

## ORIGINAL ARTICLE

# The dentine-sealer interface: Modulation of antimicrobial effects by irrigation

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## Abstract

**Aim:** Assess whether sodium hypochlorite (NaOCl) or chlorhexidine (CHX) and two irrigation protocols may alter the antibacterial properties of dentine and three endodontic sealers using a novel *ex vivo* tooth model.

**Methodology:** Prior to antibacterial testing, the tooth model was validated by means of scanning electron microscopy (SEM) to evaluate the separation between dentine and sealer surfaces. Root blocks prepared from extracted human roots were pre-treated with 17% EDTA + 0.9% saline and subsequently treated with 1% NaOCl (G1), 2% CHX (G2) or no irrigant (G3). Two irrigation protocols were further investigated, “1% NaOCl + 17% EDTA” (P1) and “1% NaOCl + 17% EDTA + 2% CHX” (P2). Following irrigation, the root blocks were either filled with AH Plus, BioRoot RCS and Pulp Canal Sealer (PCS), or left empty. All groups were incubated for 1, 7 and 28 days. Direct contact tests for planktonic *E. faecalis* and 48 h *E. faecalis* biofilms were performed at the level of dentine and sealer surfaces. Statistical analysis was performed on the bacterial survival between irrigants (G1, G2 and G3) and between irrigation protocols (P1 and P2);  $p < .05$ .

**Results:** The model was considered reproducible as SEM examination of dentine samples indicated consistent separation between dentine and sealer surfaces. Irrigation with CHX (G2) and irrigation protocol P2 enhanced the antibacterial properties of dentine without sealer application as well as dentine in contact with all three sealers tested, especially against planktonic *E. faecalis*. G2 and P2 also improved the antibacterial effect of AH Plus surfaces for all three incubation times. No irrigation groups (G1, G2) or irrigation protocols (P1, P2) altered the antibacterial properties of BioRoot RCS surfaces against planktonic bacteria or biofilms. Only BioRoot RCS surfaces eliminated the planktonic *E. faecalis* in all irrigation groups (G1, G2, G3) and protocols (P1, P2) investigated whilst PCS surfaces eliminate *E. faecalis* in biofilms in all groups up to 7 days.

**Conclusions:** The tooth model was reproducible. CHX improved the antibacterial activity upon both sealer and dentine surfaces. Amongst sealers, BioRoot RCS was less affected by NaOCl and CHX, and exhibited high antibacterial properties regardless the irrigation applied.

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## KEYWORDS

antibacterial activity, biofilm, endodontic sealer, planktonic bacteria, root canal irrigants, tooth model

## INTRODUCTION

Apical periodontitis is the inflammatory response to infection of the root canal system by planktonic or biofilm-associated microorganisms (Chan et al., 2013; Nair, 2006; Ricucci & Siqueira, 2010; Siqueira & Rôças, 2009).

Root canal treatment reduces the bacterial load of the infected root canal, which subsequently reduces inflammation of periapical tissues and promotes periapical healing. Mechanical instrumentation removes residual bacteria, pulp tissue and debris, and shapes the root canal walls to facilitate effective irrigation and obturation (Carrotte, 2004). However, mechanical debridement leaves untouched areas (Peters, 2004) and numerous irrigation regimens are used to aid the mechanical debridement in removing bacteria and necrotic pulp tissue (Haapasalo et al., 2014). Sodium hypochlorite (NaOCl) is the irrigant most frequently used for chemical treatment of the root canal system (Haapasalo et al., 2014). Whilst it has both antimicrobial and tissue dissolving properties, it lacks substantive antimicrobial activity (Dametto et al., 2005; Khademi et al., 2006). It is used clinically in concentrations ranging from 0.5% to 6% (Gomes et al., 2001; Haapasalo et al., 2014; Zehnder, 2006). Chlorhexidine digluconate (CHX) binds to hard dental tissues (substantivity) and thus confers lasting antimicrobial properties (up to 12 weeks) to dentine when used as an irrigation solution (Carrilho et al., 2010; Rosenthal et al., 2004). As such, it may serve as an adjunct antimicrobial agent to NaOCl, and has been proposed for use as a final rinse of the root canal system (Haapasalo et al., 2014). CHX acts against gram-positive bacteria, gram-negative bacteria and fungi, and has both bacteriostatic and bactericidal effects depending on its concentration (Carrilho et al., 2010; Rosenthal et al., 2004). To dissolve the smear layer produced during root canal treatment, chelating agents such as ethylenediaminetetraacetic acid (EDTA) are often used as adjunctive irrigation (Haapasalo et al., 2014; Zehnder, 2006).

Endodontic sealers with various chemistries are used in endodontics with the ultimate goal to effectively seal the endodontic space and prevent the ingress of bacteria. In addition, they entomb bacteria, preventing their access to nutrients and they may also possess antibacterial properties (Ørstavik, 1988).

Most of the *in vitro/ex vivo* study designs in the literature investigate instrumentation, irrigation and obturation as separate entities (Du et al., 2014; Keleş et al.,

2014; Prestegard et al., 2014; Velozo et al., 2020; Wang et al., 2012; Zuolo et al., 2018). In the clinical situation, they are strongly related to each other (Donnermeyer et al., 2019; Fernandes Zancan et al., 2021; Zancan et al., 2021). Clinically, different irrigation protocols are often combined with various obturation materials (AlShwaimi et al., 2016; Haapasalo et al., 2014; Šimundić Munitić et al., 2019). Both dentine and many sealers have antibacterial properties (Arias-Moliz & Camilleri, 2016; Kapralos et al., 2018; Wang et al., 2014). The irrigants used may affect the chemistry of dentine and sealer surfaces and compromise or enhance their antimicrobial properties (Arias-Moliz & Camilleri, 2016). There is scant scientific data about the potential interactions between sealers and irrigation regimes in the root canal system in terms of antimicrobial properties. One study investigated the effect of final irrigation with water, EDTA and phosphate buffered saline (PBS) on the antibacterial efficacy of BioRoot RCS (Septodont), MTA Fillapex (Angelus) and AH Plus (Dentsply International) in an *ex vivo* dentine model; all three sealers exhibited the highest antibacterial activity after irrigation with EDTA followed by water (Arias-Moliz & Camilleri, 2016). However, the effects of common/standard irrigation solutions such as NaOCl or CHX were not investigated. A recent study used the dentine infection model to investigate the role of smear layer in the antimicrobial action of four root canal sealers (AH Plus, BioRoot RCS, MTA Fillapex, TotalFill; Brasseler USA) using NaOCl as the main irrigant; BioRoot RCS was the most effective sealer and the presence of smear layer did not affect its activity (Zancan et al., 2021). Another study has investigated the combined antibacterial effect of NaOCl and root canal sealers against *E. faecalis* biofilms in dentinal tubules (Du et al., 2015), whilst two studies have assessed the residual antimicrobial activity of CHX after root canal obturation with gutta-percha/AH26 and Resilon/RealSeal SE following different methodologies (Bolhari et al., 2015; Rosenthal et al., 2004).

The aim of this study was to use an *ex vivo* tooth model to assess whether residual presence of 1% NaOCl or 2% CHX may augment or reduce the antibacterial properties of dentine and three endodontic sealers. A second aim was to compare whether/how residuals from two irrigation protocols namely, “1% NaOCl followed by 17% EDTA (1% NaOCl + 17% EDTA)” and “1% NaOCl followed by 17% EDTA and 2% CHX (1% NaOCl + 17% EDTA + 2% CHX)” could alter the antibacterial effect of dentine or sealers.

The primary null hypothesis is that 1% NaOCl and/or 2% CHX will not affect the antimicrobial properties of

neither dentine nor sealer surfaces. A second hypothesis is that the two irrigation protocols will not present differences in their antimicrobial efficacy upon neither dentine nor sealer surfaces.

## MATERIALS AND METHODS

### Endodontic sealers and irrigating solutions

An epoxy resin-based sealer, AH Plus (Dentsply International), a calcium-silicate based sealer, BioRoot™ RCS (Septodont), and a zinc oxide eugenol sealer, Pulp Canal Sealer (PCS; Kerr Corporation), were tested. The following irrigation liquids were used: 1% NaOCl (Lot # 13678, Nordenta), 2% CHX (20% in water diluted in sterile distilled water and standardized to 2%, Lot # BCBS7878V, Sigma-Aldrich), 17% EDTA (Lot # 19120, Pulpdent).

### Tooth model

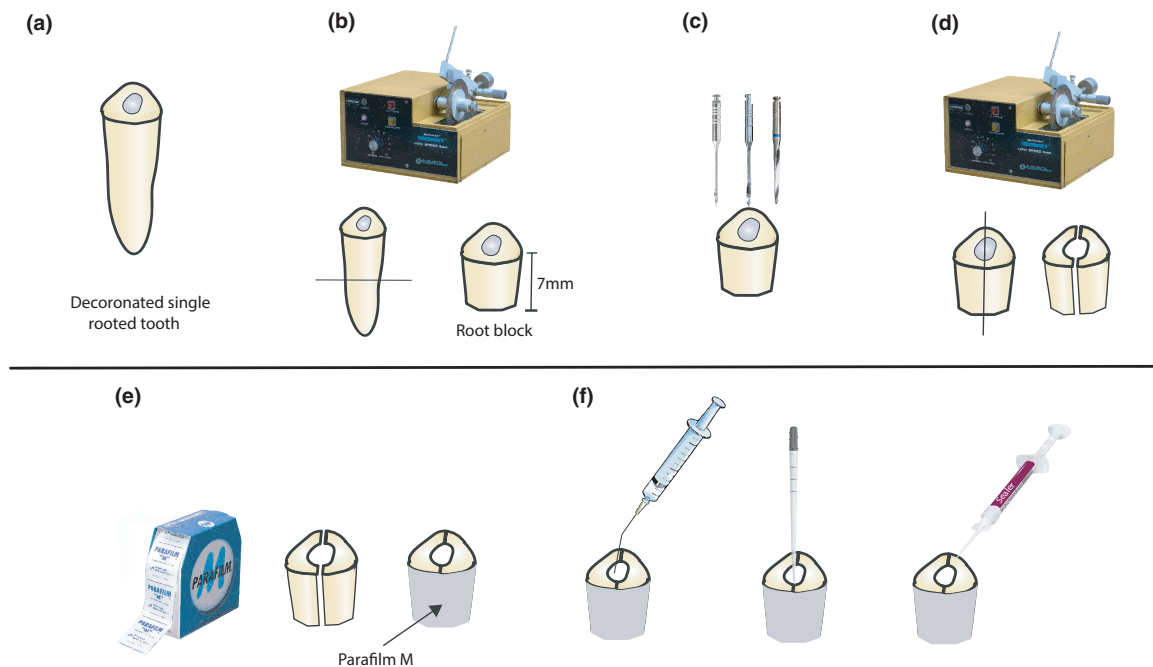
#### Preparation of root blocks

Extracted human teeth were collected from a bio-bank (“2013/413 NIOM tannbank”) approved by the Regional

Committees for Medical and Health Research Ethics (REC, application number 28748), Norway. All teeth were decoronated and their roots were horizontally sectioned at the apical parts, at a level to form root blocks with a standardized length of 7 mm, using a precision cutting machine (Buehler 11-1280-160 Isomet Low Speed Saw, Buehler; Figure 1a,b). The roots were instrumented with ProTaper rotary files (Dentsply Maillefer) up to size F4, and further enlarged with fibre post drill (3 M Relyx Fiber Post Drill No 3, 3 M, St. Paul, MN, USA; Figure 1c). Oval-shape root canals were prepared measuring approximately 4 mm at the largest diameter (semi-major axis). Irrigation with 2 ml of 1% NaOCl was followed between the changes of the rotary files and a last rinse with 0.9% saline using a 27 gauge Monoject 3cc Endodontic Syringe (CardinalHealth). The root blocks were further segmented (dichotomized) vertically with the use of the diamond saw and the two segments were repositioned and held tightly together by wrapping them up with the use of Parafilm M (Bemis; Figure 1d,e).

#### Irrigation regimes and obturation

The power calculation using G\*Power 3.1 (Heinrich Heine University) to calculate the sample size of each



**FIGURE 1** Schematic representation of teeth preparation. All teeth were decoronated (a) and their roots were horizontally sectioned at the apical parts, at a level to form root blocks with a standardized length of 7 mm, using a precision cutting machine (b). The root canals were instrumented with rotary files and further enlarged with fibre post drill (c). The roots were further segmented (dichotomized) vertically with the use of the diamond saw (d) and the two segments were repositioned and held tightly together by wrapping them up with the use of Parafilm M (e). After irrigation, the tested sealers were mixed according to the manufacturer's instructions and placed inside the root canal blocks (f)

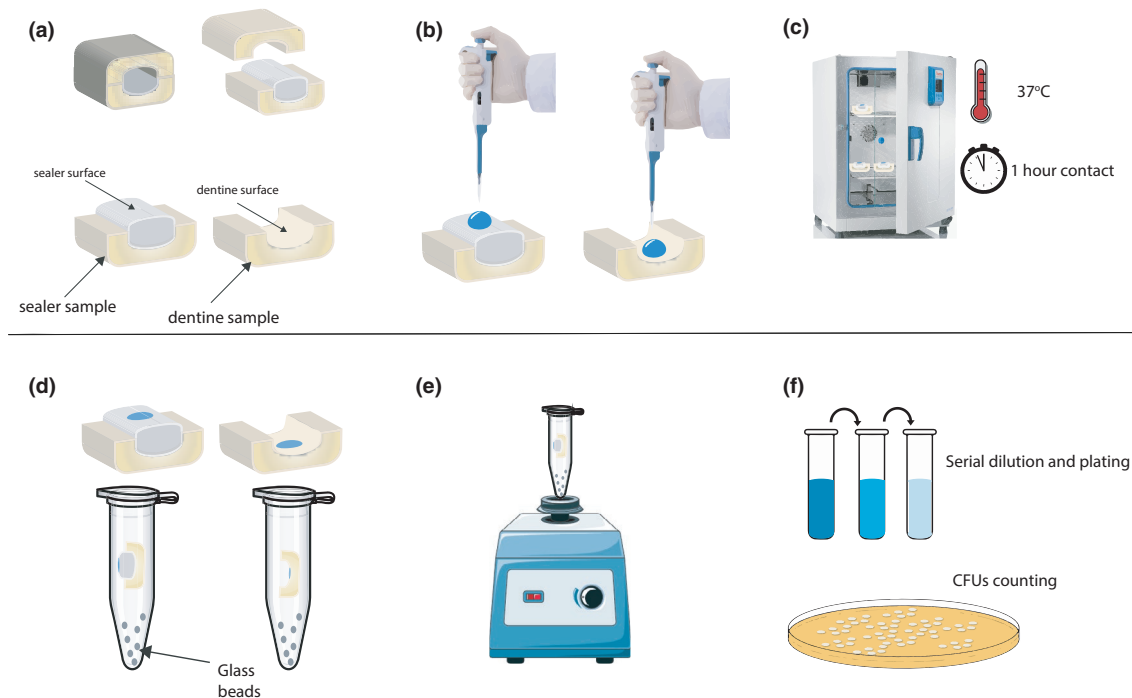
experimental condition (both the residual effect of 1% NaOCl, 2% CHX and the antibacterial effect of two irrigation protocols, with and without sealer placement) indicated at least seven root blocks in each assay (planktonic bacteria and bacteria in biofilms) (effect size  $f = 0.40$ ,  $\alpha$  error probability = 0.05). Thus, nine root blocks ( $n = 9$ ) were used for each experimental condition.

The residual effect of 1% NaOCl (G1) and 2% CHX (G2) as well as the antibacterial effect of two irrigation protocols, Protocol 1 (P1: 1% NaOCl + 17% EDTA) and Protocol 2 (P2: 1% NaOCl + 17% EDTA + 2% CHX), were tested upon dentine which had been in contact with sealers as well as the sealers facing the subjacent dentine (Figure 2a). The antibacterial properties of sealers without any irrigant applications were investigated (G3). In addition, the antibacterial effect of the irrigation solutions and protocols were evaluated on dentine without sealer application. All irrigants were applied from the top of the root blocks formed after tight repositioning of the two segments (Figure 1d,e). In G1, G2 and G3, dentine was pre-treated with 17% EDTA for 5 min (removal of smear layer), rinsed with 2 ml 0.9% saline and dried with paper points (size

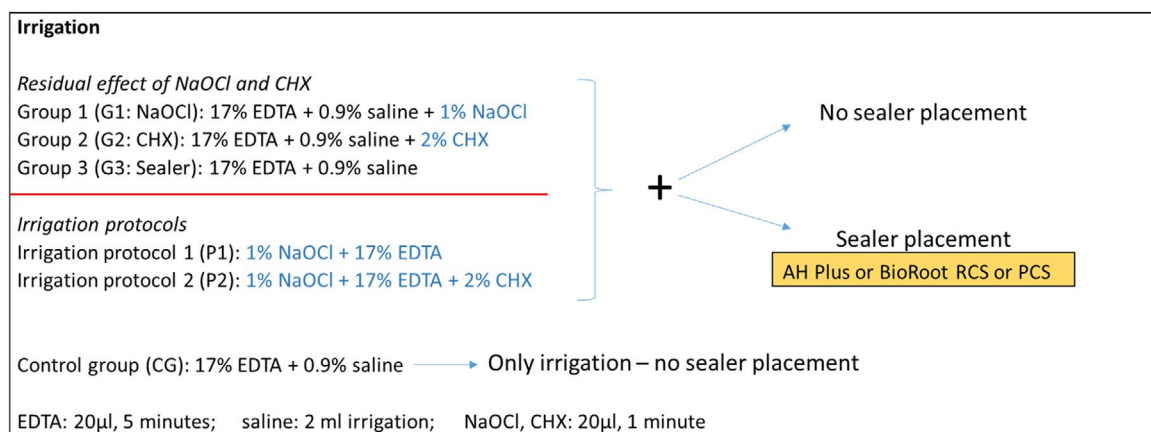
40, Reciproc blue, VDW). In P1 and P2, 17% EDTA was not used as dentine pre-treatment but NaOCl was the first irrigant.

Root blocks treated with 17% EDTA for 5 min and subsequently with saline served as controls. The root canals of the blocks were meticulously dried with paper points between irrigation with different liquids and before placement of sealers to avoid interactions (Rossi-Fedeles et al., 2012). The volumes of the irrigation solutions, their application time and sequence of use as well as the placement of sealers are shown in Table 1.

The tested sealers were mixed according to the manufacturer's instructions and placed inside the root canal blocks (Figure 1f). The root blocks were incubated for 24 h (1 day), 7 and 28 days at 37°C 100% humidity. After the incubation period, each root was unwrapped from the Parafilm M and the root segments were gently detached/debonded with the use of a scalpel that was applied in the narrow space formed along their contact surfaces. The sealers were gently exposed and retrieved intact from the dentine walls they had been in contact with. This procedure enabled to expose the sealer surface having been in contact with the dentinal walls.



**FIGURE 2** Schematic representation of planktonic assay. After separating the twin root segments to reveal dentine and sealer, the whole bulk of the sealer was adhered to one segment whilst the adjacent segment was macroscopically free of sealer remnants (a). An amount of 5  $\mu$ l *E. faecalis* bacterial suspension was carefully placed upon the dentine (dentine samples) and the sealer surface (dentine-sealer sample), or only upon the dentine surface in irrigation groups without sealer and control group (b). The specimens were incubated at 37°C for 1 h, whilst complete evaporation of the suspension's liquid was inspected (c). The sealer samples and their adjacent dentine samples were separately transferred in vials containing 500  $\mu$ l PBS and were vigorously vibrated with glass beads (d and e). Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates incubated overnight at 37°C, 5% CO<sub>2</sub> supplemented atmosphere (f)

**TABLE 1** Sequence of irrigation liquids and their application time. Allocation of groups based on last irrigants and irrigation

Hereafter, the dentine segment, which has been in contact with sealers, will be referred as dentine sample and its surface as dentine surface (Figure 2a). The exposed sealer on its dentine segment will be referred as dentine-sealer sample and the exposed surface as dentine-sealer surface. The area between the sealer and the dentinal walls will be referred as sealer-dentine interface.

### Internal validity of split tooth model—evaluation of dentine surfaces

Before antibacterial testing, the tooth model was internally validated by assessing its reproducibility. After separating the twin root segments to reveal dentine and sealer, the whole bulk of the sealer was adhered to one segment whilst the adjacent segment was macroscopically free of sealer remnants.

To assess the type of failure on the sealer-dentine interface (adhesive: complete separation of sealer from dentine, cohesive: rupture of material bulk within the sealer, or a mix) and identify any sealer remnants on dentinal walls, scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) were performed. Briefly, two root blocks for each sealer were mounted on aluminium stubs, carbon coated (Agar Scientific), and viewed with the scanning electron microscope (TM4000Plus, Hitachi). Accelerating voltage ranged between 5 and 15 kV and the probe current between 125 and 300 pA. High magnification EDS chemical analysis was carried out at 15 kV and a working distance of 8.5 mm. Scanning electron micrographs at high magnification in the backscatter electron mode were captured, and EDS was performed in selected spots and rectangular areas of the samples. Furthermore, elemental maps at the same levels were performed and each element was marked out/designated in a different colour. EDS was also performed

over sealers prepared in circular samples to define their elemental profile. At this point, it is emphasized that the analysis regards root blocks that were incubated for 24 h, when the sealers were at the most premature stage of setting compared to 7 and 28 days set materials and therefore were more prone to deform during separation of tooth segments leading to possible cohesive type of failure. Moreover, all root blocks were pre-treated with 17% EDTA for 5 min aiming for constant background of dentinal tubules, as smear layer did not allow to distinguish between tooth structure and sealer remnants.

Elements that are traced both on sealer and tooth surfaces were evaluated to not be indicative of sealer remnants on the dentine. For example, the movement of calcium from the sealer to the tooth could not be monitored by the elemental mapping because both sealers and tooth structure contain calcium. Thus, those unique elements that could only be traced in sealers were guiding to identify the presence of sealer residues upon dentine (Figure S1): zirconium (Zr) and tungsten (W) for AH Plus; silicon (Si), chlorine (Cl) and Zr for BioRoot RCS; zinc (Zn) for PCS.

## Antibacterial assays

### Bacteria and media

The antibacterial properties of both dentine and dentine-sealer surfaces were assessed on the previously described *ex vivo* tooth model. *Enterococcus faecalis* American Type Cell Culture Collection (ATCC) 19434 was grown overnight for 18 h in Tryptone Soya Broth (TSB) at 37°C, 5% CO<sub>2</sub> supplemented atmosphere. The bacteria were suspended in PBS to an optical density at 600 nanometres (OD<sub>600</sub>) of 1.0, corresponding to approximately 2 × 10<sup>8</sup> Colony Forming Units (CFU)/ml (Figure 3a). The

antibacterial properties were assessed in both planktonic bacteria and bacteria in young biofilms.

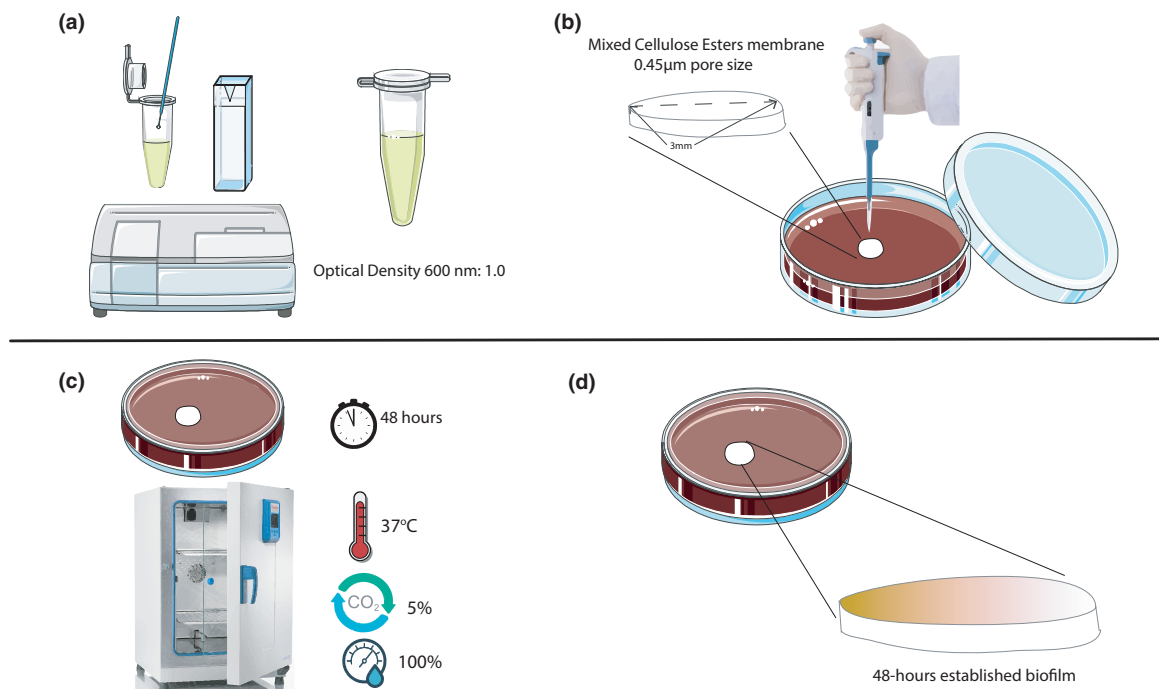
### Planktonic bacteria—Direct Contact Test (DCT)

An amount of 5  $\mu\text{l}$  from *E. faecalis* suspension was carefully placed upon the dentine (dentine sample) and the dentine-sealer surface (dentine-sealer sample), and only upon the dentine surface in irrigation groups without sealer placement and control group (Figure 2b). The specimens were incubated at 37°C for 1 h, whilst complete evaporation of the suspension's liquid was inspected (Figure 2c). The dentine-sealer samples and their adjacent dentine samples were separately transferred into vials containing 500  $\mu\text{l}$  PBS and were vigorously vibrated with glass beads (Figure 2d,e). Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates incubated overnight at 37°C, 5% CO<sub>2</sub> supplemented atmosphere (Figure 2f). Carryover effect of the method was also assessed. Briefly, an amount of 5  $\mu\text{l}$  from the bacterial suspension was placed into vials containing 500  $\mu\text{l}$  PBS together with dentine and dentine-sealer samples derived from all groups. These samples were vigorously vibrated with glass beads. Possible carryover effect was measured after serial dilutions and CFUs were

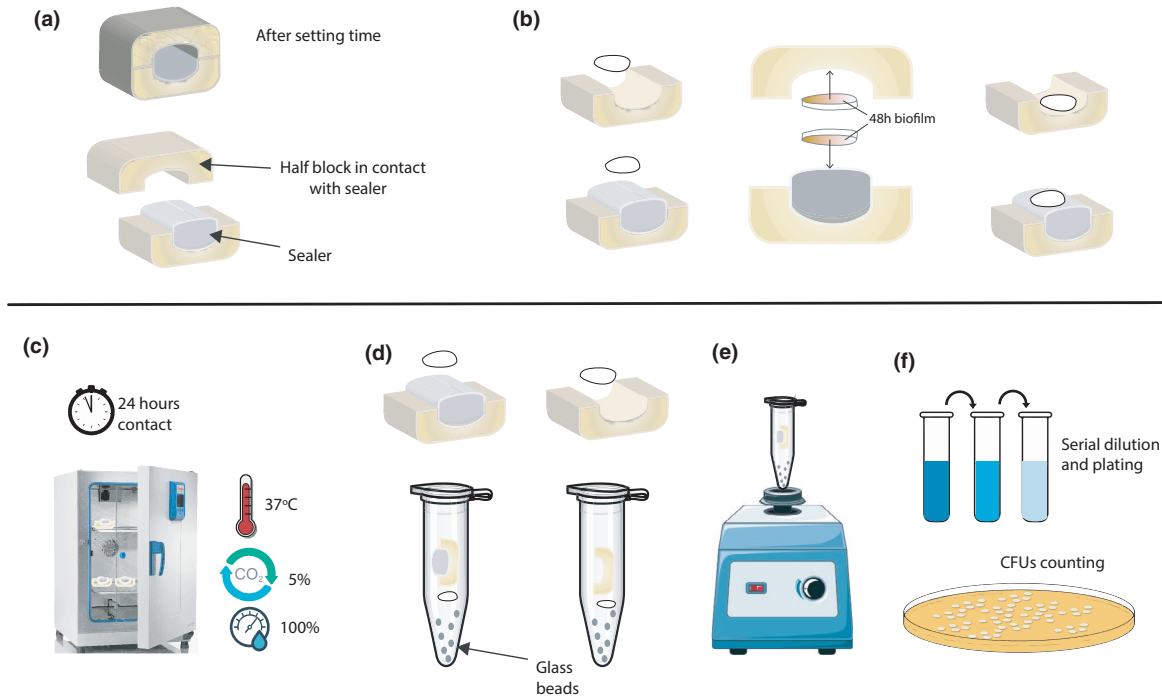
calculated as described previously. Experiments for potential carryover effect were performed in triplicate.

### Bacteria in biofilms—Direct Contact Test (DCT)

Membrane filters (MF-Millipore™ Membrane Filter, 0.45- $\mu\text{m}$  pore, Merck) were cut in circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2  $\mu\text{l}$  of each bacterial inoculum OD<sub>600</sub> 1.0 was applied upon the outer surface of membranes (Figure 3a,b). The agar plates were incubated at 37°C in a 5% CO<sub>2</sub> supplemented atmosphere for 48 h and mono-species biofilms were established (Figure 3c). The biofilm formation was verified with the use of confocal laser scanning microscopy (Figure 3d). The filter membranes were positioned upon the dentine and sealers with the established biofilms facing their surfaces (Figure 4b). The specimens were wrapped with Parafilm M to secure the membrane filters upon the surfaces and placed at 37°C in a 5% CO<sub>2</sub> supplemented atmosphere for 24 h (Figure 4c). After incubation time, the Parafilm M was removed, and a droplet of 10  $\mu\text{l}$  sterile distilled water was transferred upon the membranes to enable a gentle detachment from the sealer and dentine. Each membrane with its corresponding dentine or dentine-sealer sample was



**FIGURE 3** Schematic representation of biofilm formation. The bacteria were suspended in PBS to an optical density at 600 nanometres (O600) of 1.0 (a). Membrane filters were cut in circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2  $\mu\text{l}$  of each bacterial inoculum OD<sub>600</sub> 1.0 was applied upon the outer surface of membranes (b). The agar plates were incubated at 37°C in a 5% CO<sub>2</sub> supplemented atmosphere for 48 h (c). The monospecies biofilms were established and verified with the use of confocal laser scanning microscopy (d)



**FIGURE 4** Schematic representation of biofilm assay. After separating the twin root segments to reveal dentine and sealer, the whole bulk of the sealer was adhered to one segment whilst the adjacent segment was macroscopically free of sealer remnants (a). The filter membranes were positioned upon the dentine and sealers with the established biofilms facing their surfaces (b). The specimens were wrapped with Parafilm M to secure the membrane filters upon the surfaces and placed at 37°C in a 5% CO<sub>2</sub> supplemented atmosphere for 24 h (c). After 24 h, each membrane with its corresponding sealer-dentine or sealer block was transferred to vials containing 5 ml PBS and vigorously vortexed with glass beads (c and d). After serial dilutions in PBS, colony forming units (CFU) were counted after incubation at 37°C in a 5% CO<sub>2</sub> supplemented atmosphere (f)

transferred to vials containing 5 ml PBS (Figure 4d) and vigorously vortexed with glass beads (Figure 4e). After serial dilutions in PBS, CFUs were counted after incubation at 37°C in a 5% CO<sub>2</sub> supplemented atmosphere (Figure 4f). Carryover effect of the method was also assessed. Filter membranes with established biofilms served as positive controls and were placed in vials containing 5 ml PBS. Dentine and dentine-sealer samples derived from all groups were put in the same vial. These samples were vigorously vibrated with glass beads. Possible carryover effect was measured after serial dilutions and CFUs were calculated as described previously. Experiments for potential carryover effect were performed in triplicate.

## Statistical analysis

The statistical analysis was performed with GraphPadPrism version 9.01 for windows (GraphPad software) using the nonparametric Kruskal–Wallis test and Dunn's post-hoc method due to absence of normal distribution ( $p < .05$ ). In the case of comparing two groups, non-parametric Mann–Whitney  $U$  test was performed ( $p < .05$ ).

- Residual effect of NaOCl and CHX on dentine and dentine-sealer surfaces
  - For dentine surfaces, comparisons between different incubation times of groups (G1, G2, G3) and the control (CG) of *E. faecalis*. In irrigation with no sealer placement, pairwise comparisons between G1: NaOCl and G2: CHX for each one of the three incubation times tested (1, 7 and 28 days). In sealer placement, pairwise comparisons between G1: NaOCl or G2: CHX and G3: Sealer for each sealer for each one of the three incubation times tested (1, 7 and 28 days).
  - For dentine-sealer surfaces, pairwise comparisons between G1: NaOCl or G2: CHX and G3: Sealer for each sealer for each one of the three incubation times tested (1, 7 and 28 days).
- Effect of irrigation protocols on dentine and dentine-sealer surfaces
  - For dentine surfaces, comparisons between different incubation times of irrigation protocols (P1, P2) and the control (CG) of *E. faecalis* ( $p < .05$ ). Both for irrigation without sealer placement and for each sealer, pairwise comparisons between P1: 1% NaOCl + 17% EDTA and P2: 1% NaOCl + 17% EDTA + 2% CHX for each one of the three incubation times tested (1,

7 and 28 days).

- For dentine-sealer surfaces, pairwise comparisons between P1: 1% NaOCl + 17% EDTA and P2: 1% NaOCl + 17% EDTA + 2% CHX for each sealer for each one of the three incubation times tested (1, 7 and 28 days).

Multiple linear regression tests were performed using SPSS 27 (SPSS Inc.) and details can be found in the Supplementary Material (Supplementary material\_multiple regression analyses).

## RESULTS

### Internal validity of tooth model

SEM examination showed adhesive mode of failure at the sealer-dentine interface. Sealer residues could be sporadically identified, but no full dentine coverage was evident in any of the surfaces investigated. A full series of SEM micrographs with elemental analysis is presented in Figure S1. AH Plus bonded to dentine with sealer tags and after separation process the whole bulk of the material was debonded. Only few sealer tags rich

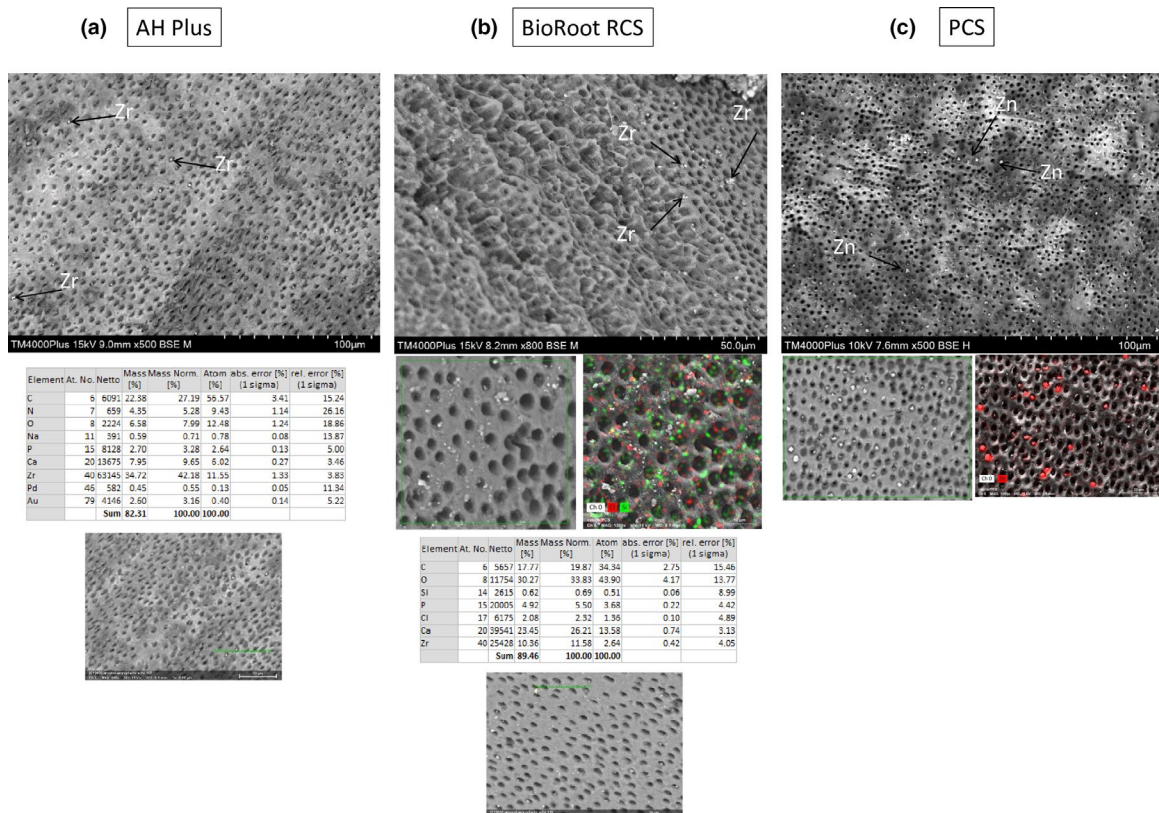
in Zr could be identified in dentinal tubules (Figure 5a). BioRoot RCS demonstrated trace elements on dentine without full coverage (Figure 5b). As for PCS, elemental analysis showed few sealer tags rich in Zn (Figure 5c). Thus, the model was considered reproducible as the SEM examination of dentine samples indicated consistent separation between dentine and dentine-sealer surfaces. This finding enabled to proceed further with antibacterial assays, testing both the dentine and dentine-sealer surfaces.

### Antibacterial properties of dentine and dentine-sealer surfaces

#### Residual effect of NaOCl and CHX on dentine and dentine-sealer surfaces

##### *Planktonic E. faecalis, dentine surfaces*

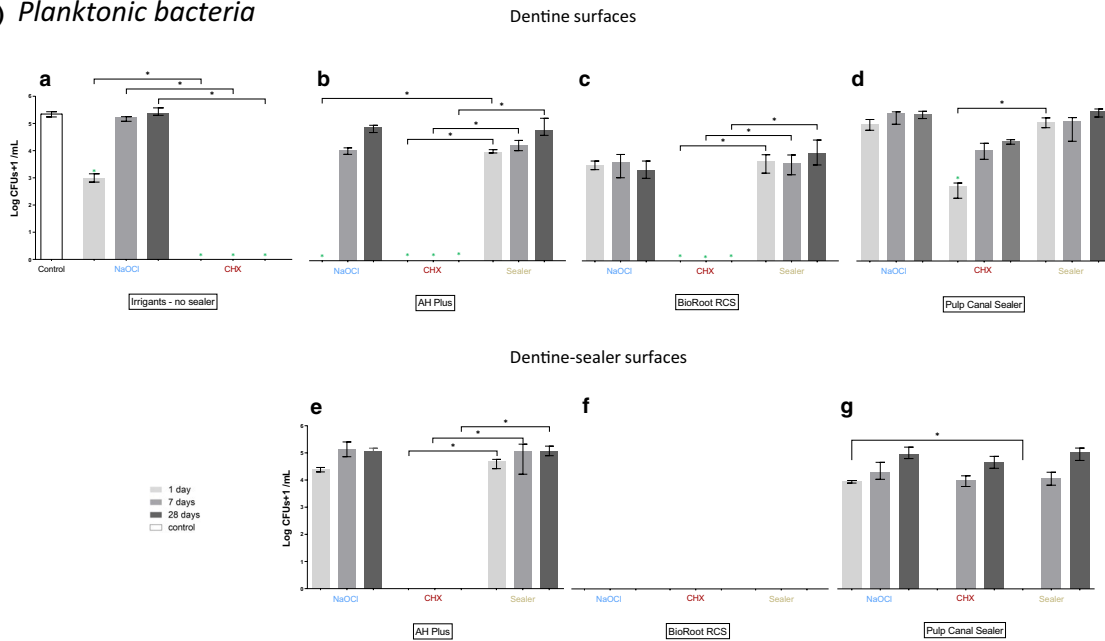
CHX (G2) eliminated *E. faecalis* on dentine without sealer placement for all three incubation times (1, 7 and 28 days). In addition, NaOCl (G1) reduced after 1 day incubation the number of surviving *E. faecalis* compared to control ( $p < .05$ ) (Figure 6Aa). CHX



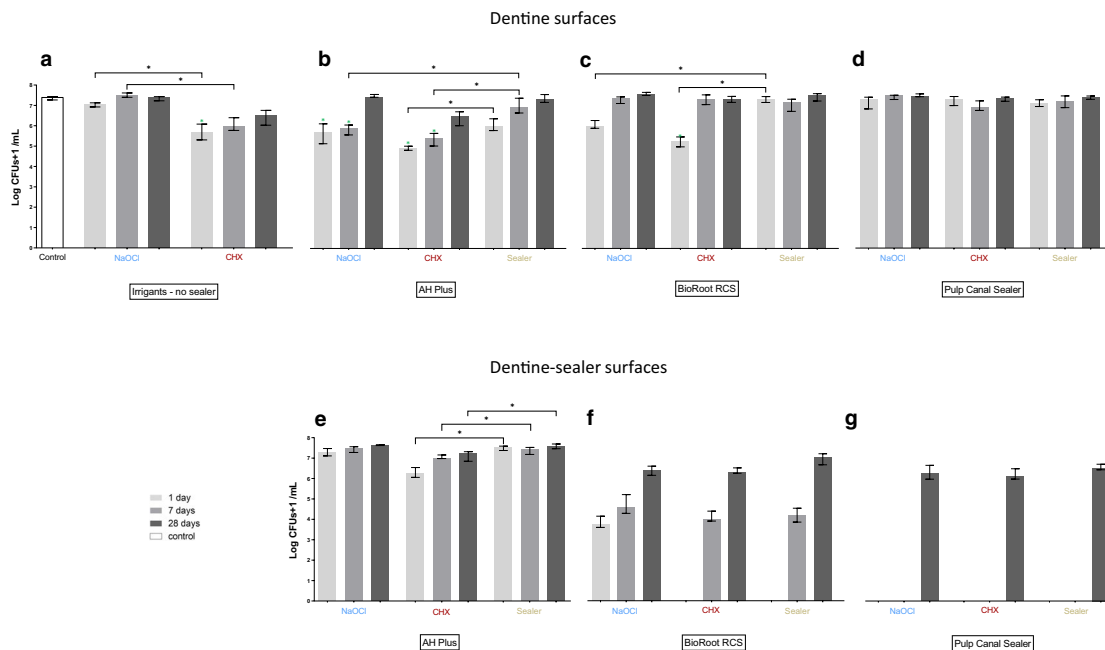
**FIGURE 5** Representative scanning electron micrographs of dentine having been in contact with the tested sealers retrieved from the split tooth model: AH Plus (a), BioRoot RCS (b) and PCS (c). The black arrows indicate sealer residues (white circular spots) rich in Zr for AH Plus/BioRoot RCS and Zn for PCS, verified by elemental analysis. Elemental mapping of dentine in contact with BioRoot RCS shows the distribution of Cl and Si. Elemental mapping of dentine in contact with PCS indicate the presence of Zn



(A) Planktonic bacteria



(B) Bacteria in Biofilms



**FIGURE 6** Residual effect of NaOCl and CHX on dentine and dentine-sealer surfaces. Median Log (CFU + 1)/ml and 25–75 interpercentile range (error bars) of *E. faecalis* in planktonic forms (A) and in biofilms (B) after direct contact with each dentine and dentine-sealer surface. For dentine surfaces, green asterisks indicate statistically significant differences between different incubation times of groups and the control of *E. faecalis* ( $p < .05$ ). For dentine and dentine-sealer surfaces, black asterisks and brackets indicate statistical differences for pairwise comparisons between G1: NaOCl or G2: CHX and G3: Sealer in each sealer for each one of the three incubation times tested (1, 7 and 28 days) ( $p < .05$ )

eliminated *E. faecalis* on dentine which had been in contact with AH Plus and BioRoot RCS for all three incubation times whilst NaOCl eliminated *E. faecalis* on dentine in contact with AH Plus for 1 day incubation (Figure 6Ab, Ac).

*Planktonic E. faecalis, dentine-sealer surfaces*

No surviving planktonic *E. faecalis* were recovered from BioRoot RCS surfaces without irrigant application (G3) as well as when both NaOCl (G1) and CHX (G2) were applied for all incubation times (Figure 6Af). AH Plus

surfaces which have been in contact with dentine treated with CHX eliminated *E. faecalis* and exhibited higher antibacterial activity than AH Plus in contact with dentine without irrigant application (G3) for all three incubation times ( $p < .05$ ; Figure 6Ae). PCS surfaces in contact with dentine without irrigant application (G3) and treated with CHX (G2) eliminated *E. faecalis* after 1 day incubation.

#### *E. faecalis* in biofilms, dentine surfaces

Dentine treated with CHX (G2) and without sealer placement significantly reduced *E. faecalis* in biofilms only for 1 day incubation of root blocks compared to control ( $p < .05$ ; Figure 6Ba). Survival of *E. faecalis* was significantly reduced upon AH Plus dentine surfaces treated with CHX (G2) and NaOCl (G1) for 1 and 7 days incubation of root blocks compared to control ( $p < .05$ ; Figure 6Bb). Dentine surfaces treated with CHX (G2) and in contact with BioRoot RCS had an antibacterial effect on *E. faecalis* biofilms only for 1 day incubation of root blocks compared to control ( $p < .05$ ; Figure 6Bc).

#### *E. faecalis* in biofilms, dentine-sealer surfaces

PCS surfaces in contact with dentine treated with NaOCl (G1), CHX (G2) and dentine without irrigant application (G3) eliminated *E. faecalis* in biofilms after 1 and 7 days incubation of root blocks (Figure 6Bg). BioRoot RCS surfaces in contact with dentine treated with CHX (G2) and also without irrigant application (G3) eliminated *E. faecalis* after 1 day incubation of root blocks (Figure 6Bf). AH Plus surfaces, which have been in contact with dentine treated with CHX (G2), exhibited higher antibacterial properties against *E. faecalis* in biofilms ( $p < .05$ ) compared to sealer without irrigant application (G3) for all three incubation times ( $p < .05$ ; Figure 6Be).

## Effect of irrigation protocols on dentine and dentine-sealer surfaces

#### *Planktonic E. faecalis, dentine surfaces*

CHX as the final irrigant (P2: NaOCl + EDTA + CHX) without sealer placement eliminated all bacteria for all three incubation times compared to control and to P1 (NaOCl + EDTA;  $p < .05$ ; Figure 7Aa). Regarding sealer placement, no surviving bacteria were observed when CHX was used as the final irrigant (P2) for all dentine surfaces for all incubation times except for those in contact with PCS after 28 days incubation (Figure 7Ab, Ac, Ad).

#### *Planktonic E. faecalis, dentine-sealer surfaces*

No surviving *E. faecalis* bacteria were retrieved from BioRoot RCS surfaces which have been in contact with

dentine treated both with P1 and P2 (Figure 7Af). When CHX was used as the last irrigant (P2), AH Plus surfaces which have been in contact with dentine eliminated *E. faecalis* and significantly reduced its numbers compared to AH Plus in contact with dentine treated with P1 for all three irrigation times ( $p < .05$ ; Figure 7Ae). PCS surfaces in contact with dentine treated with P1 eliminated *E. faecalis* after 1 day incubation, whilst treatment with P2 did after both 1 and 7 days incubation.

#### *E. faecalis* in biofilms, dentine surfaces

Dentine treated with CHX as last irrigant (P2) and without sealer placement significantly reduced *E. faecalis* in biofilms only for 1 day incubation of root blocks compared to control ( $p < .05$ ) and for both 1 and 7 days incubation compared to P1 ( $p < .05$ ; Figure 7Ba). Dentine surfaces irrigated with CHX (P2) and in contact with AH Plus for 1 day were the only amongst the tested sealers to show antibacterial properties against biofilms compared to control ( $p < .05$ ) (Figure 7Bb). In contact with PCS, dentine surfaces treated with CHX as last irrigant (P2) exhibited higher antibacterial efficacy than dentine treated with (P1) after 1 and 7 days incubation of root blocks ( $p < .05$ ; Figure 7Bd).

#### *E. faecalis* in biofilms, dentine-sealer surfaces

PCS surfaces in contact with dentine treated with P2 eliminated *E. faecalis* after 1 and 7 days incubation of root blocks. When CHX was used as the last irrigant (P2), AH Plus surfaces which have been in contact with dentine significantly reduced *E. faecalis* compared to AH Plus in contact with dentine treated with P1 for all three irrigation times ( $p < .05$ ; Figure 7Be).

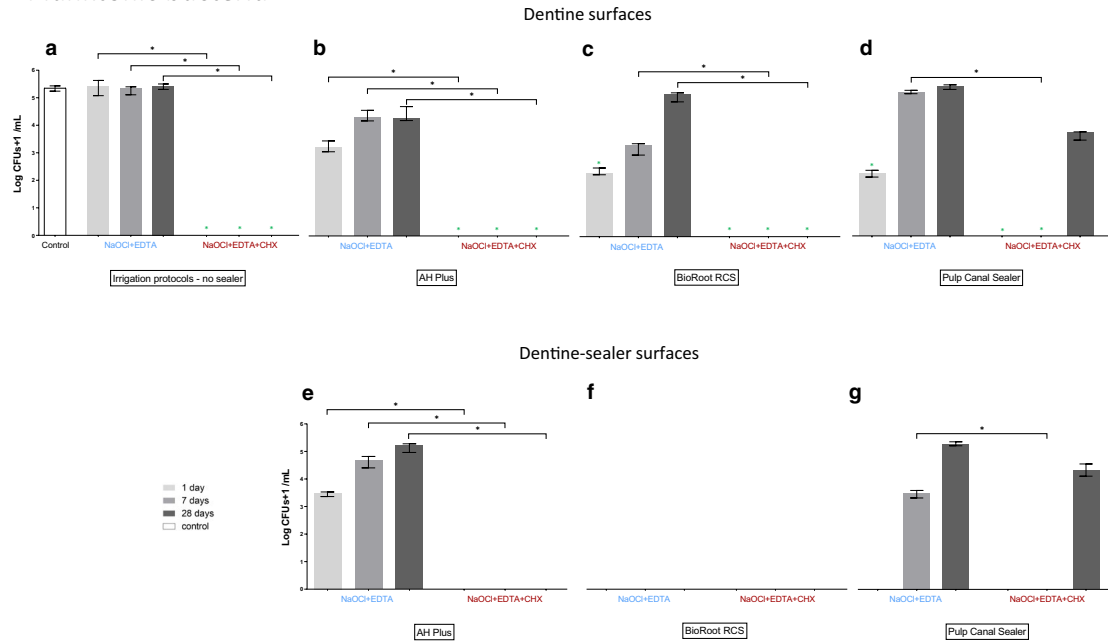
No carryover effect was detected in both planktonic bacteria and biofilms assay (data not shown). The numeric data for both assays are shown in Tables S1, S2 and S3. In addition, the pairwise comparisons between groups (G1 or G2 with G3) and irrigation protocols (P1 with P2) for each one of the incubation times tested (1, 7 and 28 days) are shown in Figures 6 and 7.

## Multiple regression analyses

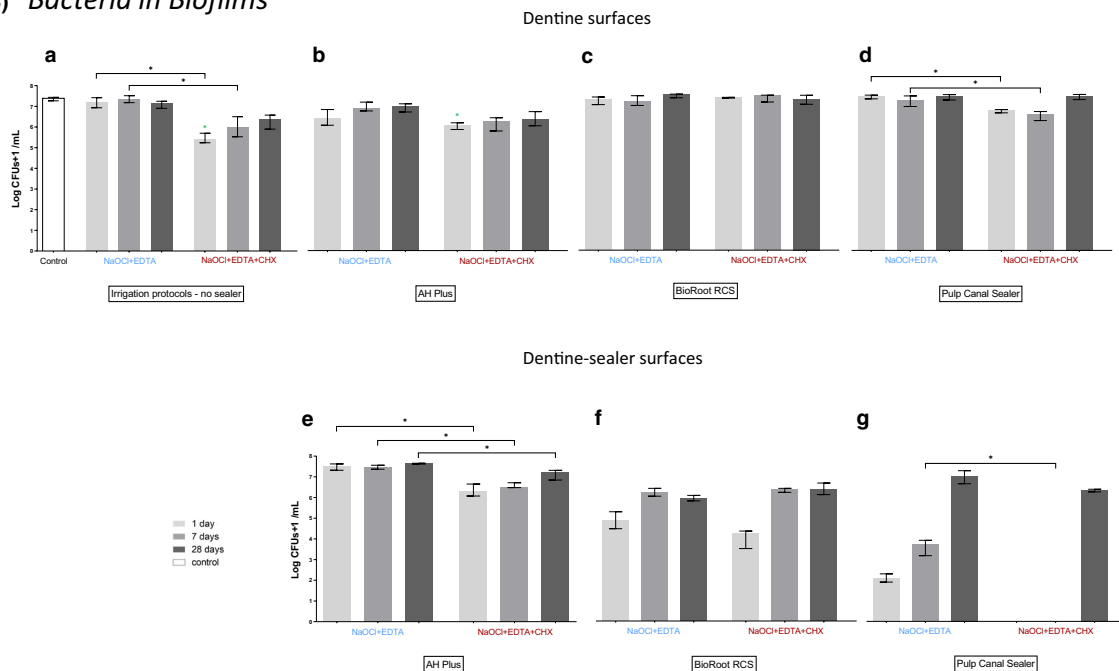
Multiple regressions were run to predict bacterial survival (CFUs) from irrigation, type of sealer, EDTA-pre-treatment, ageing period and substrate. All the tested assumptions were met for all the regression analyses performed.

The multiple regression model (1) statistically significantly predicted bacterial survival (CFUs),  $F(5, 642) = 127.654$ ,  $p < .001$ , adj.  $R^2 = .50$ . All five variables added statistically significantly to the prediction,  $p < .001$ .

## (A) Planktonic bacteria



## (B) Bacteria in Biofilms



**FIGURE 7** Antibacterial effect of two irrigation protocols on dentine and dentine-sealer surfaces. Median Log (CFU + 1)/ml and 25–75 interpercentile range (error bars) of *E. faecalis* in planktonic forms (A) and in biofilms (B) after direct contact with each dentine and dentine-sealer surface. For dentine surfaces, green asterisks indicate statistically significant differences between different incubation times of groups and the control of *E. faecalis* ( $p < .05$ ). For dentine and dentine-sealer surfaces, black asterisks and brackets indicate statistical differences for pairwise comparisons between P1: 1% NaOCl + 17% EDTA and P2: 1% NaOCl + 17% EDTA + 2% CHX in each sealer for each one of the three incubation times tested (1, 7 and 28 days;  $p < .05$ )

The multiple regression model (2) statistically significantly predicted bacterial survival (CFUs),  $F(5, 642) = 110.426$ ,  $p < .001$ , adj.  $R^2 = .46$ . All five variables added statistically

significantly to the prediction,  $p < .001$ . Models' fit, regression coefficients and standard errors for both models (1) and (2) can be found in Table S4. Models' fit, regression

coefficients and standard errors for models (3) to (18) can be found in Tables S5, S6, S7 and S8, respectively. Detailed information regarding the interpretation of the multiple regression models can be found in the Supplementary Material (Supplementary material\_multiple regression analyses).

## DISCUSSION

Bacterial infection of the root canal involves the pulp space, pulp canal walls, the dentinal tubules and the interface between endodontic sealers and dentine in cases of reinfection or presence of persistent bacteria (Ricucci & Siqueira, 2010; Ricucci et al., 2009). Irrigation solutions and the use of endodontic sealers with various chemistries may affect the antimicrobial properties of both dentine and sealer surfaces and ultimately the outcome of the root canal treatment (Arias-Moliz & Camilleri, 2016).

In this study, a split tooth model was developed to examine the residual antimicrobial effect of two irrigants and two clinical irrigation protocols at the level of sealer to dentine interface. More explicitly, both the dentine and the sealers that had been in contact with dentine were assessed for their antibacterial properties.

The split tooth model was first verified for its applicability by means of SEM and elemental analysis to secure complete separation of the sealer bulk from dentine. The SEM examination showed no indications of cohesive failure, which would have resulted in dentine surfaces, covered with sealer after separation. There was complete separation of the sealers from dentine at the sealer-dentine interface (adhesive type of failure), and the chemical analyses of the surfaces similarly indicated separation of sealers from dentin. The model was therefore considered suitable for investigating surface characteristics after separation.

Only a few studies have investigated the interaction between endodontic sealers and irrigation solutions. A recent study showed enhanced antibacterial efficacy of AH Plus, BioRoot RCS and PCS after exposure to 2% chlorhexidine digluconate against both planktonic bacteria and bacteria in biofilms (Kapralos et al., 2020). Previous studies have used a dentine infection model (*ex vivo* model for infection of dentinal tubuli) to assess the effectiveness of either endodontic irrigants (Du et al., 2014; Huang et al., 2019; Wang et al., 2012) or root canal sealers inside the dentinal tubuli (Prestegard et al., 2014). Our study is the first to measure the combined antibacterial effect of irrigation and endodontic sealers on dentine walls and sealer surfaces simultaneously.

To assess the viability of planktonic bacteria and mono-species biofilms grown upon membranes after

contact with sealer and dentine surfaces, a DCT and a quantitative tool based on microbiological culturing (the plate count method, CFUs counts) were chosen to assess bacterial viability. The DCT is widely used replacing the agar diffusion test (ADT) due to limitations of the latter: semiquantitative nature, restriction to distinguish between bacteriostatic and bactericidal activity, limitation to detect the activity of insoluble components (Eldeniz et al., 2006; Faria-Júnior et al., 2013; Weiss et al., 1996). The CFU counts are an universally accepted laboratory technique and enable comparisons between experiments (Swimberghe et al., 2019).

The use of a mono-species biofilm model is an evident limitation of our study. Irrigants and root canal sealers should also be tested in more complex environments such as multispecies biofilms (Du et al., 2015). Even though simplified laboratory models do not represent the clinical reality of the infected root canal, they constitute valuable tools to preliminary assess the antibacterial effect of irrigation solutions and endodontic materials as they can be standardized and controlled. Their set up is easy and reproducible, and they allow high experimental throughput (Swimberghe et al., 2019). The objective of this study was to develop and use a suitable tooth model for testing the antibacterial properties of both endodontic sealers and their adjacent dentinal walls after exposure with CHX and NaOCl. The lack of standardized methods in testing of antimicrobial properties of sealers is a challenge (Camilleri et al., 2020; Wang et al., 2014). A standardized tooth model may provide new insights into the antibacterial activity of endodontic materials.

In this study, *E. faecalis* in planktonic form and in biofilms was used as the test organism. This bacterium occurs particularly in cases of persistent apical periodontitis (Sunde et al., 2002; Sundqvist et al., 1998). Numerous *in vitro* and *ex vivo* studies have used *E. faecalis* to test the antibacterial properties of endodontic materials (AlShwaimi et al., 2016; Šimundić Munitić et al., 2019; Swimberghe et al., 2019). Furthermore, *E. faecalis* can colonize dentine and form biofilms on different substrates including root canal filling materials (George et al., 2010; Guerreiro-Tanomaru et al., 2013).

For investigating the antibacterial properties against *E. faecalis* in biofilms, we used a previously established 48 h-grown biofilm model modified by a substrate of mixed cellulose esters (MCE) membrane filters. A 48 h biofilm under static conditions cannot be considered as a mature biofilm. However, based on the results of the study, the 48 h biofilms did challenge the antibacterial efficacy of the endodontic sealers, even for BioRoot RCS and PCS that exhibited the highest antibacterial activity. In previous studies, *E. faecalis* biofilms were grown on biological substrates such as bovine dentine or human dentine

(Faria-Júnior et al., 2013; Wang et al., 2014). Nevertheless, the tested sealers may firmly adhere on dentine leading to partial retrieval of bacteria or possible carryover effect. In our study, the SEM examination showed substantial separation of the sealers from the dentine. In addition, the high hydrophilicity of MCE membrane filters enabled an easy separation of the filter with the biofilm from the sealers, thus minimizing the disruption of the biofilm. The reproducibility of our method in retrieving the bacteria from the MCE membranes is reflected also by the consistency in values of our controls.

An endodontic sealer is meant to seal, any sealer that exerts effects after it is set, i.e. is not inert at that time, and may become leaky. MicroCT analysis has revealed a higher void volume for BioRoot RCS compared to AH Plus (Viapiana et al., 2016) and hydraulic calcium-silicate cements have been reported incapable to produce a fluid-tight seal (De-Deus et al., 2007). Most sealers maintain their antibacterial properties throughout the setting process (Kapralos et al., 2018; Ørstavik, 2005). Amongst irrigants investigated, CHX can bind to dentine and be gradually released. This may contribute to prolonged antibacterial properties (Carrilho et al., 2010; Haapasalo et al., 2014). In this study, the incubation time ranged from 1 day up to 28 days to assess the potential long lasting antibacterial effect of irrigation on sealers. Regarding antibacterial activity of the sealers against biofilms, a short contact time may not be adequate and representative of the full antibacterial capacity of materials. Therefore, we tested the antibacterial properties against established biofilms for 24 h contact time.

Sealers with different chemistry were chosen to assess any specificity in the interactions with the tested irrigants. AH Plus, an epoxy resin-based root canal sealer, has been thoroughly investigated and is commonly used as a benchmark for comparisons (Ørstavik, 2005; Zhou et al., 2015). BioRoot RCS, a hydraulic calcium-silicate based sealer, has both potent antibacterial (Arias-Moliz & Camilleri, 2016) and biological (cytotoxicity; Jung et al., 2019) properties. The sealer is highly susceptible to the environmental conditions due to its hydraulic properties and the formation of calcium hydroxide during hydration process (Kebudi Benezra et al., 2017). PCS, a zinc-oxide eugenol sealer, has been used in endodontics for decades and possesses antibacterial properties. In our study, the sealers were applied in bulk without a gutta-percha core.

To assess the isolated effect of 1% NaOCl and 2% CHX on antibacterial properties, the smear layer was removed with the use of 17% EDTA and the root blocks were rinsed in between with saline solution to avoid any additional interactions between EDTA and NaOCl-CHX (Rossi-Fedele et al., 2012). As clinical procedures most often entail the use of several irrigation liquids, two relevant irrigation

protocols were also tested: 1% NaOCl + 17% EDTA and 1% NaOCl + 17% EDTA + 2% CHX. Only treatment with CHX, both in group 2 and in irrigation protocol 2, eliminated the planktonic bacteria on dentine surfaces in all incubation times up to 28 days. This result corroborates earlier literature on CHX's ability to possess long-lasting antibacterial properties due to substantivity (Carrilho et al., 2010; Rosenthal et al., 2004; Souza et al., 2018). In this study, 1% NaOCl had inferior antibacterial properties to 2% CHX that can be potentially attributed to its low concentration; *in vitro* studies indicate that higher percentage of NaOCl could result in increased antibacterial properties (Gomes et al., 2001; Tirali et al., 2009). However, clinical findings suggest no significant differences in antimicrobial properties of NaOCl in different concentrations (0.5%–5.25%; Byström & Sundqvist, 1985; Soares & Pires Júnior, 2006). Moreover, a recent randomized clinical study reported similar clinical outcomes for high (5%) and low (1%) NaOCl concentrations (Verma et al., 2019). Toxicity of NaOCl to periapical tissues as well as its deleterious effect on the integrity of dentine structure and on the collagen matrix is concentration dependent, with higher concentrations being more irritating (Farook et al., 2014; Marending et al., 2007; Pashley et al., 1985; Zancan et al., 2021). Thus, in our study, 1% NaOCl was preferred to higher percentages as low NaOCl concentrations have been shown to combine both antimicrobial properties and low cytotoxicity. Application of CHX (G2 and P2) managed to reduce significantly the numbers of *E. faecalis* in biofilms only after 1-day incubation period, confirming that biofilms are more resistant than their planktonic counterparts (Bjarnsholt, 2013).

AH Plus possesses antibacterial properties mainly during setting of the material (Kapralos et al., 2018; Zhang et al., 2009). We also found persistent antibacterial activity of AH Plus unexposed to CHX or NaOCl (G3). However, AH Plus and dentine surfaces exerted antibacterial properties against both *E. faecalis* planktonic bacteria and biofilms when CHX was applied (G2 and P2). A previous study on the antibacterial properties of AH Plus modified with CHX showed improved efficacy compared to unmodified sealer (Bailón-Sánchez et al., 2014). In addition, both short- (1 min) and long-term (24 h) application of 2% CHX on AH Plus surfaces improved the sealer's antibacterial performance against planktonic *E. faecalis* in an *in vitro* study (Kapralos et al., 2020). Exposed to NaOCl AH Plus dentine surface (G1) eliminated the planktonic *E. faecalis* after 1 day of incubation, and reduced *E. faecalis* in biofilms after 1 and 7 days incubation, confirming the additive effect of NaOCl and AH Plus shown in an *ex vivo* study (Du et al., 2015).

BioRoot RCS sealer surfaces eliminated planktonic *E. faecalis* in all groups (G1, G2, G3, P1, P2) and incubation

times. The proposed antibacterial mechanism of BioRoot RCS is based on hydration of tricalcium silicate-based cements (Cuesta et al., 2018; Long et al., 2020). Hydration of tricalcium silicates leads to the formation of calcium hydroxide which in contact with water releases calcium ions ( $\text{Ca}^{+2}$ ) and hydroxyl ions ( $\text{OH}^-$ ) raising the pH and contributing to the antibacterial activity (Kapralos et al., 2020; Xuereb et al., 2015). BioRoot RCS was found to be strongly antibacterial against *E. faecalis*, especially after a final irrigation with EDTA, in an *ex vivo* intratubular infection tooth model study (Arias-Moliz & Camilleri, 2016). Our results corroborated these findings: a final application of EDTA (P1) increased the antibacterial properties of BioRoot RCS. Even though EDTA has been found to interact with the tricalcium silicate and reduce or eliminate the formed calcium hydroxide (Arias-Moliz & Camilleri, 2016; Lee et al., 2007), the antibacterial properties of the sealer were not compromised in this study. This can partially be explained as EDTA chelates calcium from the sealer and the dentine, providing more free calcium thus increasing the antibacterial activity (Arias-Moliz & Camilleri, 2016). Moreover, the residual effect of CHX (G2 and P2) enhanced the antibacterial efficacy of BioRoot RCS dentine surfaces. Previous studies on Biodentine, another tricalcium silicate cement, showed improved antibacterial properties when mixed with CHX compared to unmodified cement (Deveci et al., 2019; Nikhil et al., 2014). At the same time, BioRoot RCS chemistry has been shown to remain unaffected under CHX irrigation (Kapralos et al., 2020). One study found that the antibacterial properties of BioRoot RCS against *E. faecalis* biofilms in dentinal tubules presented fluctuations over time (Alsubait et al., 2019); another concluded that BioRoot RCS had moderate antibacterial properties using a modified DCT (Poggio et al., 2017). Two recent studies showed strong antimicrobial activity for BioRoot RCS, as the sealer did not allow any biofilm accumulation (Long et al., 2020) and presented the highest microbial killing (Bose et al., 2020) amongst the investigated sealers. Variable results for the antibacterial properties of BioRoot RCS seem most likely due to differences in methodology (Alsubait et al., 2019; Arias-Moliz & Camilleri, 2016; Poggio et al., 2017).

In this study, PCS exhibited antibacterial properties mainly on sealer surfaces, which had been in contact with dentine and high efficacy against *E. faecalis* biofilms. This indicates that PCS may exhibit moderate constant antibacterial properties, related to the gradual release of eugenol (Hauman & Love, 2003; Marchese et al., 2017), given that in biofilm assays the contact time of dentine or dentine-sealer surfaces with bacteria was 24 h. In addition, a new study demonstrated a decrease in *E. faecalis* live bacteria upon PCS surfaces after an

initial biofilm formation, which may be correlated to the release of zinc (Long et al., 2020). On the contrary, the antibacterial effect of the PCS upon dentine was weak especially against biofilms. This could be attributed to the pronounced shrinkage that PCS displays stored at 100% humidity (Camilleri & Mallia, 2011), which might lead to loose (non-tight) contact with the dentinal walls and thus compromised antibacterial properties. Moreover, a zinc-oxide eugenol impression material exhibited reduction in dimensions after disinfection with aqueous CHX and NaOCl solutions (Amin et al., 2009). Previous studies on zinc oxide eugenol cements as PCS have demonstrated improved antibacterial activity after mixing with CHX (Nambu, 1984; Tchaou et al., 1996). In our study, treatment with CHX (G2 and P2) conferred antibacterial properties on dentine walls against planktonic *E. faecalis*.

Although many *in vitro* and *ex vivo* studies have demonstrated a wide range of antibacterial efficacy amongst endodontic materials, clinical studies indicate no significant differences amongst different endodontic sealers and irrigation solutions regarding clinical outcome (Ng et al., 2011; Zandi et al., 2019; Zavattini et al., 2020). The success of endodontic treatment is multifactorial, with each distinct procedural step playing a significant role and contributing to the overall therapeutic result. The potential antimicrobial clinical advantages of endodontic sealers need to be addressed in clinical studies.

Further studies assessing the combined antibacterial properties of various endodontic filling materials and irrigants both at the sealer-to-dentine interface and in the dentinal tubules should be performed using multispecies biofilms at different stage of maturity in *ex vivo* tooth models.

## CONCLUSIONS

The split tooth model developed for this study was reproducible. The hypotheses were rejected: NaOCl and CHX affected to various extent the antimicrobial properties of both dentine and sealer surfaces and the two irrigation protocols differed in antimicrobial efficacy. Overall, CHX improved the antibacterial activity in relation to sealer and dentine surfaces.

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## CONFLICT OF INTEREST

The authors have explicitly stated that there are no conflicts of interest.

## ETHICAL APPROVAL

Extracted human teeth were collected from a bio-bank (“2013/413 NIOM tannbank”) approved by the Regional Committees for Medical and Health Research Ethics (REC, application number 28748), Norway.

## AUTHOR CONTRIBUTIONS

**Vasileios Kapralos** – main author, the conception and design of the study, drafting the article, acquisition of data, statistical analysis and interpretation of data. **Håkon Valen** – close follow-up and supervision of the experimental process, design of the study, revising the article critically, final approval of the version to be submitted. **Andreas Koutroulis** – contribution to the study design, revising the article critically, final approval of the version to be submitted. **Josette Camilleri** – close follow-up and supervision of the experimental process, design of the study, revising the article, final approval of the version to be submitted. **Dag Ørstavik** – experience and overview, design of the study, interpretation of data, revising the article critically, final approval of the version to be submitted. **Pia Titterud Sunde** – close follow-up and main responsibility, supervision of the experimental process, design of the study, interpretation of data, revising the article critically, final approval of the version to be submitted.

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## REFERENCES

- AlShwaimi, E., Bogari, D., Ajaj, R., Al-Shahrani, S., Almas, K. & Majeed, A. (2016) In vitro antimicrobial effectiveness of root canal sealers against *Enterococcus faecalis*: a systematic review. *Journal of Endodontics*, 42, 1588–1597.
- Alsubait, S., Albader, S., Alajlan, N., Alkhunaini, N., Niazy, A. & Almahdy, A. (2019) Comparison of the antibacterial activity of calcium silicate- and epoxy resin-based endodontic sealers against *Enterococcus faecalis* biofilms: a confocal laser-scanning microscopy analysis. *Odontology*, 107, 513–520.
- Amin, W.M., Al-Ali, M.H., Al Tarawneh, S.K., Taha, S.T., Saleh, M.W. & Ereifij, N. (2009) The effects of disinfectants on dimensional accuracy and surface quality of impression materials and gypsum casts. *Journal of Clinical Medicine Research*, 1, 81–89.
- Arias-Moliz, M.T. & Camilleri, J. (2016) The effect of the final irrigant on the antimicrobial activity of root canal sealers. *Journal of Dentistry*, 52, 30–36.
- Bailón-Sánchez, M.E., Baca, P., Ruiz-Linares, M. & Ferrer-Luque, C.M. (2014) Antibacterial and anti-biofilm activity of AH plus with chlorhexidine and cetrimide. *Journal of Endodontics*, 40, 977–981.
- Bjarnsholt, T. (2013) The role of bacterial biofilms in chronic infections. *APMIS*, 121(Suppl. 136), 1–51.
- Bolhari, B., Dehghan, S., Sharifian, M.R., Bahador, A. & Pirmoazen, S. (2015) Effect of different obturation materials on residual antimicrobial activity of 2% chlorhexidine in dentin at different time intervals: an ex vivo study. *Journal of Dentistry*, 12, 720–728.
- Bose, R., Ioannidis, K., Foschi, F., Bakhsh, A., Kelly, R.D., Deb, S. et al. (2020) Antimicrobial effectiveness of calcium silicate sealers against a nutrient-stressed multispecies biofilm. *Journal of Clinical Medicine*, 9, 2722.
- Byström, A. & Sundqvist, G. (1985) The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *International Endodontic Journal*, 18, 35–40.
- Camilleri, J., Arias Moliz, T., Bettencourt, A., Costa, J., Martins, F., Rabadjeva, D. et al. (2020) Standardization of antimicrobial testing of dental devices. *Dental Materials*, 36, e59–e73.
- Camilleri, J. & Mallia, B. (2011) Evaluation of the dimensional changes of mineral trioxide aggregate sealer. *International Endodontic Journal*, 44, 416–424.
- Carrilho, M.R., Carvalho, R.M., Sousa, E.N., Nicolau, J., Breschi, L., Mazzoni, A. et al. (2010) Substantivity of chlorhexidine to human dentin. *Dental Materials*, 26, 779–785.
- Carrotte, P. (2004) Endodontics: part 7. Preparing the root canal. *British Dental Journal*, 197, 603–613.
- Chan, L.T., Zhong, S., Naqvi, A.R., Self-Fordham, J., Nares, S., Bair, E. et al. (2013) MicroRNAs: new insights into the pathogenesis of endodontic periapical disease. *Journal of Endodontics*, 39, 1498–1503.
- Cuesta, A., Zea-Garcia, J.D., Londono-Zuluaga, D., De la Torre, A.G., Santacruz, I., Vallcorba, O. et al. (2018) Multiscale understanding of tricalcium silicate hydration reactions. *Scientific Reports*, 8, 8544.
- Dametto, F.R., Ferraz, C.C., Gomes, B.P., Zaia, A.A., Teixeira, F.B. & de Souza-Filho, F.J. (2005) In vitro assessment of the immediate and prolonged antimicrobial action of chlorhexidine gel as an endodontic irrigant against *Enterococcus faecalis*. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*, 99, 768–772.
- De-Deus, G., Reis, C., Brandão, C., Fidel, S. & Fidel, R.A.S. (2007) The ability of Portland Cement, MTA, and MTA Bio to prevent through-and-through fluid movement in repaired furcal perforations. *Journal of Endodontics*, 33, 1374–1377.
- Deveci, C., Tuzuner, T., Cinar, C., Odabas, M.E. & Buruk, C.K. (2019) Short-term antibacterial activity and compressive strength of biodynamic containing chlorhexidine/cetrimide mixtures. *Nigerian Journal of Clinical Practice*, 22, 227–231.
- Donnermeyer, D., Vahdat-Pajouh, N., Schafer, E. & Dammaschke, T. (2019) Influence of the final irrigation solution on the push-out bond strength of calcium silicate-based, epoxy resin-based and silicone-based endodontic sealers. *Odontology*, 107, 231–236.
- Du, T., Wang, Z., Shen, Y., Ma, J., Cao, Y. & Haapasalo, M. (2014) Effect of long-term exposure to endodontic disinfecting solutions on young and old *Enterococcus faecalis* biofilms in dentin canals. *Journal of Endodontics*, 40, 509–514.
- Du, T., Wang, Z., Shen, Y., Ma, J., Cao, Y. & Haapasalo, M. (2015) Combined antibacterial effect of sodium hypochlorite and root canal sealers against *Enterococcus faecalis* biofilms in dentin canals. *Journal of Endodontics*, 41, 1294–1298.

- Eldeniz, A.U., Hadimli, H.H., Ataoglu, H. & Ørstavik, D. (2006) Antibacterial effect of selected root-end filling materials. *Journal of Endodontics*, 32, 345–349.
- Faria-Júnior, N.B., Tanomaru-Filho, M., Berbert, F.L. & Guerreiro-Tanomaru, J.M. (2013) Antibiofilm activity, pH and solubility of endodontic sealers. *International Endodontic Journal*, 46, 755–762.
- Farook, S.A., Shah, V., Lenouvel, D., Sheikh, O., Sadiq, Z. & Cascarini, L. (2014) Guidelines for management of sodium hypochlorite extrusion injuries. *British Dental Journal*, 217, 679–684.
- Fernandes Zancan, R., Hadis, M., Burgess, D., Zhang, Z.J., Di Maio, A., Tomson, P. et al. (2021) A matched irrigation and obturation strategy for root canal therapy. *Scientific Reports*, 11, 4666.
- George, S., Basrani, B. & Kishen, A. (2010) Possibilities of gutta-percha-centered infection in endodontically treated teeth: an in vitro study. *Journal of Endodontics*, 36, 1241–1244.
- Gomes, B.P., Ferraz, C.C., Vianna, M.E., Berber, V.B., Teixeira, F.B. & Souza-Filho, F.J. (2001) In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. *International Endodontic Journal*, 34, 424–428.
- Guerreiro-Tanomaru, J.M., de Faria-Júnior, N.B., Duarte, M.A., Ordinola-Zapata, R., Graeff, M.S. & Tanomaru-Filho, M. (2013) Comparative analysis of *Enterococcus faecalis* biofilm formation on different substrates. *Journal of Endodontics*, 39, 346–350.
- Haapasalo, M., Shen, Y., Wang, Z. & Gao, Y. (2014) Irrigation in endodontics. *British Dental Journal*, 216, 299–303.
- Hauman, C.H. & Love, R.M. (2003) Biocompatibility of dental materials used in contemporary endodontic therapy: a review. Part 2. Root-canal-filling materials. *International Endodontic Journal*, 36, 147–160.
- Huang, X., Haapasalo, M., Wang, Z., Hancock, R.E.W., Wei, X. & Shen, Y. (2019) Effect of long-term exposure to peptides on mono- and multispecies biofilms in dentinal tubules. *Journal of Endodontics*, 45, 1522–1528.
- Jung, S., Libricht, V., Sielker, S., Hanisch, M.R., Schäfer, E. & Dammaschke, T. (2019) Evaluation of the biocompatibility of root canal sealers on human periodontal ligament cells ex vivo. *Odontology*, 107, 54–63.
- Kapralos, V., Koutroulis, A., Ørstavik, D., Sunde, P.T. & Rukke, H.V. (2018) Antibacterial activity of endodontic sealers against planktonic bacteria and bacteria in biofilms. *Journal of Endodontics*, 44, 149–154.
- Kapralos, V., Rukke, H.V., Ørstavik, D., Koutroulis, A., Camilleri, J. & Sunde, P.T. (2020) Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate. *Dental Materials*, 37, 249–263.
- Kebudi Benezra, M., Schembri Wismayer, P. & Camilleri, J. (2017) Influence of environment on testing of hydraulic sealers. *Scientific Reports*, 7, 17927.
- Keleş, A., Alcin, H., Kamalak, A. & Versiani, M.A. (2014) Micro-CT evaluation of root filling quality in oval-shaped canals. *International Endodontic Journal*, 47, 1177–1184.
- Khademi, A.A., Mohammadi, Z. & Havaee, A. (2006) Evaluation of the antibacterial substantivity of several intra-canal agents. *Australian Endodontic Journal*, 32, 112–115.
- Lee, Y.L., Lin, F.H., Wang, W.H., Ritchie, H.H., Lan, W.H. & Lin, C.P. (2007) Effects of EDTA on the hydration mechanism of mineral trioxide aggregate. *Journal of Dental Research*, 86, 534–538.
- Long, J., Kreft, J.U. & Camilleri, J. (2020) Antimicrobial and ultrastructural properties of root canal filling materials exposed to bacterial challenge. *Journal of Dentistry*, 93, 103283.
- Marchese, A., Barbieri, R., Coppo, E., Orhan, I.E., Daglia, M., Nabavi, S.F. et al. (2017) Antimicrobial activity of eugenol and essential oils containing eugenol: a mechanistic viewpoint. *Critical Reviews in Microbiology*, 43, 668–689.
- Marending, M., Luder, H.U., Brunner, T.J., Knecht, S., Stark, W.J. & Zehnder, M. (2007) Effect of sodium hypochlorite on human root dentine—mechanical, chemical and structural evaluation. *International Endodontic Journal*, 40, 786–793.
- Nair, P.N. (2006) On the causes of persistent apical periodontitis: a review. *International Endodontic Journal*, 39, 249–281.
- Nambu, T. (1984) Study on antibacterial root canal sealer containing chlorhexidine dihydrochloride. II. Investigation of antibacterial activity and follow-up study on clinical usage. *Dental Materials Journal*, 3, 288–311.
- Ng, Y.L., Mann, V. & Gulabivala, K. (2011) A prospective study of the factors affecting outcomes of nonsurgical root canal treatment: part 1: periapical health. *International Endodontic Journal*, 44, 583–609.
- Nikhil, V., Madan, M., Agarwal, C. & Suri, N. (2014) Effect of addition of 2% chlorhexidine or 10% doxycycline on antimicrobial activity of biodentine. *Journal of Conservative Dentistry*, 17, 271–275.
- Ørstavik, D. (1988) Antibacterial properties of endodontic materials. *International Endodontic Journal*, 21, 161–169.
- Ørstavik, D. (2005) Materials used for root canal obturation: technical, biological and clinical testing. *Endodontic Topics*, 12, 25–38.
- Pashley, E.L., Birdsong, N.L., Bowman, K. & Pashley, D.H. (1985) Cytotoxic effects of NaOCl on vital tissue. *Journal of Endodontics*, 11, 525–528.
- Peters, O.A. (2004) Current challenges and concepts in the preparation of root canal systems: a review. *Journal of Endodontics*, 30, 559–567.
- Poggio, C., Trovati, F., Ceci, M., Colombo, M. & Pietrocola, G. (2017) Antibacterial activity of different root canal sealers against *Enterococcus faecalis*. *Journal of Clinical and Experimental Dentistry*, 9, e743–e748.
- Prestegard, H., Portenier, I., Ørstavik, D., Kayaoglu, G., Haapasalo, M. & Endal, U. (2014) Antibacterial activity of various root canal sealers and root-end filling materials in dentin blocks infected ex vivo with *Enterococcus faecalis*. *Acta Odontologica Scandinavica*, 72, 970–976.
- Ricucci, D., Siqueira, J.F. Jr., Bate, A.L. & Pitt Ford, T.R. (2009) Histologic investigation of root canal-treated teeth with apical periodontitis: a retrospective study from twenty-four patients. *Journal of Endodontics*, 35, 493–502.
- Ricucci, D. & Siqueira, J.F. Jr. (2010) Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. *Journal of Endodontics*, 36, 1277–1288.
- Rosenthal, S., Spangberg, L. & Safavi, K. (2004) Chlorhexidine substantivity in root canal dentin. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*, 98, 488–492.
- Rossi-Fedele, G., Doğramaci, E.J., Guastalli, A.R., Steier, L. & de Figueiredo, J.A. (2012) Antagonistic interactions between sodium hypochlorite, chlorhexidine, EDTA, and citric acid. *Journal of Endodontics*, 38, 426–431.



- Šimundić Munitić, M., Poklepić Peričić, T., Utrobičić, A., Bago, I. & Puljak, L. (2019) Antimicrobial efficacy of commercially available endodontic bioceramic root canal sealers: a systematic review. *PLoS One*, 14, e0223575.
- Siqueira, J.F. Jr. & Rôças, I.N. (2009) Diversity of endodontic microbiota revisited. *Journal of Dental Research*, 88, 969–981.
- Soares, J.A. & Pires Júnior, D.R. (2006) Influence of sodium hypochlorite-based irrigants on the susceptibility of intracanal microbiota to biomechanical preparation. *Brazilian Dental Journal*, 17, 310–316.
- Souza, M.A., Menon, C.Z., Nery, L.F., Bertol, C.D., Rossato-Grando, L.G. & Cecchin, D. (2018) Effect of root canal preparation techniques on chlorhexidine substantivity on human dentin: a chemical analysis. *Clinical Oral Investigations*, 22, 859–865.
- Sunde, P.T., Olsen, I., Debelian, G.J. & Tronstad, L. (2002) Microbiota of periapical lesions refractory to endodontic therapy. *Journal of Endodontics*, 28, 304–310.
- Sundqvist, G., Figdor, D., Persson, S. & Sjögren, U. (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*, 85, 86–93.
- Swimberghe, R.C.D., Coenye, T., De Moor, R.J.G. & Meire, M.A. (2019) Biofilm model systems for root canal disinfection: a literature review. *International Endodontic Journal*, 52, 604–628.
- Tchaou, W.S., Turng, B.F., Minah, G.E. & Coll, J.A. (1996) Inhibition of pure cultures of oral bacteria by root canal filling materials. *Pediatric Dentistry Journal*, 18, 444–449.
- Tirali, R.E., Turan, Y., Akal, N. & Karahan, Z.C. (2009) In vitro antimicrobial activity of several concentrations of NaOCl and Octenisept in elimination of endodontic pathogens. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*, 108, e117–e120.
- Velozo, C., Silva, S., Almeida, A., Romeiro, K., Vieira, B., Dantas, H. et al. (2020) Shaping ability of XP-endo Shaper and ProTaper Next in long oval-shaped canals: a micro-computed tomography study. *International Endodontic Journal*, 53, 998–1006.
- Verma, N., Sangwan, P., Tewari, S. & Duhan, J. (2019) Effect of different concentrations of sodium hypochlorite on outcome of primary root canal treatment: a randomized controlled trial. *Journal of Endodontics*, 45, 357–363.
- Viapiana, R., Moynadeh, A.T., Camilleri, L., Wesselink, P.R., Tanomaru-Filho, M. & Camilleri, J. (2016) Porosity and sealing ability of root fillings with gutta-percha and BioRoot RCS or AH Plus sealers. Evaluation by three ex vivo methods. *International Endodontic Journal*, 49, 774–782.
- Wang, Z., Shen, Y. & Haapasalo, M. (2012) Effectiveness of endodontic disinfecting solutions against young and old *Enterococcus faecalis* biofilms in dentin canals. *Journal of Endodontics*, 38, 1376–1379.
- Wang, Z., Shen, Y. & Haapasalo, M. (2014) Dentin extends the antibacterial effect of endodontic sealers against *Enterococcus faecalis* biofilms. *Journal of Endodontics*, 40, 505–508.
- Weiss, E.I., Shalhav, M. & Fuss, Z. (1996) Assessment of antibacterial activity of endodontic sealers by a direct contact test. *Endodontics & Dental Traumatology*, 12, 179–184.
- Xuereb, M., Vella, P., Damidot, D., Sammut, C.V. & Camilleri, J. (2015) In situ assessment of the setting of tricalcium silicate-based sealers using a dentin pressure model. *Journal of Endodontics*, 41, 111–124.
- Zancan, R.F., Di Maio, A., Tomson, P.L., Duarte, M. & Camilleri, J. (2021) The presence of smear-layer affects the antimicrobial action of root canal sealers. *International Endodontic Journal*, 54, 1369–1382.
- Zandi, H., Petronijevic, N., Mdala, I., Kristoffersen, A.K., Enersen, M., Rôças, I.N. et al. (2019) 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. *Journal of Endodontics*, 45, 1089–1098.e5.
- Zavattini, A., Knight, A., Foschi, F. & Mannocci, F. (2020) Outcome of root canal treatments using a new calcium silicate root canal sealer: a non-randomized clinical trial. *Journal of Clinical Medicine*, 9, 782.
- Zehnder, M. (2006) Root canal irrigants. *Journal of Endodontics*, 32, 389–398.
- Zhang, H., Shen, Y., Ruse, N.D. & Haapasalo, M. (2009) Antibacterial activity of endodontic sealers by modified direct contact test against *Enterococcus faecalis*. *Journal of Endodontics*, 35, 1051–1055.
- Zhou, H.M., Du, T.F., Shen, Y., Wang, Z.J., Zheng, Y.F. & Haapasalo, M. (2015) In vitro cytotoxicity of calcium silicate-containing endodontic sealers. *Journal of Endodontics*, 41, 56–61.
- Zuolo, M.L., Zaia, A.A., Belladonna, F.G., Silva, E.J.N.L., Souza, E.M., Versiani, M.A. et al. (2018) Micro-CT assessment of the shaping ability of four root canal instrumentation systems in oval-shaped canals. *International Endodontic Journal*, 51, 564–571.

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