11), contributes to its detrimental effects on bacterial cells [109]. The antimicrobial action of NaOCl is extensively studied, and combined with its organic dissolution properties, it establishes it as the gold standard for irrigation in Endodontics. However, a variety of deleterious effects are induced by NaOCl reactions on dentine tissue, which are closely associated with the risk of tooth fracture under subsequent load from treatment operation or mastication [19,111,112]. Research suggests that the greater the concentration of NaOCl used, the more pronounced its effect on dentine mineral content [19]. This raises concerns regarding periapical tissue irritation, which contradicts the contemporary medical principle of "primum non nocere", meaning "first, do no harm" [18–20].

The PRO type utilized, BRPX, is recognized for its high concentration of bioactive compounds, with Artepillin-C (3,5-Diprenyl-4-hydroxycinnamic acid) being its main marker. Additionally, caffeic acid, p-coumaric acid, drupanin, and culifolin were identified like in a previous study [47]. The presence of total flavonoids and phenols is responsible for PRO's antimicrobial and immunomodulatory effect [113,114], demonstrating broad-spectrum efficacy, including against oral bacteria [32,38,40]. Caffeic acid exhibits several pharmacological effects, including anticariogenic potential [115], while Artepillin C, one of the phenolic compounds, has bacteriostatic action [116]. The constituents of PRO facilitate an antimicrobial mechanism by inhibiting ATP synthesis, reducing microbial mobility, increasing cell membrane permeability, disrupting cell membrane potential, and eliciting a host immune response [113,116]. In the present study, it is possible to infer that the antimicrobial effect promoted by PRO was mainly generated by disrupting the bacterial membrane. In the intratubular test, the 10 % PRO applied via the standardized delivery system and flow rate in the main root canal walls reached the larger dentine tubules (superficial dentine) and affected bacterial membrane permeability, disrupting the biofilm, and creating a passage for the constituents to reach bacteria deeply lodged in dentinal tubules (deep dentine).

In this study, a standardized sample obtained from *Copaifera reticulata Ducke*, extracted in the Brazilian Amazon region, was utilized for the analysis. This sample is primarily composed of sesquiterpenoids such as caryophyllenes and diterpene acids, particularly copalic and polyalthic acids, which are known to confer significant biological activities to the oil-resin [47,53,117]. Previous research has suggested that the antimicrobial properties of COR may be attributed to its lipophilic nature [53,118]. Diterpenes have been implicated in inducing bacterial lysis and disrupting cell membranes due to their structural lipophilic characteristics [119]. Moraes *et al.* showed the effectiveness of *Copaifera pubiflora* oleoresin, and its associated acids against *S. mutans* and *Porphyromonas gingivalis*. It was demonstrated that higher concentrations of *C. pubiflora* oleoresin could disrupt the bacterial membrane, leading to compromised membrane integrity and permeability, resulting in the loss of nucleic acids (DNA and RNA) and proteins, ultimately leading to cell

death [53]. Linking these findings to our present study, 10 % COR disrupted the bacterial membrane due to its lipophilic specificity in both sets of tests (direct contact and intratubular). Like the results obtained for 10 % PRO, 10 % COR exhibited the ability to penetrate deeply into dentinal tubules and promote disinfection.

Considering the results obtained for the PRO + COR combination (ratio 1:1), it is plausible to hypothesize that the mixture of compounds preserves the antimicrobial properties of each individual component, potentially leading to effectivity against *E. faecalis* and *S. mutans* biofilms. However, these effects did not demonstrate a significant difference compared to other treatments in the study. To achieve optimal efficacy, it would be essential to determine the ideal concentration of both PRO and COR to establish conditions capable of effectively disinfecting the root canal system during irrigation procedures. Various factors such as the delivery system, flow rate, exposure time, and volume application can significantly influence the efficacy of disinfection [8,60–62].

In the present study, a 1:1 mixture from 10 % stock solutions of PRO and COR was selected and applied in the main root canal as a closed-end system, where there is no contact with live tissues. This choice was informed by the anti-inflammatory properties demonstrated by both PRO and COR. Furthermore, the PRO + COR combination has been found to promote proliferation of human periodontal ligament fibroblasts which are the cells present around the apical area and are involved in the tissue response generated by endodontic materials [47]. Despite NaOCl's gold standard status in Endodontics, an alternative solution with anti-inflammatory properties for disinfection in the root canal system may prove beneficial for adjunctive steps such as final rinse or ultrasonic activation use. It could also aid in reducing the volume of NaOCl used during RCT, thus aiming to mitigate destructive effects on dentine tissue and facilitate the repair of apical periodontitis. However, additional research is necessary to fully comprehend the effects of PRO, COR, and PRO + COR on the mechanical properties of dentine tissue.

Regarding intracanal medication, Otosporin® showed the highest bacterial viability in the direct contact test, with a median between the control and the CH paste. However, there is limited literature on the action of Otosporin® against biofilms. Primarily used to treat otitis and ear infections, Otosporin® targets pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Gram-negative bacilli, and anaerobes [120]. Nevertheless, this medication is not specifically formulated or indicated for targeting biofilms. The limited action of Otosporin® in the direct contact test may be due to the possibility of a larger amount of EPS matrix protecting bacteria [31]. In the intratubular scope, Otosporin® exhibited antimicrobial action like the other treatments. However, these results should be interpreted with caution since in clinical conditions, Otosporin® is often restricted to the pulp chamber space (delivered within embedded cottons) after the removal of the affected portion of the pulp [47,98]. In the present study, all main root canal extensions evaluated were filled with Otosporin® to standardize the medication conditions in the intratubular test, which likely contributed to the results.

It is also important to highlight that chemomechanical preparation is the main mechanism responsible for the elimination of biofilms during complete RCT [9–12]. Some intracanal medications, such as CH paste, are recommended after chemomechanical preparation, particularly in cases of dental pulp necrosis and apical periodontitis to enhance root canal disinfection, preventing reinfection being a physic-chemical barrier in the root canal, and eliminating any remaining bacteria [30–34]. PRO, COR, and PRO + COR demonstrated comparable antimicrobial activity to CH paste in both the direct contact and intratubular tests. It is noteworthy that the selection of MBC concentrations was used on a prolonged exposure period of the biofilms to the medications (7 days). Higher concentrations than MBC could be used as intracanal medication. However, MBC concentrations were chosen considering the longer period of contact between the root canal walls and medications employed. A stronger action would be expected to occur within a shorter period of contact, such as in irrigation procedures.

The present study corroborates previous findings regarding the antimicrobial efficacy of propolis (PRO), as demonstrated in earlier research [38–40]. Cunha Neto *et al.* conducted an intratubular assay revealing that both CH and PRO were comparably effective to a triple antibiotic paste containing ciprofloxacin, metronidazole, and minocycline, diluted in propylene glycol [39]. Pereira *et al.* further supported these findings by showing that the combination of CH with PRO in propylene glycol achieved significant disinfection of dentine tubules [32]. In contrast, to the best of our knowledge, there is not literature available regarding the intratubular action of copaiba oil-resin (COR). However, considering the documented antimicrobial and anti-inflammatory properties of both PRO and COR, they could serve as alternative intracanal medications to mitigate the potential negative effects of CH, particularly its toxicity [35].

In deeper dentin tubules, PRO + COR was found to be less effective compared to CH paste. This observation is not surprising, considering that dentinal tubules are significantly smaller spaces, and the substances have different antimicrobial mechanisms of action and diffusion. The antibacterial effect of CH pastes is primarily attributed to the nonspecific bactericidal action of high alkalinity attributed to its pH [121]. However, in clinical scenarios, the alkalinity and antimicrobial properties of CH pastes may be compromised when combined with an intense buffering system [31]. Regarding PRO + COR, it is possible to hypothesize that this combination may form larger molecular complexes that are not as readily able to diffuse into deeper dentin tubules. Meanwhile, CH paste acted by releasing ions in contact with the main root canal walls.

Vehicles associated with CH are known to be harmless to microorganisms; however, they can influence the properties of these medications [31]. Since the vehicles used for CH paste can impact ionic release, they directly influence pH values [122] and exert biological activities on both bacteria and tissues [123]. Propylene glycol, as a viscous vehicle, induces a more favorable release of calcium and hydroxyl ions compared to aqueous vehicles like distilled water and saline solution, resulting in a higher pH [31,83,84,122]. In addition, the non-antimicrobial action of propylene glycol was proved in an intratubular model with similar superficial and deep bacterial viabilities to not treated group [124,125]. Despite that, the penetration of intracanal medication through difficult-to-reach areas remains a challenge for effectively eliminating remaining bacteria [126].

Strategies have been proposed to improve the penetration and efficacy of intracanal medications. These include the incorporation of natural substances into CH paste [32] and the utilization of ultrasonic agitation techniques [126,127]. The latter method has shown promise in minimizing the duration of medication within the root canal, thereby optimizing the decontamination process during RCT [126]. Considering the potential of natural antimicrobial compounds evaluated in the present study, employing ultrasonic agitation could be a promising approach to address these challenges.

4.3. Immediate antibiofilm effect tests

This model aimed to evaluate the removal and characteristics of biofilms by means of OCT and CFU/cm^2 counting after irrigant application on statically grown biofilms confected on saliva-coated dentine discs. This approach enables access to additional data concerning the working mechanism of each substance when in contact with oral biofilms, as CLSM is commonly employed to assess the effects of chemical treatments on biofilms, focusing mainly on bacterial killing and not biofilm removal [8,61]. To achieve this goal, the biofilm model with clinical isolates of *S. oralis* J22 and *A. naeslundii* T14V-J1 was selected, with some modifications performed. This dual-species biofilm presents well-established characteristics in the literature [8,60–62,81]. Both bacteria create coaggregation-mediated interactions, forming robust biofilms from the earliest point, and are involved in persistent endodontic infections [81,128].

In contrast to the irrigation solution test conducted in direct contact settings, the immediate antibiofilm action involved gently pipetting a minimal volume of solution (40 μL) over the biofilm samples, which were then left undisturbed for 30 s. This test, designed based on the confirmed antimicrobial action of solutions, aimed to simulate a realistic contact surface area between the biofilm substrate and solutions while omitting convection. Consequently, only the diffusion-induced chemical effects could be investigated [60,61].

When assessing the potential biofilm reduction promoted by natural compounds compared to NaOCl, it's preferable to employ minimal volumes. This is because rinsing with large volumes of NaOCl renders the test incapable of providing a comparison for biofilm reduction since the biofilms could be completely dissolved. This arises due to the dissolution properties and fast chlorine consumption generated by NaOCl [19,109,110]. In the present study, a concentration of 2 % NaOCl was selected such as the employed in previous studies using this dual-species biofilm model [8,60–62,108].

Besides structural differences between differently grown biofilms, resistant spots and layer stratification within the same biofilm can be present. Regions with high bacterial density, fluffy top layers, and compacted basal layers have been described [61,62,129]. For instance, young static biofilms confected in 4 days can simulate a fluffier top layer, while models performed on constant depth film fermenters could represent the basal cell-dense layer or resistant spots of an oral biofilm [8,61,81].

In this study, several pilots were performed to select a 7-day static biofilm model that could provide stratification on standardized samples and represent a biofilm that is not entirely compact compared to those from a constant depth film fermenter model. This approach allowed for the consideration of an intermediary biofilm model that could provide a feasible visualization of the effects provoked by contacts between irrigants and samples once highly dense bacterial biofilms, characterized by reduced water and EPS content, proved resistant to effective removal by potent biocides like NaOCl [61]. In the present study, 2 % NaOCl caused the most significant biofilm reduction presenting the lowest biofilm mean thickness (height) after 30 s of application, followed by 10 % COR, PRO + COR (1:1), and finally 10 % PRO. The natural antimicrobial solutions were not different between them. While solutions may possess biofilm reduction properties, NaOCl exhibits the fastest reaction when in contact with biofilm surfaces at an early stage. However, it's important to highlight its instability, especially when applied statically since the reactive components of NaOCl are progressively deactivated upon contact with organic substrates, such as the biofilm [110,130]. This differs from natural antimicrobial compounds, which act on matrix permeability and bacterial membrane disruption via interactions provided by their organic constituents [32,38,40,47,53,113–119].

Multiple assessments can be conducted to analyze biofilm structures using OCT [68]. In addition to biofilm height, parameters such as roughness coefficient (Ra*) and porosity are evaluated. Ra* is particularly useful for comparing the structure of different biofilms [67], making it applicable for biofilms subjected to various treatments. In the present study, the highest roughness coefficient was observed after NaOCl contact, followed by PRO + COR (with means about 0.5), indicating an increase in irregularity at the biofilm interface compared to the controls (Ra* approximately 0.3). Given the demonstrated effectiveness of NaOCl in biofilm reduction, the finding of the highest roughness coefficient after NaOCl treatment aligns with expectations.

Meanwhile, porosity is an important structural parameter that considers irregularities within the biofilm, reflecting its internal structure. In addition, porosity is directly connected to transport processes within and through the biofilm [68,131,132]. Interestingly, the group treated with 10 % PRO exhibited the highest porosity (mean above 2), which was statistically different from the other groups. The porosity results may be linked to structural organization of biofilms (mainly of water, matrix, and microorganisms) which determines the susceptibility of biofilms to biocides. The existence of a highly negatively charge within the biofilm matrix is ensured through hydrophobic interactions, and provides a barrier to antimicrobial diffusion, shielding the biofilm from chemical stresses [133–136].

In a previous study involving dense *S. oralis* J22 with *A. naeslundi* T14V-J1 biofilms grown in a constant depth film fermenter, it was found that 2 % chlorhexidine, an antimicrobial agent, was ineffective in chemically removing the biofilm structure. Instead, it prompted a re-arrangement of the biofilm structure. It was suggested that the observed structural rearrangement could be attributed to attractive forces between EPS and the solution, with originally negatively charged EPS relocating upwards, attracted by, and bound to positively charged chlorhexidine [61].

In the present study, the potential for biofilm re-arrangements, with charged EPS moving upwards, is considered, particularly given the increased porosity observed, especially for 10 % PRO. However, unlike chlorhexidine in the previous study, a reduction in biofilm thickness was observed with all tested solutions beyond an antimicrobial action confirmed by CFU/cm^2 counting. It is plausible to hypothesize that the chemical attack exerted by the natural compounds could induce both biofilm reduction and re-arrangement of the subjacent cell layer as an early response of the biofilm to chemical stress, exhibiting more open spaces potentially due to their ability to induce disruption in the matrix and bacterial membrane [113,116].

In terms of CFU/cm^2 counting, 2 % NaOCl exhibited the most significant disinfection compared to natural antimicrobial compounds. However, it's worth noting that in root canal irrigation, higher volumes of solutions and longer exposure periods, as seen in direct contact and intratubular assays, are applied. Additionally, mechanical forces such as shear stress and irrigation flow rate can have a significant impact on biofilm removal. The scope of the present study focused on investigating the chemical disinfection performance via different working models. Further investigations are necessary to explore the disinfection efficacy of natural antimicrobial compounds while simulating mechanical forces in RCT.

4.4. Clinical translation of working models

To bridge the present findings with clinical applications, it is essential to consider the varying levels of contamination and the diverse status of microorganisms within the root canal system, which depend on the condition of the dental pulp tissue. When the vital dental pulp tissue is exposed to microorganisms, the lack of timely intervention allows microbial colonization to cause pulp necrosis and the spread of infection throughout the root canal system, affecting the periapical region of the teeth [137]. The activation of the host immune response triggers local acute and/or chronic inflammation, leading to resorption and destruction of periapical tissues and, consequently, the formation of periapical lesions [138–140], consisting in the apical periodontitis disease. In other words, it is an inflammatory response triggered by pathogens and their toxins within the root canal system [10,102].

Bacteria are the most prevalent and dominant microorganisms in endodontic microbiota [102], typically forming sessile, multispecies communities—biofilms—attached to the dentinal walls of the root canal system [141,142]. However, microorganisms adapt to various environmental conditions through a wide range of physiological and morphological changes, with biofilms progressing through distinct stages of formation [142–144]. In this context, the present study identified interesting correlating trends.

In macrodilution models, the contact surface between bacteria and the tested substance occurs in a liquid environment, wherein is important to consider the planktonic state of bacteria and their behavior in response to potential antimicrobials. Translating to endodontic infections, planktonic microorganisms may originate from sources such as dental caries, accidental trauma, inadequate restorations, pulp exposure, or other irritants [145–147]. The adhesion of planktonic microorganisms to a substrate is a prerequisite for the subsequent formation of

an organized and dense biofilm. Planktonic bacteria can coexist with biofilms, particularly in fluid-filled areas, where some bacteria may disperse from the biofilm into the surrounding environment as planktonic cells [142]. This dispersal is a natural process that enables bacteria to colonize new areas, spread infection, or adapt to environmental changes [143]. Then, it is important to detect antimicrobial action against the planktonic phase before testing on a sessile biofilm structure. For example, the determination of MIC and MBC values for natural compounds, such as PRO and COR, as well as conventional used substances, yielded promising results and laid the foundation for the subsequent models employed and tested.

In models where the contact surface between the evaluated substance and the biofilm was relatively large, such as biofilms on dentine discs, direct contact was considered. Conversely, intratubular analyses assessed the antimicrobial activity of natural compounds against bacteria within dentine tubules, mimicking a biofilm packed in smaller structures of the root canal. In these cases, the most significant contact occurred between the evaluated substance and the dentine root canal walls. Once microorganisms are organized into dense biofilms, the microbiome exhibits variations depending on the type of infection, which is closely associated with the condition of apical periodontitis [102]. Dense bacterial biofilm models have been employed to more accurately mimic the basal layer of the biofilm, as demonstrated with *S. mutans* and *E. faecalis* or *S. oralis* and *A. naeslundii*. The latter has been particularly used to investigate short contact periods between tested substances and biofilm surfaces.

In cases of primary apical periodontitis caused by infection of the necrotic dental pulp, mixed bacterial communities predominantly consist of obligate anaerobic species, with 20–30 species often forming the core microbiome [102,141,148]. To combat infection biofilms during RCT, the use of effective antimicrobial volumes and concentrations is recommended [61,108], as chemomechanical preparation plays a critical role in achieving disinfection [7,11]. When tested as root canal irrigants, PRO, COR, and their combination PRO + COR demonstrated significant antimicrobial activity, comparable to that of NaOCl in both direct contact and intratubular evaluated models.

Is also important to highlight the bacteria species selected for the models employed in this study. Considering that, the post-treatment apical periodontitis is characterized by the persistence of a periapical lesion caused by persistent or secondary intra-radicular infections. These infections often involve a combination of anaerobic and facultative bacteria, indicating a mixed infection [102,149]. Among the frequently detected bacteria in post-treatment infections, *Streptococcus* and *Actinomyces* species are abundant, with *E. faecalis* being one of the most prevalent species identified in the root canals of teeth with post-treatment apical periodontitis [149–151].

The establishment of an intratubular contamination protocol involving *S. mutans* and *E. faecalis* is also innovative, as it provided the *in vitro* coexistence of a cariogenic/root canal pathogen (*S. mutans*) [76] with another pathogen (*E. faecalis*) that exhibits greater capability for dentinal tubule penetration, collagen adhesion, and survival under harsh conditions [31,74,75], consisting of a biofilm that can represents an infection in both primary and post-treatment apical periodontitis. In managing post-treatment apical periodontitis, the use of intracanal medication is strongly recommended [32]. The findings of the present study highlight the antimicrobial potential of natural compounds, which were evaluated after 7 days of contact with biofilms and compared to Otosporin® and CH pastes. Notably, for the intratubular biofilm model, higher concentrations of natural compounds could be further explored to achieve deeper bacterial eradication. Additionally, strategies such as ultrasonic agitation have shown promising results, particularly with 10 % PRO as irrigation solution [152]. This approach facilitates the delivery of irrigation solutions or intracanal medications deep into the dentine mass [152].

In the *S. oralis* and *A. naeslundii* models, the primary goal was to investigate biofilm removal capacity. It was suggested that exposure times longer than 30 seconds are required to achieve a chemical effect like that of NaOCl. Clinically, the complex root canal anatomy further complicates biofilm removal, and any remaining biofilms after treatment may survive [8]. Additionally, it is known that increasing the volume of NaOCl used can achieve greater biofilm removal in less time [60]. However, in a clinical context, prolonged exposure of dentine to the strong oxidative action of NaOCl should be avoided [18], provided that sufficient irrigation volume is available for effective root canal disinfection [60,111].

The present findings support the potential of PRO, COR, and PRO + COR as adjunctive strategies to complement root canal system disinfection. These natural compounds not only facilitate biofilm removal but may also help minimize adverse effects on the physico-mechanical properties of dentine. However, some limitations include the need to develop optimal delivery systems for PRO and COR, given their hydrophobic characteristics. Additionally, it is important to further investigate the antimicrobial activity of these natural compounds in combination with mechanical forces, as employed during root canal chemo-mechanical preparation, since this process is crucial and plays a significant role in the removal of oral biofilms in RCT [7,11].

4.5. Future perspectives

This study reaffirms the significant challenges associated with biofilm removal and the potential harmful effects of conventional root canal decontamination techniques, underscoring the pressing need for new and alternative antimicrobial agents. The demand for natural products is growing, driven by alarming projections of increased human mortality from infections caused by resistant bacteria [135,153], alongside a rising consumer preference for health-conscious and sustainable solutions. These trends are further supported by the economic and ecological benefits of utilizing natural resources, as well as the increasing global emphasis on environmental sustainability, ethical consumption, and the need for natural bioactive compounds [153].

By contributing to future medical advancements, this study lays the groundwork for the safe and effective use of natural compounds, integrating them with emerging technologies to enhance root canal disinfection and promote the healing of apical periodontitis. However, besides biological investigation [47], further research is required to optimize the application of natural compounds, focusing particularly on achieving deeper biofilm penetration. The development of drug carriers presents a promising solution for this challenge, enabling the creation of formulations that ensure the presence of the primary active agents in PRO, COR, or their combination PRO + COR, thereby ensuring their efficacy in endodontic applications.

5. Conclusion

Highlighting the range of assays performed, our study examined the effects of natural antimicrobial compounds, PRO and COR, as well as their combination PRO + COR, against endodontic pathogens using macrodilution, direct contact, and intratubular analysis with a traced dual-species biofilm. The results demonstrate that these compounds can promote disinfection comparable to available agents in Endodontics. In an immediate condition, OCT analysis revealed similar reductions in biofilm thickness for PRO, COR, and PRO + COR, corroborated by CFU/cm^2 counting. Notably, NaOCl exhibited the greater reduction in biofilm thickness and the most significant antimicrobial action. These findings suggest the antimicrobial potential of PRO, COR, and PRO + COR in various tested models, emphasizing the possibility for complementary approaches in RCT with these natural products.

Funding sources

This work was supported by the Coordination of Higher Education and Post-Graduation (CAPES), FAPESP (grants 2010/20186-3; 2020/

12726-0), and the Abel Tasman Talent Program (ATTP) of the Graduate School of Medical Sciences of the University of Groningen.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors express their gratitude to all members of the research team for their valuable contributions to this work.

Data availability

Data will be made available on request.

References

[1] C.S. Tibúrcio-Machado, C. Michelon, F.B. Zanatta, M.S. Gomes, J.A. Marin, C. A. Bier, The global prevalence of apical periodontitis: a systematic review and meta-analysis, Int. Endod. J. 54 (5) (2021) 712–735, https://doi.org/10.1111/iej.13467.

[2] S.R. Hernández, J.F. Siqueira, Jr, D.D. Voigt, G. Soimu, S.C. Brasil, J. C. Provenzano, I. Mdala, F.R.F. Alves, I.N. Rôças, Bacteriologic conditions of the apical root canal system of teeth with and without posttreatment apical periodontitis: a correlative multianalytical approach, J. Endod. 50 (2) (2024) 154–163, https://doi.org/10.1016/j.joen.2023.11.005.

[3] S. Kakehashi, Hr Stanley, Fitzgerald, Rj. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats, Oral. Surg. Oral. Med. Oral. Pathol. 20 (1965) 340–349, https://doi.org/10.1016/0030-4220(65)90166-0.

[4] S. Friedman, Prognosis of healing in treated teeth with endodontic infections, in: A.F. Fouad (Ed.), Endodontic microbiology, 2nd ed, Wiley & Sons, 2017, pp. 341–384, https://doi.org/10.1002/9781119080343.ch15.

[5] P.E. Kolenbrander, R.J.Jr Palmer, S. Periasamy, N.S. Jakubovics, Oral multispecies biofilm development and the key role of cell-cell distance, Nat. Rev. Microbiol 8 (7) (2010) 471–840, https://doi.org/10.1038/nrmicro2381.

[6] D. Ricucci, J.F.Jr Siqueira, Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings, J. Endod. 36 (8) (2010) 1277–1288, https://doi.org/10.1016/j.joen.2010.04.007.

[7] A.R. Pérez, D. Ricucci, G.C.S. Vieira, J.C. Provenzano, F.R.F. Alves, M. F. Marceliano-Alves, I.N. Rôças, J.F.Jr Siqueira, Cleaning, shaping, and disinfecting abilities of 2 instrument systems as evaluated by a correlative micro-computed tomographic and histobacteriologic approach, J. Endod. 46 (6) (2020) 846–857, https://doi.org/10.1016/j.joen.2020.03.017.

[8] T.C. Pereira, R.J.B. Dijkstra, X. Petridis, P.K. Sharma, W.J. van de Meer, L.W. M. van der Sluis, F.B. de Andrade, Chemical and mechanical influence of root canal irrigation on biofilm removal from lateral morphological features of simulated root canals, dentine discs and dentinal tubules, Int Endod. J. 54 (1) (2021) 112–129, https://doi.org/10.1111/iej.13399.

[9] H. Zandi, N. Petronijevic, I. Mdala, A.K. Kristoffersen, M. Enersen, I.N. Rôças, J.F. Jr Siqueira, D. Ørstavik, Outcome of endodontic retreatment using 2 root canal irrigants and influence of infection on healing as determined by a molecular method: a randomized clinical trial, J. Endod. 45 (9) (2019) 1089–1098.e5, https://doi.org/10.1016/j.joen.2019.05.021.

[10] J.F.Jr Siqueira, I.N. Rôças, Clinical implications and microbiology of bacterial persistence after treatment procedures, J. Endod. 34 (11) (2008) 1291–1301.e3, https://doi.org/10.1016/j.joen.2008.07.028.

[11] J.F.Jr Siqueira, A.R. Pérez, M.F. Marceliano-Alves, J.C. Provenzano, S.G. Silva, F. R. Pires, G.C.S. Vieira, I.N. Rôças, F.R.F. Alves, What happens to unprepared root canal walls: a correlative analysis using micro-computed tomography and histology/scanning electron microscopy, Int. Endod. J. 51 (5) (2018) 501–508, https://doi.org/10.1111/iej.12753.

[12] F.C. Martinho, B.P. Gomes, Quantification of endotoxins and cultivable bacteria in root canal infection before and after chemomechanical preparation with 2.5% sodium hypochlorite, J. Endod. 34 (3) (2008) 268–272, https://doi.org/10.1016/j.joen.2007.11.015.

[13] M.A. Versiani, G.B. Leoni, L. Steier, G. De-Deus, S. Tassani, J.D. Pécora, M.D. de Sousa-Neto, Micro-computed tomography study of oval-shaped canals prepared with the self-adjusting file, Reciproc, WaveOne, and ProTaper universal systems, J. Endod. 39 (8) (2013) 1060–1066, https://doi.org/10.1016/j.joen.2013.04.009.

[14] C.E. Radcliffe, L. Potouridou, R. Qureshi, N. Habahbeh, A. Qualtrough, H. Worthington, D.B. Drucker, Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms Actinomyces israelii, A. naeslundii, Candida albicans and Enterococcus faecalis, Int. Endod. J. 37 (7) (2004) 438–446, https://doi.org/10.1111/j.1365-2591.2004.00752.x.

[15] A.L.B. Li, M. Markvart, P.V. Abbott, Effect of different concentrations of sodium hypochlorite on the compressive strength of endodontically treated roots, J. Endod. 48 (3) (2022) 370–374, https://doi.org/10.1016/j.joen.2021.12.006.

[16] B.H. Sen, P.R. Wesselink, M. Türkün, The smear layer: a phenomenon in root canal therapy, Int Endod. J. 28 (3) (1995) 141–148, https://doi.org/10.1111/j.1365-2591.1995.tb00289.x.

[17] A. Oyarzún, A.M. Cordero, M. Whittle, Immunohistochemical evaluation of the effects of sodium hypochlorite on dentin collagen and glycosaminoglycans, J. Endod. 28 (3) (2002) 152–156, https://doi.org/10.1097/00004770-200203000-00002.

[18] L.S. Gu, X.Q. Huang, B. Griffin, B.R. Bergeron, D.H. Pashley, L.N. Niu, F.R. Tay, Primum non nocere - the effects of sodium hypochlorite on dentin as used in endodontics, Acta Biomater. 61 (2017) 144–156, https://doi.org/10.1016/j.actbio.2017.08.008.

[19] H. Xu, Z. Ye, A. Zhang, F. Lin, J. Fu, A.S.L. Fok, Effects of concentration of sodium hypochlorite as an endodontic irrigant on the mechanical and structural properties of root dentine: a laboratory study, Int Endod. J. 55 (10) (2022) 1091–1102, https://doi.org/10.1111/iej.13800.

[20] C.M. Bramante, J.A. Duque, B.C. Cavenago, R.R. Vivan, A.S. Bramante, F.B. de Andrade, M.A. Duarte, Use of a 660-nm laser to aid in the healing of necrotic alveolar mucosa caused by extruded sodium hypochlorite: a case report, J. Endod. 41 (11) (2015) 1899–1902, https://doi.org/10.1016/j.joen.2015.07.011.

[21] E.G. Trevino, A.N. Patwardhan, M.A. Henry, G. Perry, N. Dybdal-Hargreaves, K. M. Hargreaves, A. Diogenes, Effect of irrigants on the survival of human stem cells of the apical papilla in a platelet-rich plasma scaffold in human root tips, J. Endod. 37 (8) (2011) 1109–1115, https://doi.org/10.1016/j.joen.2011.05.013.

[22] N.B. Ruparel, F.B. Teixeira, C.C. Ferraz, A. Diogenes, Direct effect of intracanal medicaments on survival of stem cells of the apical papilla, J. Endod. 38 (10) (2012) 1372–1375, https://doi.org/10.1016/j.joen.2012.06.018.

[23] J.F. Siqueira, Jr, H.P. Lopes, Mechanisms of antimicrobial activity of calcium hydroxide: a critical review, Int Endod. J. 32 (5) (1999) 361–369, https://doi.org/10.1046/j.1365-2591.1999.00275.x.

[24] M. Naseri, L. Eftekhar, F. Gholami, M. Atai, O. Dianat, The effect of calcium hydroxide and nano-calcium hydroxide on microhardness and superficial chemical structure of root canal dentin: an ex vivo study, J. Endod. 45 (9) (2019) 1148–1154, https://doi.org/10.1016/j.joen.2019.06.002.

[25] P.C. Foreman, I.E. Barnes, Review of calcium hydroxide, Int Endod. J. 23 (6) (1990) 283–297, https://doi.org/10.1111/j.1365-2591.1990.tb00108.x.

[26] D.D. Rice, A. Grandhi, G.D. Roque-Torres, U. Oyoyo, L.K. Bakland, The effect of calcium hydroxide pastes on isolated vital nerve fibers, J. Endod. 50 (3) (2024) 355–361, https://doi.org/10.1016/j.joen.2023.11.011.

[27] E.K. Sirén, E. Kerosuo, E. Lavonius, J.H. Meurman, M. Haapasalo, Ca(OH)2 application modes: in vitro alkalinity and clinical effect on bacteria, Int Endod. J. 47 (7) (2014) 628–638, https://doi.org/10.1111/iej.12199.

[28] Z. Mohammadi, P.M. Dummer, Properties and applications of calcium hydroxide in endodontics and dental traumatology, Int Endod. J. 44 (8) (2011) 697–730, https://doi.org/10.1111/j.1365-2591.2011.01886.x.

[29] F. Elmsmari, J.A. González Sánchez, F. Duran-Sindreu, R. Belkadi, M. Espina, M. L. García, E. Sánchez-López, Calcium hydroxide-loaded PLGA biodegradable nanoparticles as an intracanal medicament, Int. Endod. J. 54 (11) (2021) 2086–2098, https://doi.org/10.1111/iej.13603.

[30] X. Roig, L. Halbaut, F. Elmsmari, R. Pareja, A. Arrien, F. Duran-Sindreu, L. M. Delgado, M. Espina, M.L. García, J.A.G. Sánchez, E. Sánchez-López, Calcium hydroxide-loaded nanoparticles dispersed in thermosensitive gel as a novel intracanal medicament, Int. Endod. J. 57 (7) (2024) 907–921, https://doi.org/10.1111/iej.14041.

[31] V.F. Pedrinha, M.R.C. Cuellar, M.C. de Barros, P.C.G. Titato, M.A. Shahbazi, P. K. Sharma, F.B. de Andrade, The vehicles of calcium hydroxide pastes interfere with antimicrobial effect, biofilm polysaccharidic matrix, and pastes' physicochemical properties, Biomedicines 10 (12) (2022) 3123, https://doi.org/10.3390/biomedicines10123123.

[32] T.C. Pereira, L.R. da Silva Munhoz Vasconcelos, M.S.Z. Graeff, M.C.M. Ribeiro, M. A.H. Duarte, F.B. de Andrade, Intratubular decontamination ability and physicochemical properties of calcium hydroxide pastes, Clin. Oral. Invest. 23 (3) (2019) 1253–1262, https://doi.org/10.1007/s00784-018-2549-0.

[33] R.F. Zancan, R.R. Vivan, M.R. Milanda Lopes, P.H. Weckwerth, F.B. de Andrade, J.B. Ponce, M.A. Duarte, Antimicrobial activity and physicochemical properties of calcium hydroxide pastes used as intracanal medication, J. Endod. 42 (12) (2016 Dec) 1822–1828, https://doi.org/10.1016/j.joen.2016.08.017.

[34] G.H. Yassen, M.M. Vail, T.G. Chu, J.A. Platt, The effect of medicaments used in endodontic regeneration on root fracture and microhardness of radicular dentine, Int Endod. J. 46 (7) (2013) 688–695, https://doi.org/10.1111/iej.12046.

[35] O. Swanljung, M.M. Vehkalahti, Root canal irrigants and medicaments in endodontic malpractice cases: a nationwide longitudinal observation, J. Endod. 44 (4) (2018) 559–564, https://doi.org/10.1016/j.joen.2018.01.003.

[36] G. Kayaoglu, H. Ömürlü, G. Akca, M. Gürel, Ö. Gençay, K. Sorkun, B. Salih, Antibacterial activity of Propolis versus conventional endodontic disinfectants against Enterococcus faecalis in infected dentinal tubules, J. Endod. 37 (3) (2011) 376–381, https://doi.org/10.1016/j.joen.2010.11.024.

[37] M. Viuda-Martos, Y. Ruiz-Navajas, J. Fernández-López, J.A. Pérez-Alvarez, Functional properties of honey, propolis, and royal jelly, J. Food Sci. 73 (9) (2008) R117–R124, https://doi.org/10.1111/j.1750-3841.2008.00966.x.

[38] F.B. Ferreira, S.A. Torres, O.P. Rosa, C.M. Ferreira, R.B. Garcia, M.C. Marcucci, B. P. Gomes, Antimicrobial effect of propolis and other substances against selected endodontic pathogens, Oral. Surg. Oral. Med Oral. Pathol. Oral. Radio. Endod. 104 (5) (2007) 709–716, https://doi.org/10.1016/j.tripleo.2007.05.019.

[39] M.M. Madhubala, N. Srinivasan, S. Ahamed, Comparative evaluation of propolis and triantibiotic mixture as an intracanal medicament against Enterococcus faecalis, J. Endod. 37 (9) (2011) 1287–1289, https://doi.org/10.1016/j.joen.2011.05.028.

[40] M.A.D. Cunha Neto, J.A. Coêlho, K.P. Pinto, M.R.C. Cuellar, M.C. Marcucci, E.J.N. L. Silva, F.B. Andrade, L.M. Sassone, Antibacterial efficacy of triple antibiotic medication with macrogol (3Mix-MP), Traditional triple antibiotic paste, calcium hydroxide, and ethanol extract of propolis: an intratubular dentin ex vivo confocal laser scanning microscopic study, J. Endod. 47 (10) (2021) 1609–1616, https://doi.org/10.1016/j.joen.2021.07.014.

[41] Z. Ahangari, M. Naseri, M. Jalili, Y. Mansouri, F. Mashhadiabbas, A. Torkaman, Effect of propolis on dentin regeneration and the potential role of dental pulp stem cell in Guinea pigs, Cell J. 13 (4) (2012) 223–228.

[42] A.C.D.S. Menezes, L.D.B. Alves, D.C. Goldemberg, A.C. de Melo, H.S. Antunes, Anti-inflammatory and wound healing effect of Copaiba oleoresin on the oral cavity: A systematic review, Heliyon 8 (2) (2022) e08993, https://doi.org/10.1016/j.heliyon.2022.e08993.

[43] D.K. Bardají, J.J. da Silva, T.C. Bianchi, D. de Souza Eugênio, P.F. de Oliveira, L. F. Leandro, H.L. Rogez, R.C. Venezianni, S.R. Ambrosio, D.C. Tavares, J.K. Bastos, C.H. Martins, Copaifera reticulata oleoresin: chemical characterization and antibacterial properties against oral pathogens, Anaerobe 40 (2016) 18–27, https://doi.org/10.1016/j.anaerobe.2016.04.017.

[44] R. da Trindade, J.K. da Silva, W.N. Setzer, *Copaifera* of the neotropics: a review of the phytochemistry and pharmacology, Int. J. Mol. Sci. 19 (5) (2018) 1511, https://doi.org/10.3390/ijms19051511.

[45] C. Herrero-Jáuregui, M.A. Casado, M. das Graças Bichara Zoghbi, R. Célia Martins-da-Silva, Chemical variability of Copaifera reticulata Ducke oleoresin, Chem. Biodivers. 8 (4) (2011) 674–685, https://doi.org/10.1002/cbdv.201000258.

[46] A.D. Garrido, R.C. Lia, S.C. França, J.F. da Silva, S. Astolfi-Filho, M.D. Sousa-Neto, Laboratory evaluation of the physicochemical properties of a new root canal sealer based on Copaifera multijuga oil-resin, Int. Endod. J. 43 (4) (2010) 283–291, https://doi.org/10.1111/j.1365-2591.2009.01678.x.

[47] V.F. Pedrinha, L.M. Santos, C.P. Gonçalves, M.T. Garcia, O.A. Lameira, C. L. Queiroga, M.C. Marcucci, M.A. Shahbazi, P.K. Sharma, J.C. Junqueira, C. R. Sipert, F.B. de Andrade, Effects of natural antimicrobial compounds propolis and copaiba on periodontal ligament fibroblasts, molecular docking, and in vivo study in Galleria mellonella, Biomed. Pharm. 171 (2024) 116139, https://doi.org/10.1016/j.biopha.2024.116139.

[48] C.C.B. Meneses, L.T. Olivi, C.N. Carvalho, G. Gavini, C.R. Sipert, Cytotoxic effect of niobium phosphate glass-based gutta-percha points on periodontal ligament fibroblasts in vitro, J. Endod. 46 (9) (2020) 1297–1301, https://doi.org/10.1016/j.joen.2020.06.016.

[49] B.J. Moncla, P.W. Guevara, J.A. Wallace, M.C. Marcucci, J.E. Nor, W.A. Bretz, The inhibitory activity of typified propolis against Enterococcus species, Z. Nat. C. J. Biosci. 67 (5-6) (2012) 249–256, https://doi.org/10.1515/znc-2012-5-603.

[50] R.P. Adams, Allured Publ. Identification of essential oil components by gas chromatography/mass spectroscopy, Corp. Carol Stream IL, 1995.

[51] J.L. Pasquel-Reátegui, L.C. dos Santos, F.M. Barrales, V.L. Grober, M.B. Soares Forte, A. Sartoratto, C.L. Queiroga, J. Martínez, Fractionation of sesquiterpenes and diterpenic acids from copaiba (Copaifera officinalis) oleoresin using supercritical adsorption, J. Supercrit. Fluids 184 (2022) 105565, https://doi.org/10.1016/j.supflu.2022.105565.

[52] European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Dieases (ESCMID). EUCAST Definitive Document E.Def 1.2, May 2000: Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clin Microbiol Infect. 2000;6(9):503-508, ⟨https://doi.org/10.1046/j.1469-0691.2000.00149.x⟩.

[53] T.D.S. Moraes, L.F. Leandro, M.B. Santiago, L. de Oliveira Silva, T.C. Bianchi, R.C. S. Veneziani, S.R. Ambrósio, S.B. Ramos, J.K. Bastos, C.H.G. Martins, Assessment of the antibacterial, antivirulence, and action mechanism of Copaifera pubiflora oleoresin and isolated compounds against oral bacteria, Biomed. Pharm. 129 (2020) 110467, https://doi.org/10.1016/j.biopha.2020.110467.

[54] M.T. Arias-Moliz, R. Ordinola-Zapata, P. Baca, M. Ruiz-Linares, E. García García, M.A. Hungaro Duarte, C. Monteiro Bramante, C.M. Ferrer-Luque, Antimicrobial activity of Chlorhexidine, Peracetic acid and Sodium hypochlorite/etidronate irrigant solutions against Enterococcus faecalis biofilms, Int Endod. J. 48 (12) (2015) 1188–1193, https://doi.org/10.1111/iej.12424.

[55] V.F. Pedrinha, M.R. Cardenas Cuellar, E.G. Velásquez-Espedilla, M.A.H. Duarte, F. B. Andrade, P.A. Rodrigues, Impact of irrigation protocols with some chelators and mechanical agitation on intratubular decontamination, Braz. Oral. Res. 35 (2021) e127, https://doi.org/10.1590/1807-3107bor-2021.vol35.0127.

[56] D.F. Oda, M.A.H. Duarte, F.B. Andrade, L.T. Moriyama, V.S. Bagnato, I.G. de Moraes, Antimicrobial action of photodynamic therapy in root canals using LED curing light, curcumin and carbopol gel, Int. Endod. J. 52 (7) (2019) 1010–1019, https://doi.org/10.1111/iej.13092.

[57] M. Haapasalo, D. Orstavik, In vitro infection and disinfection of dentinal tubules, J. Dent. Res. 66 (8) (1987) 1375–1379, https://doi.org/10.1177/00220345870660081801.

[58] F.B. Andrade, M.P. Arias, A.G. Maliza, M.A. Duarte, M.S. Graeff, P.A. Amoroso-Silva, R.Z. Midena, I.G. Moraes, A new improved protocol for in vitro intratubular dentinal bacterial contamination for antimicrobial endodontic tests: standardization and validation by confocal laser scanning microscopy, J. Appl. Oral. Sci. 23 (6) (2015) 591–598, https://doi.org/10.1590/1678-775720140261.

[59] J. Ma, Z. Wang, Y. Shen, M. Haapasalo, A new noninvasive model to study the effectiveness of dentin disinfection by using confocal laser scanning microscopy, J. Endod. 37 (10) (2011) 1380–1385, https://doi.org/10.1016/j.joen.2011.06.018.

[60] X. Petridis, F.H. Busanello, M.V.R. So, R.J.B. Dijkstra, P.K. Sharma, L.W.M. van der Sluis, Factors affecting the chemical efficacy of 2% sodium hypochlorite against oral steady-state dual-species biofilms: exposure time and volume application, Int Endod. J. 52 (8) (2019) 1182–1195, https://doi.org/10.1111/iej.13102.

[61] F.H. Busanello, X. Petridis, M.V.R. So, R.J.B. Dijkstra, P.K. Sharma, L.W.M. van der Sluis, Chemical biofilm removal capacity of endodontic irrigants as a function of biofilm structure: optical coherence tomography, confocal microscopy and viscoelasticity determination as integrated assessment tools, Int Endod. J. 52 (4) (2019) 461–474.

[62] M.M.B. Borges, R.J.B. Dijkstra, F.B. de Andrade, M.A.H. Duarte, M. Versluis, L.W. M. van der Sluis, X. Petridis, The response of dual-species bacterial biofilm to 2% and 5% NaOCl mixed with etidronic acid: a laboratory real-time evaluation using optical coherence tomography, Int Endod. J. 55 (7) (2022) 758–771, https://doi.org/10.1111/iej.13754.

[63] J. Hou, C. Wang, R.T. Rozenbaum, N. Gusnaniar, E.D. de Jong, W. Woudstra, G. I. Geertsema-Doornbusch, J. Atema-Smit, J. Sjollema, Y. Ren, H.J. Busscher, H. C. van der Mei, Bacterial density and biofilm structure determined by optical coherence tomography, Sci. Rep. 9 (1) (2019) 9794, https://doi.org/10.1038/s41598-019-46196-7.

[64] N.A. Otsu, Threshold selection method from gray-level histograms, IEEE Trans. Syst. Man Cyber (9) (1979) 62–66.

[65] M.J. Romeu, M. Lima, L.C. Gomes, E.D. Jong, J. Morais, V. Vasconcelos, M.F. R. Pereira, O.S.G.P. Soares, J. Sjollema, F.J. Mergulhão, The use of 3D optical coherence tomography to analyze the architecture of cyanobacterial biofilms formed on a carbon nanotube composite, Polymers 14 (20) (2022) 4410, https://doi.org/10.3390/polym14204410.

[66] M.J. Romeu, P. Alves, J. Morais, J.M. Miranda, E.D. de Jong, J. Sjollema, V. Ramos, V. Vasconcelos, F.J.M. Mergulhão, Biofilm formation behaviour of marine filamentous cyanobacterial strains in controlled hydrodynamic conditions, Environ. Microbiol 21 (11) (2019) 4411–4424, https://doi.org/10.1111/1462-2920.14807.

[67] R. Murga, P.S. Stewart, D. Daly, Quantitative analysis of biofilm thickness variability, Biotechnol. Bioeng. 45 (6) (1995) 503–510, https://doi.org/10.1002/bit.260450607.

[68] M. Wagner, H. Horn, Optical coherence tomography in biofilm research: a comprehensive review, Biotechnol. Bioeng. 114 (7) (2017) 1386–1402, https://doi.org/10.1002/bit.26283.

[69] Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk Susceptibility Tests, 11th ed.; Approved Standard, CLSI Document M02-A11; CLSI: Wayne, PA, USA, 2012; p. 32, ISBN 1-56238-782-0.

[70] L. Giardino, L. Generali, P. Savadori, M.C. Barros, L.L. de Melo Simas, J. Pytko-Polończyk, W. Wilkoński, V. Ballal, F.B. Andrade, Can the concentration of citric acid affect its cytotoxicity and antimicrobial activity? Dent. J. 10 (8) (2022) 148, https://doi.org/10.3390/dj10080148.

[71] I.N. Rôças, J.F.Jr Siqueira, Identification of bacteria enduring endodontic treatment procedures by a combined reverse transcriptase-polymerase chain

reaction and reverse-capture checkerboard approach, J. Endod. 36 (1) (2010) 45–52, https://doi.org/10.1016/j.joen.2009.10.022.
[72] L.C.L. Nardello, E.T. Pinheiro, G. Gavini, L.C. Prado, R.X. Romero, B.P.F. A. Gomes, M.C. Skelton-Macedo, Nature and prevalence of bacterial taxa persisting after root canal chemomechanical preparation in permanent teeth: a systematic review and meta-analysis, J. Endod. 48 (5) (2022) 572–596, https://doi.org/10.1016/j.joen.2022.01.016.
[73] G. Sundqvist, D. Figdor, S. Persson, U. Sjögren, Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment, Oral. Surg. Oral. Med Oral. Pathol. Oral. Radio. Endod. 85 (1) (1998) 86–93, https://doi.org/10.1016/s1079-2104(98)90404-8.
[74] C.M. Ferrer-Luque, I. Bejarano, M. Ruiz-Linares, P. Baca, Reduction in Enterococcus faecalis counts - a comparison between rotary and reciprocating systems, Int Endod. J. 47 (4) (2014) 380–386, https://doi.org/10.1111/iej.12158.
[75] R.M. Love, Enterococcus faecalis–a mechanism for its role in endodontic failure, Int Endod. J. 34 (5) (2001) 399–405, https://doi.org/10.1046/j.1365-2591.2001.00437.x.
[76] M.I. Klein, J. Xiao, B. Lu, C.M. Delahunty, J.R. Yates, 3rd, H. Koo, Streptococcus mutans protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics, PLoS One 7 (9) (2012) e45795, https://doi.org/10.1371/journal.pone.0045795.
[77] A.R. Lima, D.R. Herrera, P.A. Francisco, A.C. Pereira, J. Lemos, J. Abranches, B.P. F.A. Gomes, Detection of Streptococcus mutans in symptomatic and asymptomatic infected root canals, Clin. Oral. Invest. 25 (6) (2021) 3535–3542, https://doi.org/10.1007/s00784-020-03676-9.
[78] A.R. Lima, T. Ganguly, A.R. Walker, N. Acosta, P.A. Francisco, R. Pileggi, J. A. Lemos, B.P.F.A. Gomes, J. Abranches, Phenotypic and genotypic characterization of streptococcus mutans strains isolated from endodontic infections, J. Endod. 46 (12) (2020) 1876–1883, https://doi.org/10.1016/j.joen.2020.09.002.
[79] H.P. Lew, S.Y. Quah, J.N. Lui, G. Bergenholtz, V.S. Hoon Yu, K.S. Tan, Isolation of alkaline-tolerant bacteria from primary infected root canals, J. Endod. 41 (4) (2015) 451–456, https://doi.org/10.1016/j.joen.2014.12.003.
[80] T. Xia, J.C. Baumgartner, Occurrence of Actinomyces in infections of endodontic origin, J. Endod. 29 (9) (2003 Sep) 549–552, https://doi.org/10.1097/00004770-200309000-00001.
[81] Y. He, B.W. Peterson, M.A. Jongsma, Y. Ren, P.K. Sharma, H.J. Busscher, H.C. van der Mei, Stress relaxation analysis facilitates a quantitative approach towards antimicrobial penetration into biofilms, PLoS One 8 (5) (2013) e63750, https://doi.org/10.1371/journal.pone.0063750.
[82] J.C. Baumgartner, W.A.Jr Falkler, Bacteria in the apical 5 mm of infected root canals, J. Endod. 17 (8) (1991) 380–383, https://doi.org/10.1016/s0099-2399(06)81989-8.
[83] M.A. Duarte, R.Z. Midena, M.A. Zeferino, R.R. Vivan, P.H. Weckwerth, F. Dos Santos, J.M. Guerreiro-Tanomaru, M. Tanomaru-Filho, Evaluation of pH and calcium ion release of calcium hydroxide pastes containing different substances, J. Endod. 35 (9) (2009) 1274–1277, https://doi.org/10.1016/j.joen.2009.05.009.
[84] S.T. Simon, K.S. Bhat, R. Francis, Effect of four vehicles on the pH of calcium hydroxide and the release of calcium ion, Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radio. Endod. 80 (4) (1995) 459–464, https://doi.org/10.1016/s1079-2104(05)80371-3.
[85] M.A. Duarte, K. Alves de Aguiar, M.A. Zeferino, R.R. Vivan, R. Ordinola-Zapata, M. Tanomaru-Filho, P.H. Weckwerth, M.C. Kuga, Evaluation of the propylene glycol association on some physical and chemical properties of mineral trioxide aggregate, Int. Endod. J. 45 (6) (2012) 565–570, https://doi.org/10.1111/j.1365-2591.2012.02012.x.
[86] J. Monteil, A. Hadj-Sassi, É . Dargelos, N. Guzman-Barrera, E. Poque, F. Leal-Calderon, Method to prepare aqueous propolis dispersions based on phase separation, Food Chem. 389 (2022) 133072, https://doi.org/10.1016/j.foodchem.2022.133072.
[87] M.C.F. de Lima, R. Ribeiro, J.E. Almeida e Silva, S.S. dos Santos Tavares, Y.C. D. de Araujo, V.F. da Veiga-Junior, Chemistry, Biological Activities, and Uses of Copaiba Oil Resins, in: H.N. Murthy (Ed.), Gums, Resins and Latexes of Plant Origin. Reference Series in Phytochemistry, Springer, Cham, 2022, https://doi.org/10.1007/978-3-030-76523-1_19-1.
[88] B.P. Binks, P.D. Fletcher, M.A. Thompson, R.P. Elliott, Influence of propylene glycol on aqueous silica dispersions and particle-stabilized emulsions, Langmuir 29 (19) (2013) 5723–5733, https://doi.org/10.1021/la4008697.
[89] N.R. Mathias, J. Crison, The use of modeling tools to drive efficient oral product design, AAPS J. 14 (3) (2012) 591–600, https://doi.org/10.1208/s12248-012-9372-3.
[90] E.S.C. Ferreira, I.V. Voroshylova, V.A. Koverga, C.M. Pereira, M.N.D.S. Cordeiro, New force field model for propylene glycol: insight to local structure and dynamics, J. Phys. Chem. B 121 (48) (2017) 10906–10921, https://doi.org/10.1021/acs.jpcb.7b08251.
[91] F.H. Xavier-Junior, N. Huang, J.J. Vachon, V.L. Rehder, E.S. do Egito, C. Vauthier, Match of solubility parameters between oil and surfactants as a rational approach for the formulation of microemulsion with a high dispersed volume of copaiba oil and low surfactant content, Pharm. Res. 33 (12) (2016) 3031–3043, https://doi.org/10.1007/s11095-016-2025-y.
[92] D. Kim, T. Ito, A. Hara, Y. Li, J. Kreth, H. Koo, Antagonistic interactions by a high H_2O_2 -producing commensal streptococcus modulate caries development by Streptococcus mutans, Mol. Oral. Microbiol 37 (6) (2022) 244–255, https://doi.org/10.1111/omi.12394.
[93] S.K. Filoche, S.A. Anderson, C.H. Sissons, Biofilm growth of lactobacillus species is promoted by actinomyces species and streptococcus mutans, Oral. Microbiol Immunol. 19 (5) (2004) 322–326, https://doi.org/10.1111/j.1399-302x.2004.00164.x.
[94] J. Krzek, M. Starek, A. Kwiecień, W. Rzeszutko, Simultaneous identification and quantitative determination of neomycin sulfate, polymixin B sulfate, zinc bacytracin and methyl and propyl hydroxybenzoates in ophthalmic ointment by TLC, J. Pharm. Biomed. Anal. 24 (4) (2001) 629–636, https://doi.org/10.1016/s0731-7085(00)00438-6.
[95] H. Koo, B.P. Gomes, P.L. Rosalen, G.M. Ambrosano, Y.K. Park, J.A. Cury, In vitro antimicrobial activity of propolis and Arnica montana against oral pathogens, Arch. Oral. Biol. 45 (2) (2000) 141–148, https://doi.org/10.1016/s0003-9969(99)00117-x.
[96] Gomes B.P. Ferraz, C.C. Garrido, F.D. Rosalen, P.L. Zaia, A.A. Teixeira, F.B. de Souza-Filho, FJ. Microbial susceptibility to calcium hydroxide pastes and their vehicles, J. Endod. 28 (11) (2002) 758–761, https://doi.org/10.1097/00004770-200211000-00003.
[97] O. Koru, F. Toksoy, C.H. Acikel, Y.M. Tunca, M. Baysallar, A. Uskudar Guclu, E. Akca, A. Ozkok Tuylu, K. Sorkun, M. Tanyuksel, B. Salih, In vitro antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens, Anaerobe 13 (3-4) (2007) 140–145, https://doi.org/10.1016/j.anaerobe.2007.02.001.
[98] L.R. Fava, Human pulpectomy: incidence of postoperative pain using two different intracanal dressings, Int. Endod. J. 25 (5) (1992) 257–260, https://doi.org/10.1111/j.1365-2591.1992.tb01159.x.
[99] A. Al-Ahmad, H. Ameen, K. Pelz, L. Karygianni, A. Wittmer, A.C. Anderson, B. Spitzmüller, E. Hellwig, Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth, J. Endod. 40 (2) (2014) 223–230, https://doi.org/10.1016/j.joen.2013.07.023.
[100] G.B. Jungermann, K. Burns, R. Nandakumar, M. Tolba, R.A. Venezia, A.F. Fouad, Antibiotic resistance in primary and persistent endodontic infections, J. Endod. 37 (10) (2011) 1337–1344, https://doi.org/10.1016/j.joen.2011.06.028.
[101] L.M. Cherian, S. Liu, K. Hon, M. Ramezanpour, H. Hu, S. Javadiyan, I. Stafford, P. J. Wormald, A.J. Psaltis, S. Vreugde, Hydrocortisone metabolism by Staphylococcus: effects on anti-inflammatory and antimicrobial action, Int Forum Allergy Rhinol. 14 (3) (2024) 711–715, https://doi.org/10.1002/alr.23242.
[102] J.F.Jr Siqueira, I.N. Rôças, Present status and future directions: microbiology of endodontic infections, Int Endod. J. 55 (3) (2022) 512–530, https://doi.org/10.1111/iej.13677.
[103] Y. Shen, Y. Gao, J. Lin, J. Ma, Z. Wang, M. Haapasalo, Methods and models to study irrigation, Endod. Top. 27 (2012) 3–34, https://doi.org/10.1111/etp.12029.
[104] J. Xiao, M.I. Klein, M.L. Falsetta, B. Lu, C.M. Delahunty, J.R. Yates, 3rd, A. Heydorn, H. Koo, The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm, PLoS Pathog. 8 (4) (2012) e1002623, https://doi.org/10.1371/journal.ppat.1002623.
[105] M.T. Arias-Moliz, C.M. Ferrer-Luque, M.P. González-Rodríguez, M.J. Valderrama, P. Baca, Eradication of Enterococcus faecalis biofilms by cetrimide and chlorhexidine, J. Endod. 36 (1) (2010) 87–90, https://doi.org/10.1016/j.joen.2009.10.013.
[106] C.M. Ferrer-Luque, M.T. Arias-Moliz, M.P. González-Rodríguez, P. Baca, Antimicrobial activity of maleic acid and combinations of cetrimide with chelating agents against Enterococcus faecalis biofilm, J. Endod. 36 (10) (2010) 1673–1675, https://doi.org/10.1016/j.joen.2010.06.009.
[107] C. Boutsioukis, M.T. Arias-Moliz, L.E. Chávez de Paz, A critical analysis of research methods and experimental models to study irrigants and irrigation systems, Int. Endod. J. 55 (2) (2022) 295–329, https://doi.org/10.1111/iej.13710.
[108] X. Petridis, F.H. Busanello, M.V.R. So, R.J.B. Dijkstra, P.K. Sharma, L.W.M. van der Sluis, Chemical efficacy of several NaOCl concentrations on biofilms of different architecture: new insights on NaOCl working mechanisms, Int. Endod. J. 52 (12) (2019) 1773–1788, https://doi.org/10.1111/iej.13198.
[109] C. Estrela, C.R. Estrela, E.L. Barbin, J.C. Spanó, M.A. Marchesan, J.D. Pécora, Mechanism of action of sodium hypochlorite, Braz. Dent. J. 13 (2) (2002) 113–117, https://doi.org/10.1590/s0103-64402002000200007.
[110] S. Tejada, P. Baca, C.M. Ferrer-Luque, M. Ruiz-Linares, M.J. Valderrama, M. T. Arias-Moliz, Influence of dentine debris and organic tissue on the properties of sodium hypochlorite solutions, Int. Endod. J. 52 (1) (2019) 114–122, https://doi.org/10.1111/iej.12986.
[111] F.M. Pascon, K.R. Kantovitz, P.A. Sacramento, M. Nobre-dos-Santos, R.M. Puppin-Rontani, Effect of sodium hypochlorite on dentine mechanical properties. A review, J. Dent. 37 (12) (2009) 903–908, https://doi.org/10.1016/j.jdent.2009.07.004.
[112] E.M. Souza, A.M. Calixto, C.N. Lima, F.G. Pappen, G. De-Deus, Similar influence of stabilized alkaline and neutral sodium hypochlorite solutions on the fracture resistance of root canal-treated bovine teeth, J. Endod. 40 (10) (2014) 1600–1603, https://doi.org/10.1016/j.joen.2014.02.028.
[113] M.A. de Sá Assis, L. de Paula Ramos, A. Abu Hasna, T.S. de Queiroz, T.C. Pereira, P.M. Nagai de Lima, A.A. Berretta, M.C. Marcucci, C.A. Talge Carvalho, L.D. de Oliveira, Antimicrobial and antibiofilm effect of brazilian green propolis aqueous extract against dental anaerobic bacteria, Molecules 27 (23) (2022) 8128, https://doi.org/10.3390/molecules27238128.
[114] S. Bouchelaghem, S. Das, R.S. Naorem, L. Czuni, G. Papp, M. Kocsis, Evaluation of total phenolic and flavonoid contents, antibacterial and antibiofilm activities of

hungarian propolis ethanolic extract against *Staphylococcus aureus*, Molecules 27 (2) (2022) 574, https://doi.org/10.3390/molecules27020574.
[115] Y. Niu, K. Wang, S. Zheng, Y. Wang, Q. Ren, H. Li, L. Ding, W. Li, L. Zhang, Antibacterial effect of caffeic acid phenethyl ester on cariogenic bacteria and streptococcus mutans biofilms, Antimicrob. Agents Chemother. 64 (9) (2020) e00251-20, https://doi.org/10.1128/AAC.00251-20.
[116] I. Przybyłek, T.M. Karpiński, Antibacterial properties of propolis, Molecules 24 (11) (2019) 2047, https://doi.org/10.3390/molecules24112047.
[117] C. Arruda, J.A. Aldana Mejía, V.P. Ribeiro, C.H. Gambeta Borges, C.H.G. Martins, R.C. Sola Veneziani, S.R. Ambrósio, J.K. Bastos, Occurrence, chemical composition, biological activities and analytical methods on Copaifera genus-a review, Biomed. Pharm. 109 (2019) 1–20, https://doi.org/10.1016/j.biopha.2018.10.030.
[118] A.L. Diefenbach, F.W.M.G. Muniz, H.J.R. Oballe, C.K. Rösing, Antimicrobial activity of copaiba oil (Copaifera ssp.) on oral pathogens: systematic review, Phytother. Res 32 (4) (2018) 586–596, https://doi.org/10.1002/ptr.5992.
[119] A. Urzúa, M.C. Rezende, C. Mascayano, L. Vásquez, A structure-activity study of antibacterial diterpenoids, Molecules 13 (4) (2008) 882–891, https://doi.org/10.3390/molecules13040822.
[120] N. Miró, Controlled multicenter study on chronic suppurative otitis media treated with topical applications of ciprofloxacin 0.2% solution in single-dose containers or combination of polymyxin B, neomycin, and hydrocortisone suspension, Otolaryngol. Head. Neck Surg. 123 (5) (2000) 617–623, https://doi.org/10.1067/mhn.2000.107888.
[121] E.K. Sirén, E. Kerosuo, E. Lavonius, J.H. Meurman, M. Haapasalo, Ca(OH)2 application modes: in vitro alkalinity and clinical effect on bacteria, Int Endod. J. 47 (7) (2014) 628–638, https://doi.org/10.1111/iej.12199.
[122] F.B.A. Ferreira, P.A.S. Souza, do, M.S. Vale, I.G. de Moraes, J.M. Granjeiro, Evaluation of pH levels and calcium ion release in various calcium hydroxide endodontic dressings, Oral. Surg. Oral. Med Oral. Pathol. Oral. Radio. Endod. 97 (3) (2004) 388–392, https://doi.org/10.1016/j.tripleo.2003.08.020.
[123] C. Estrela, G.B. Sydney, L.L. Bammann, O. Felippe Júnior, Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria, Braz. Dent. J. 6 (2) (1995) 85–90.
[124] E.V. Cruz, K. Kota, J. Huque, M. Iwaku, E. Hoshino, Penetration of propylene glycol into dentine, Int Endod. J. 35 (4) (2002) 330–336, https://doi.org/10.1046/j.1365-2591.2002.00482.x.
[125] T.C. Pereira, L.R. Vasconcelos, M.S. Graeff, M.A. Duarte, C.M. Bramante, F. B. Andrade, Intratubular disinfection with tri-antibiotic and calcium hydroxide pastes, Acta Odontol. Scand. 75 (2) (2017) 87–93, https://doi.org/10.1080/00016357.2016.1256427.
[126] F.B. de Andrade, L.R.S.M. Vasconcelos, T.C. Pereira, R.B. Garcia, C.M. Bramante, M.A.H. Duarte, Ultrasonic agitation reduces the time of calcium hydroxide antimicrobial effect and enhances its penetrability, J. Mater. Sci. Mater. Med 32 (12) (2021) 150, https://doi.org/10.1007/s10856-021-06607-6.
[127] M.P. Arias, A.G. Maliza, R.Z. Midena, M.S. Graeff, M.A. Duarte, F.B. Andrade, Effect of ultrasonic streaming on intra-dentinal disinfection and penetration of calcium hydroxide paste in endodontic treatment, J. Appl. Oral. Sci. 24 (6) (2016) 575–581, https://doi.org/10.1590/1678-775720150553.
[128] R.J.Jr Palmer, S.M. Gordon, J.O. Cisar, P.E. Kolenbrander, Coaggregation-mediated interactions of streptococci and actinomyces detected in initial human dental plaque, J. Bacteriol. 185 (11) (2003) 3400–3409, https://doi.org/10.1128/JB.185.11.3400-3409.2003.
[129] D. De Beer, R. Srinivasan, P.S. Stewart, Direct measurement of chlorine penetration into biofilms during disinfection, Appl. Environ. Microbiol 60 (12) (1994) 4339–4344, https://doi.org/10.1128/aem.60.12.4339-4344.1994.
[130] H.K. Haapasalo, E.K. Sirén, T.M. Waltimo, D. Ørstavik, M.P. Haapasalo, Inactivation of local root canal medicaments by dentine: an in vitro study, Int Endod. J. 33 (2) (2000) 126–131, https://doi.org/10.1046/j.1365-2591.2000.00291.x.
[131] N. Derlon, M. Peter-Varbanets, A. Scheidegger, W. Pronk, E. Morgenroth, Predation influences the structure of biofilm developed on ultrafiltration membranes, Water Res. 46 (10) (2012) 3323–3333, https://doi.org/10.1016/j.watres.2012.03.031.
[132] L. Fortunato, A. Qamar, Y. Wang, S. Jeong, Suzana Pereira Nunes. In-situ assessment of biofilm formation in submerged membrane system using optical coherence tomography and computational fluid dynamics, J. Membr. Sci. 521 (2017) 84–94, https://doi.org/10.1016/j.memsci.2016.09.004.
[133] H. Koo, M.L. Falsetta, M.I. Klein, The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm, J. Dent. Res 92 (12) (2013) 1065–1073, https://doi.org/10.1177/0022034513504218.
[134] R.A. Harper, G.H. Carpenter, G.B. Proctor, R.D. Harvey, R.J. Gambogi, A. R. Geonnotti, R. Hider, S.A. Jones, Diminishing biofilm resistance to antimicrobial nanomaterials through electrolyte screening of electrostatic interactions, Colloids Surf. B Biointerfaces 173 (2019) 392–399, https://doi.org/10.1016/j.colsurfb.2018.09.018.
[135] R.T. Rozenbaum, O.C.J. Andrén, H.C. van der Mei, W. Woudstra, H.J. Busscher, M. Malkoch, P.K. Sharma, Penetration and accumulation of dendrons with different peripheral composition in *Pseudomonas aeruginosa* Biofilms, Nano Lett. 19 (7) (2019) 4327–4333, https://doi.org/10.1021/acs.nanolett.9b00838.
[136] F. Aldeek, C. Mustin, L. Balan, T. Roques-Carmes, M.P. Fontaine-Aupart, R. Schneider, Surface-engineered quantum dots for the labeling of hydrophobic microdomains in bacterial biofilms, Biomaterials 32 (23) (2011) 5459–5470, https://doi.org/10.1016/j.biomaterials.2011.04.019.
[137] G. Conrads, I. About, Pathophysiology of dental caries, Monogr. Oral. Sci. 27 (2018) 1–10, https://doi.org/10.1159/000487826.
[138] Jakovljevic A., Nikolic N., Jacimovic J., Pavlovic O., Milicic B., Beljic-Ivanovic K., Miletic M., Andric M., Milasin J. Prevalence of Apical Periodontitis and Conventional Nonsurgical Root Canal Treatment in General Adult Population: An Updated Systematic Review and Meta-analysis of Cross-sectional Studies Published between 2012 and 2020. J Endod. 2020;46(10):1371-1386.e8. Erratum in: J Endod. 2021;47(2):336, ⟨https://doi.org/10.1016/j.joen.2020.07.007⟩.
[139] D. Ricucci, J.F.Jr Siqueira, Fate of the tissue in lateral canals and apical ramifications in response to pathologic conditions and treatment procedures, J. Endod. 36 (1) (2010) 1–15, https://doi.org/10.1016/j.joen.2009.09.038.
[140] J.C. Farges, B. Alliot-Licht, E. Renard, M. Ducret, A. Gaudin, A.J. Smith, P. R. Cooper, Dental pulp defence and repair mechanisms in dental caries, Mediat. Inflamm. 2015 (2015) 230251, https://doi.org/10.1155/2015/230251.
[141] J.F.Jr Siqueira, Endodontic infections: concepts, paradigms, and perspectives, Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radio. Endod. 94 (3) (2002) 281–293, https://doi.org/10.1067/moe.2002.126163.
[142] S. Jayakumar, D. Sridhar, B.M. John, K. Arumugam, P. Ponnusamy, H. Pulidindi, Biofilm in endodontic infection and its advanced therapeutic options - an updated review, J. Pharm. Bioallied Sci. 16 (2) (2024) S1104–S1109, https://doi.org/10.4103/jpbs.jpbs_394_23.
[143] C. Rollet, L. Gal, J. Guzzo, Biofilm-detached cells, a transition from a sessile to a planktonic phenotype: a comparative study of adhesion and physiological characteristics in Pseudomonas aeruginosa, FEMS Microbiol Lett. 290 (2) (2009) 135–142, https://doi.org/10.1111/j.1574-6968.2008.01415.x.
[144] A. Zhao, J. Sun, Y. Liu, Understanding bacterial biofilms: from definition to treatment strategies, Front Cell Infect. Microbiol 13 (2023) 1137947, https://doi.org/10.3389/fcimb.2023.1137947.
[145] Kahler B., Taha N.A., Lu J., Saoud T.M. Vital pulp therapy for permanent teeth with diagnosis of irreversible pulpitis: biological basis and outcome. Aust Dent J. 2023;68 Suppl 1:S110-S122, ⟨https://doi.org/10.1111/adj.12997⟩.
[146] L.G. Levin, A.S. Law, G.R. Holland, P.V. Abbott, R.S. Roda, Identify and define all diagnostic terms for pulpal health and disease states, J. Endod. 35 (12) (2009) 1645–1657, https://doi.org/10.1016/j.joen.2009.09.032.
[147] H.F. Duncan, Present status and future directions-Vital pulp treatment and pulp preservation strategies, Int Endod. J. 55 (3) (2022) 497–511, https://doi.org/10.1111/iej.13688.
[148] G. Sundqvist, Taxonomy, ecology, and pathogenicity of the root canal flora, Oral. Surg. Oral. Med Oral. Pathol. 78 (4) (1994) 522–530, https://doi.org/10.1016/0030-4220(94)90047-7.
[149] M. Sakamoto, J.F. Siqueira, Jr, I.N. Rôças, Y. Benno, Molecular analysis of the root canal microbiota associated with endodontic treatment failures, Oral. Microbiol Immunol. 23 (4) (2008) 275–281, https://doi.org/10.1111/j.1399-302X.2007.00423.x.
[150] E.T. Pinheiro, B.P. Gomes, C.C. Ferraz, E.L. Sousa, F.B. Teixeira, F.J. Souza-Filho, Microorganisms from canals of root-filled teeth with periapical lesions, Int. Endod. J. 36 (1) (2003) 1–11, https://doi.org/10.1046/j.1365-2591.2003.00603.x.
[151] B.P. Gomes, E.T. Pinheiro, R.C. Jacinto, A.A. Zaia, C.C. Ferraz, F.J. Souza-Filho, Microbial analysis of canals of root-filled teeth with periapical lesions using polymerase chain reaction, J. Endod. 34 (5) (2008) 537–540, https://doi.org/10.1016/j.joen.2008.01.016.
[152] M.C. Barros, V.F. Pedrinha, F.E. Oliveira, M.C. Marcucci, B.P.F.A. Gomes, L. D. Oliveira, F.B. Andrade, Decrease from main root canal and intratubular *Fusobacterium nucleatum* and its endotoxin after ultrasonic activation of conventional and alternative irrigation solutions, Biofouling 40 (10) (2024) 904–914, https://doi.org/10.1080/08927014.2024.2426765.
[153] F.J. Álvarez-Martínez, E. Barrajón-Catalán, V. Micol, Tackling antibiotic resistance with compounds of natural origin: a comprehensive review, Biomedicines 8 (10) (2020) 405, https://doi.org/10.3390/biomedicines8100405.