Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate

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Dental Materials

Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate.

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Abstract:	Objectives: Assess the antibacterial, physical and chemical properties of AH Plus, BioRoot RCS and Pulp Canal Sealer (PCS) in contact with 2% chlorhexidine digluconate (CHX) used as final irrigant prior to root canal obturation. Methods: The antimicrobial properties were investigated by direct contact tests for planktonic and biofilm growth of <i>E. faecalis</i>, <i>S. mutans</i>, <i>S. epidermidis</i> and <i>S. aureus</i> in vitro. The setting time, wettability, microhardness and surface roughness were also assessed. The sealers were studied in no contact, 1-minute (short-term) and continuous contact (long-term) with CHX. Chemical characterization of sealers was performed by scanning electron microscopy, X-ray diffraction analysis and Fourier-transform infrared spectroscopy after CHX or saline used as the last irrigant in an ex vivo tooth model and in endo-training blocks. Results: CHX increased the antibacterial activity of all the sealers investigated against planktonic bacteria and biofilms with PCS exerting the highest antimicrobial activity with and without the presence of CHX. The setting of AH Plus and BioRoot RCS was retarded, while for PCS accelerated in the presence of CHX. AH Plus and PCS were more hydrophilic after contact with CHX, whilst BioRoot RCS was hydrophobic in a time-dependent manner. The microhardness of sealers was compromised and the surface roughness increased after CHX exposure for AH Plus and BioRoot RCS, and decreased for PCS. CHX did not affect the sealers' chemistry, but PCS that exhibited two extra phases. Significance: CHX improved the antibacterial efficacy of endodontic sealers but further evidence is needed to confirm its suitability as a final irrigant prior to root canal obturation.
Response to Reviewers:	

V. Kapralos
DDS, Doctoral Research Fellow
Section of Endodontics
Institute of Clinical Dentistry

April 30, 2020

Dear Prof Watts,

I would like to submit an original research paper entitled ' **Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate** ' to journal of Dental Materials for consideration for publication. We highly consider Dental Materials the most suitable peer reviewed journal for the submission of our manuscript, based on its on-going interest in the field of Endodontics and material science. Our work is conveying new knowledge within these areas and conforms with the requirements for reporting novel research, presenting direct clinical translation of the experimental studies.

The work carried out is original and the manuscript is not concurrently under consideration for publication in another journal. All of the named authors were involved in the work leading to the publication of the paper and have read the paper before it is submitted for publication. All authors have no conflict of interest.

Kind Regards

Vasileios Kapralos

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Dear Prof Watts,

We would like to thank you and the reviewers for taking the time to evaluate our manuscript entitled *“Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate”*. Comments from the referees were particularly constructive and raised interesting issues both in terms of the methodological process followed in the study and the interpretation of our results.

In the section below, a point-by-point response to the comments of the reviewers is provided as indicated. Modifications to the manuscript according to suggestions have been made and are highlighted throughout the text. We hope to have adequately addressed all the comments and issues raised. Additionally, some minor alterations introduced in the manuscript appear highlighted as well. These changes do not affect the meaning of the originally submitted manuscript in any part.

Reply to Editor’s and Reviewers’ comments

General comment

This is a well written, well designed, and thoroughly conducted translational study evaluating the effects of root canal irrigation with chlorhexidine on the properties of distinct endodontic sealers.

General reply

Thank you for your positive comment on our study. Indeed, we consider that investigating the effects of root canal irrigation with chlorhexidine on the properties of endodontic sealers may give insight into the customization of clinical protocols regarding irrigation techniques and filling materials.

1. Concern of the reviewer: *Material and Methods: 'A standardized CHX volume (μ L) to sealer diameter (mm) ratio (2 μ L/mm) was used for both physical and antibacterial testing' I did not quite understand how was this done? Please explain and add a reference.*

Our response: Thank you for your comment. This sentence refers to the amount of CHX applied on the surface of each sealer specimen for testing antimicrobial and physical properties. Hydraulic cements and sealers are not inert and interact with the media they are placed in. Like in bioactivity studies the volume of the solution used in relation to the size of the specimen is important as this affects the reactivity of the surface. For bioactivity and surface interactions this has been shown by Zadpoor 2014 (Zadpoor AA. [Relationship between in vitro apatite-forming ability measured using simulated body fluid and in vivo bioactivity of biomaterials](#). Mater Sci Eng C Mater Biol Appl. 2014 Feb 1;35:134-43).

Moreover, the three sealers tested in our study present different hydrophilicity; if we apply the same amount of liquid on their surfaces, the sealers will be wetted to different extents. For example, the same amount of CHX as a drop spreads effortlessly on BioRoot RCS (hydrophilic material) compared to AH Plus and PCS (hydrophobic materials), which demand higher volumes of CHX to achieve the same drop spreading and thus sample coverage. To address this, the CHX drops were evenly spread with a sterile plastic inoculation loop as we have described in 'Materials and methods' section. In addition, different devices were used to test antibacterial and physical properties. Briefly, for testing planktonic bacteria, the bottoms (6 mm diameter) of a 96-well microtiter plate (Costar, Flat bottom, Ultra low attachment, Corning Inc, Corning, NY, USA) were coated with each sealer by using a small size round ended dental instrument. As for biofilms, caps (4 mm diameter) of 0.2-mL polypropylene thin wall PCR tubes (Axygen, Corning, NY) were filled with endodontic sealers. Regarding physical properties, the materials were mixed and placed into cylindrical molds measuring 10 mm in diameter. Hence, to face the issue of various hydrophilicity among the tested sealers as well as the different sizes of the devices, the upcoming guiding principle regarding the CHX volume and the surface area of the samples was followed: our purpose was to apply as less CHX was possible in order to sufficiently cover the surface area of the sealers and imitate the clinical scenario. Taking into account the different levels of hydrophilicity and the devices' diameters, we performed a pre-experimental evaluation of the ideal CHX volumes to be applied.

Fixed CHX volumes (μL) were applied on the sealers for each assay in order to cover the whole surface area of the samples:

- Physical properties: 25 μL CHX to 10 mm diameter sealer surface ($\approx 0.32 \mu\text{L CHX/mm}^2$)

- Planktonic assay: 15 μl CHX to 6 mm diameter sealer surface ($\approx 0.53 \mu\text{l CHX/mm}^2$)
- Biofilms assay: 10 μl CHX to 4 mm diameter sealer surface ($\approx 0.79 \mu\text{l CHX/mm}^2$)

We have now written a new sentence that elaborates more on the purpose of using different amounts of CHX for the different assays. We also provide now the exact amounts of CHX volumes that were applied in the different assays as well as the CHX volume (μl) to sealer surface area (mm^2), wherever it was omitted in the original manuscript.

Revised text: - *‘Regarding physical and antibacterial testing, standard CHX volumes were used with the ultimate aim to sufficiently cover the surface area of the sealers. Taking into account the different levels of hydrophilicity of sealers, the guiding principle was to apply as less CHX was possible to achieve full coverage of the tested materials in order to imitate the clinical scenario.’* (Materials and methods section < 1st paragraph, page 3, lines 6-10)

- *‘A fixed amount of 15 μl CHX was applied on the sealer surfaces ($\approx 0.53 \mu\text{l CHX/mm}^2$)’*
(Materials and methods section, 2.1 Antibacterial assays < 2nd paragraph, page 4, lines 4-5)

- *‘A fixed amount of 10 μl CHX was applied on the sealer surfaces with a pipette and evenly spread with a sterile plastic inoculation loop ($\approx 0.79 \mu\text{l CHX/mm}^2$).’* (Materials and methods section, 2.1 Antibacterial assays < 3rd paragraph, page 5-6, lines 14-16)

- *‘..... and evenly spread with a sterile plastic inoculation loop ($\approx 0.32 \mu\text{l CHX or water/mm}^2$)’*
(Materials and methods section, 2.2 Assessment of Physical Properties < 2nd paragraph, page 7, lines 10-11)

- Figure legend of figure S3: *‘A fixed amount of 10 μl CHX was applied on the sealer surfaces.’*

- Figure S3b: not applicable text

2. Concern of the reviewer: Assessment of setting time: please add a reference that used this method. What if a partial indentation was left on the specimen? Would the material still be considered set?

Our response: Thank you for the specific comment. The assessment of setting time was performed according to ISO 6876 (2012) standards. The appropriate reference has been now added in the 1st sentence of '2.2.1 Assessment of setting time' part in 'Materials and methods' section.

ISO 6876 (5.4.3 procedure) indicates the following: *"When the setting time stated by the manufacturer approaches, carefully lower the Gilmore-type indenter vertically on to the horizontal surface of the sealer. If an indentation is visible, raise the needle, clean the needle tip and lower the needle to a new position on the surface of the sealer. Repeat this operation until indentations cease to be visible. Record the time, from the end of mixing, at which this occurs."* Based on the above statement, if a partial indentation was left on the specimen the material would be considered unset.

Indeed, the last sentence of materials and methods (2.2.1 Assessment of setting time) regarding setting time was vague and has been now modified.

Revised text: - *'The setting times of the sealers were analyzed in compliance to ISO 6876 (2012) [27]'* (Materials and methods section, 2.2.1 Assessment of setting time < 1st paragraph, page 7, line 1)

- *'The sealers were considered as set when the indenter was lowered gently onto the material surface and did not leave any visible round indentation on it.'* (Materials and methods section, 2.2.1 Assessment of setting time < 1st paragraph, pages 7-8, lines 6-8)

3. Concern of the reviewer: Microhardness test: how many indentations were performed on each sample? What was the distancing among indentations?

Our response: Thank you for commenting this. At the beginning of '2.2 Assessment of Physical Properties' part in the 'Materials and methods' section we indicate that all the experiments regarding physical properties were performed in triplicates with at least three parallel samples. Regarding microhardness, at least two independent indentations were performed on each sample. The distancing among the measurement spots was at 5 mm taking into account that our samples were measuring 10 mm in diameter. Thus, the microscopical fields did not overlap each other and the indentations were performed on

nonintersecting areas on the samples. We are now describing in more detail how the indentations were performed.

Revised text: *'At least two independent indentations at a distance of 5 mm selecting non-overlapping microscopical regions were performed on each sample and the Vickers hardness number (VHN) was recorded.'* (Materials and methods section, 2.2.3 Microhardness Test < 1st paragraph, page 8, lines 4-6)

4. Concern of the reviewer: *Assessment of Surface roughness: was average roughness evaluated? Please specify roughness testing parameters such cut-off value.*

Our response: Thank you for indicating this. Indeed, the average arithmetic roughness (Ra) was evaluated and it is now clarified in the text. We also specify more testing parameters.

Revised text: - *'The vertical axis range was set at 1 mm and a resolution of 16 nm was used. The average arithmetic roughness (Ra) was recorded.'* (Materials and methods section, 2.2.4 Assessment of surface roughness < 1st paragraph, pages 8-9, lines 6-7)

5. Concern of the reviewer: *Sealer characterization in ex vivo tooth model and endo training blocks: what type of teeth were used (any single rooted tooth - anterior or posterior)?*

Our response: Thank you for the indication. Anterior and posterior single rooted teeth with one root canal were used. This part has been modified accordingly in the text.

Revised text: - *'Twenty four, single-rooted anterior and posterior human teeth with one root canal.....'* (Materials and methods section, 2.3 Sealer characterization in ex vivo tooth model and endo training blocks < 2nd paragraph, page 9, line 1)

6. Concern of the reviewer: *I suggest the authors elaborate more on differences in planktonic antibacterial properties within different bacteria. I found for this property, the antibacterial activity inconsistent among different strains, especially for BioRoot RCS.*

Our response: Thank you for indicating this. Among the different bacterial species, *S. mutans* control was the one with the weakest growth both in planktonic and biofilm assays. This might explain *S. mutans*' higher susceptibility to BioRoot RCS and PCS with and without CHX compared to the other three bacterial species.

At this point we would like to elaborate more on the bacteria selected to be studied. There is either scant or even no scientific data regarding antibacterial properties of these sealers especially for BioRoot RCS and PCS against *S. mutans*, *S. epidermidis* and *S. aureus*. As we mentioned in discussion section, due to differences in bacteria tested in previous studies and the current, no direct comparisons can be performed and the extrapolation of conclusions cannot be carried out. The rationale behind the selection of bacterial species to study, was to use the most commonly tested species (*E. faecalis*) and two other species retrieved from endodontic infections that have been studied only in planktonic forms before; *S. mutans* was added, as explained in the discussion part, in order to examine if there would be any difference in susceptibility between bacteria associated with post treatment endodontic infection and species that are not retrieved from such infection. Bacteria of the same Gram stain test can be more comparable; thus, *S. mutans* was selected as a gram positive bacterial species.

Indeed, we agree with the esteemed reviewer that further elaboration is needed regarding differences in planktonic antibacterial properties within different bacteria. We have now revised the table 1 that shows the planktonic antibacterial properties as well as the corresponding text in 'results' and 'discussion' section.

Revised text: - '*No surviving planktonic bacteria of any species tested....*' (Results section, 3.1 Antibacterial Properties < 1st paragraph, page 12, line 1)

- '*or without saline for all tested species. BioRoot RCS without contact with CHX did not significantly reduce the numbers of surviving bacteria compared to controls except for S. mutans (p<0.05). Saline application on BioRoot RCS rendered the sealer to lose its antibacterial properties compared to control (p>0.05). PCS without CHX and saline did not exhibit any antibacterial properties against E. faecalis, S. epidermidis and S. aureus except for S. mutans. PCS with saline application eliminated all the bacteria of any species (p<0.05).*' (Results section, 3.1 Antibacterial Properties < 1st paragraph, page 12, lines 4-10)

- *'Additionally, all the four bacterial species investigated are gram-positive; the comparisons between bacteria of the same Gram stain may be more accurate as among others they share similar characteristics regarding their cell envelope and thus susceptibility to antimicrobial agents [38].'* (Discussion section < 2nd paragraph, page 17, lines 13-16)

- *'Among the different bacterial species, S.mutans control was the one with the weakest growth both in planktonic and biofilm assays which is in accordance with the results of a study investigating antibacterial properties of sealers against the same four bacterial species [39]. This might explain S.mutans' higher susceptibility to BioRoot RCS and PCS with and without CHX compared to the other three bacterial species.'* (Discussion section < 7th paragraph, page 20, lines 1-5)

7. Concern of the reviewer: Pg 21, Pg 2, Lines 1-2: This statement needs revision. I do not think that in the reference article (Marashdeh et al., 2019) bond strength was even evaluated.

Our response: Thank you for commenting this. Indeed, the designated text needs revision. We have now amended the introductory sentence and changed the reference.

Revised text: - *'Microhardness of a material is a measure of multiple properties. It can be used as an indicator of the setting process as well as to show how different setting conditions can affect the overall surface strength of a material [78]'* (Discussion section <11th paragraph, page 22, lines 1-3)

8. Concern of the reviewer: Is it possible to reduce the number of cited references, over 100 references seem a lot.

Our response: We have now reduced the number of cited references.

Revised text: Not applicable.

9. Concern of the reviewer: *All Tables should be similarly formatted.*

Our response: Thank you for the indications. All tables have been now similarly formatted.

Revised text: Not applicable.

Highlights

- Pulp canal sealer exerted the highest antibacterial activity
- Chlorhexidine increased the antibacterial activity of all sealers investigated
- BioRoot was the most affected sealer by CHX in terms of physicochemical properties
- Chlorhexidine did not affect AH Plus and BioRoot as shown by surface characterization
- Two extra phases were observed for Pulp Canal Sealer under chlorhexidine irrigation

Abstract

Objectives: Assess the antibacterial, physical and chemical properties of AH Plus, BioRoot RCS and Pulp Canal Sealer (PCS) in contact with 2% chlorhexidine digluconate (CHX) used as final irrigant prior to root canal obturation.

Methods: The antimicrobial properties were investigated by direct contact tests for planktonic and biofilm growth of *E. faecalis*, *S. mutans*, *S. epidermidis* and *S. aureus in vitro*. The setting time, wettability, microhardness and surface roughness were also assessed. The sealers were studied in no contact, 1-minute (short-term) and continuous contact (long-term) with CHX. Chemical characterization of sealers was performed by scanning electron microscopy, X-ray diffraction analysis and Fourier-transform infrared spectroscopy after CHX or saline used as the last irrigant in an *ex vivo* tooth model and in endo-training blocks.

Results: CHX increased the antibacterial activity of all the sealers investigated against planktonic bacteria and biofilms with PCS exerting the highest antimicrobial activity with and without the presence of CHX. The setting of AH Plus and BioRoot RCS was retarded, while for PCS accelerated in the presence of CHX. AH Plus and PCS were more hydrophilic after contact with CHX, whilst BioRoot RCS was hydrophobic in a time-dependent manner. The microhardness of sealers was compromised and the surface roughness increased after CHX exposure for AH Plus and BioRoot RCS, and decreased for PCS. CHX did not affect the sealers' chemistry, but PCS that exhibited two extra phases.

Significance: CHX improved the antibacterial efficacy of endodontic sealers but further evidence is needed to confirm its suitability as a final irrigant prior to root canal obturation.

Keywords: chlorhexidine, endodontic sealer, antibacterial activity, planktonic bacteria, biofilm, physicochemical properties, surface characterization

Title:

Antimicrobial and physicochemical characterization of endodontic sealers after exposure to chlorhexidine digluconate.

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Declarations of interest: none

1. Introduction

The primary aim of root canal treatment of teeth with apical periodontitis is to eliminate the microbial load from the root canal system and promote periapical healing [1, 2]. Meticulous mechanical debridement of the root canal system significantly reduces the bacterial load and is considered important in canal disinfection [3]. However, complete elimination of all microorganisms is challenging, as viable bacteria potentially remain on the dentin walls and inside dentinal tubules, both in planktonic forms and biofilms [4, 5]. About 35% of the root canal area is left untouched when conventional rotary and hand instruments are used [6]. Therefore, disinfection with irrigation solutions during root-canal treatment and thereafter obturation of the root canal are important factors to reduce the amount and growth of residual bacteria [7].

Different irrigation solutions such as sodium hypochlorite (NaOCl), chlorhexidine digluconate (CHX), 17% ethylene diamine tetracetic acid (EDTA), citric acid and MTAD are used in endodontic treatments [8, 9]. NaOCl is widely used, as it dissolves organic material and has antibacterial properties [9]. CHX has antibacterial properties but does not dissolve organic tissue. Unlike NaOCl, it has the ability to be absorbed, bind to dentin and be gradually released (substantivity), which may contribute to a prolonged antibacterial effect [9, 10]. CHX is a cationic substance that kills the bacteria by acting at the microbial cell wall or outer membrane [11].

Endodontic sealers play an important role in obturation of the root canal system as they may provide a seal, which prevents the penetration of bacteria. Sealers are meant to entomb residual bacteria, prevent leakage of nutrients and ideally possess antibacterial properties [12, 13]. Sealers with numerous chemical compositions are used in endodontics,

such as zinc oxide eugenol- (ZOE), resin-, silicone- and calcium silicate- based materials [14]. AH Plus (Dentsply Maillefer, Ballaigues, Switzerland) is a resin-based root canal sealer that is frequently used as a benchmark for comparisons [15, 16]. BioRoot RCS (Septodont, Saint-Maur-des-Fossés, France) is a hydraulic calcium-silicate based sealer and it possesses both antibacterial [17] and biological properties [18]. Its hydraulic nature and the formation of calcium hydroxide as part of the hydration process makes the sealer very susceptible to the environment it is placed in [19]. Pulp Canal Sealer (Kerr Corporation, Romulus, MI, USA) is a traditional eugenol containing sealer that has been in clinical use for decades possessing antibacterial properties, but controversial biocompatibility [20].

Several studies have addressed the effect of irrigation solutions on sealers' properties such as sealing ability, microleakage, and wettability [12, 21-23]. However, there is scant information regarding the effect of irrigation solutions on sealers' antibacterial properties. To the best of our knowledge only one study has investigated the effect of final irrigation with water, EDTA and phosphate buffered saline (PBS) on antibacterial activity of BioRoot RCS, MTA Fillapex (Angelus, Londrina, Brazil) and AH Plus. The irrigation solution affected the antibacterial properties of all three sealers as they exhibited the highest antibacterial activity after irrigation with EDTA followed by water [17].

Remnants of irrigation solutions are present in the root canal system after completion of chemo-mechanical root canal preparation [24, 25]. CHX in 2% concentration is often used in endodontics as a final irrigant before placement of endodontic sealers [26]. Due to binding to dentin and subsequent release of CHX, this may influence the sealers' properties. Many different procedures for debridement and irrigation of the root canal are described in the literature. Debridement and irrigation **are** important to reduce the amount of bacteria in the

root canal, however the most efficacious combination of irrigation solutions and obturating materials is not known.

The primary aim of this *in vitro* study was to assess the antibacterial, physical (physicomechanical) and chemical properties of AH Plus, BioRoot RCS and Pulp Canal Sealer after exposure to 2% chlorhexidine digluconate. An *ex vivo* tooth model and endo training blocks were used to simulate irrigation procedures. The null hypothesis tested was that sealers' antibacterial and physicochemical properties would not be affected after contact with CHX.

2. Materials and methods

An epoxy resin-based sealer, AH Plus (Dentsply Maillefer, Ballaigues, Switzerland), a calcium silicate-based sealer, BioRoot™ RCS (Septodont, Saint-Maur-des-Fossés, France), and a zinc oxide eugenol-based sealer, Pulp Canal Sealer (PCS) (Kerr Corporation, Romulus, MI, USA) were tested. The materials were mixed according to manufacturer's instructions. Chlorhexidine digluconate, 20% in water solution, (Lot # BCBS7878V, Sigma-Aldrich, St. Louis, MO, USA) was diluted in sterile distilled water (water) and standardized to 2%. Regarding physical and antibacterial testing, standard CHX volumes were used with the ultimate aim to sufficiently cover the surface area of the sealers. Taking into account the different levels of hydrophilicity of sealers, the guiding principle was to apply as less CHX was possible to achieve full coverage of the tested materials in order to imitate the clinical scenario. CHX was used as a last irrigant in both tooth model and endo training blocks for assessing chemical properties.

2.1 Antibacterial assays

All experiments were conducted in triplicate and with three independent parallels for each material investigated. *Enterococcus faecalis* American Type Cell Culture Collection (ATCC) 19434, *Streptococcus mutans* ATCC 700610, *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus aureus* Newman were grown overnight for 18 hours in Tryptone Soya Broth (TSB) at 37° C, 5% CO₂ supplemented atmosphere. The bacteria were suspended in Phosphate Buffered Saline to an optical density at 600 nanometers (OD₆₀₀) of 1.0, corresponding to approximately 2 x 10⁸ Colony Forming Units (CFUs)/ml. The sealers were tested against both planktonic bacteria and bacteria in biofilms for three different groups of exposure to CHX: group 1, no CHX (no contact with CHX); group 2, short CHX (1-minute contact with CHX); group 3, long CHX (continuous contact with CHX along with setting process).

A direct contact test (DCT) was used to investigate the antibacterial activity of sealer surfaces against planktonic bacteria. Briefly, the bottoms of a 96-well microtiter plate (Costar, Flat bottom, Ultra low attachment, Corning Inc, Corning, NY, USA) were coated with each sealer by using a small size round ended dental instrument. A fixed amount of 15 µl CHX was applied on the sealer surfaces ($\approx 0.53 \mu\text{l CHX/mm}^2$). The same amount of CHX within the same application times was also transferred on uncoated wells serving as negative controls. The CHX drop was applied upon the sealers with a pipette and evenly spread with a sterile plastic inoculation loop. In short-term exposure groups, after 1 minute of contact with CHX, the drop was removed with a pipette and the sealers were placed in a dry incubator at 37°C for 20 minutes to let any liquid excess dry out, before allowed to set. Subsequently, half of the samples were covered with 300 µl saline. All the materials were

stored in humidified atmosphere at 37°C and incubated for 24 hours. After incubation, the supernatant saline solution was removed from the wells and all surfaces proceeded for testing. The sealer surfaces were tested either with or without saline application, constituting two experimental conditions. An amount of 10 µl from each bacterial suspension was carefully placed on the surface of the materials. Another 10 µl from the same bacterial suspension was transferred to uncoated wells serving as positive control. The specimens were incubated at 37°C for 1 hour, while complete evaporation of the suspension's liquid was inspected. Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates incubated overnight at 37°C, 5% CO₂ supplemented atmosphere (Supplementary Figure S1).

For biofilm assay, membrane filters (MF-Millipore™ Membrane Filter, 0.45 µm pore, Merck, Darmstadt, Germany) were cut in circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2 µl of each bacterial inoculum OD₆₀₀ 1.0 was applied upon the outer surface of membranes. The agar plates were incubated at 37°C in a 5% CO₂ supplemented atmosphere for 48 hours and monospecies biofilms were established and verified with the use of confocal laser scanning microscopy (CLSM; Olympus FluoView FV1200, Olympus Corp, Tokyo, Japan) (Figure 1). The Syto-9/Propidium iodide (PI) staining (FilmTracer™ LIVE/DEAD Biofilm Viability kit, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to color the biofilms upon membranes. A diode laser emitting at 473 nanometres (nm) was used. The scanning was performed from the top of the biofilm to the membrane surface using a 60x water lens, 0.5 µm step size, and a format of 512 x 512 pixels corresponding to an area of 88 x 88 µm (Figure S2). Caps of 0.2-mL polypropylene thin wall PCR tubes (Axygen, Corning, NY) were cut and filled with the mixed sealers, and a glass microscope slide was applied on the cap to obtain smooth surfaces. A fixed amount of 10 µl

CHX was applied on the sealer surfaces with a pipette and evenly spread with a sterile plastic inoculation loop ($\approx 0.79 \mu\text{l CHX}/\text{mm}^2$). After incubation period, the filter membranes were positioned upon the sealers with the established biofilms facing their surfaces. Membranes also covered uncoated bottoms, serving as positive controls. Parafilm M (Bemis Inc, Neenah, WI, USA) was applied around the caps to ensure tight contact between membranes and sealer surfaces. The specimens were placed at 37°C in a 5% CO₂ supplemented atmosphere for 24 hours. After 24 hours, a droplet of 10 μl water was transferred upon the membranes to enable gentle detachment from the sealer and the caps' bottoms. Each membrane with its corresponding cap was transferred to vials containing 5 ml PBS and vigorously vortexed with glass beads. After serial dilutions in PBS, CFUs were counted after overnight incubation at 37°C in a 5% CO₂ supplemented atmosphere (Figure S3). Carry over 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. Sealer specimens inside caps were allowed to set independently, as it was aforementioned, for 24 hours at 37°C in a humidified chamber and then 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.

2.2 Assessment of Physical Properties

The physical properties were assessed by testing the setting time, wettability (contact angle measurements), microhardness, and surface roughness of sealers. The experiments were performed in triplicates with at least three parallel samples.

Cylindrical specimens, measuring 10 mm in diameter and 2 mm in height, were prepared into molds for each sealer. For setting time, wettability and microhardness tests, after preparation the sealers were incubated in 100% humidified atmosphere at 37°C and allowed to set for 24 hours with and without contact with 2% CHX or water. Five groups were formed according to exposure to CHX or water: group 1, no CHX/water (no contact with CHX or water); group 2, short CHX (1 minute contact time with CHX); group 3, long CHX (continuous contact with CHX along with the setting process); group 4, short water (1 minute contact time with water); group 5, long water (continuous contact with water along with the setting process). For both CHX and water exposure groups, a drop of 25 µl was applied upon the sealers with a pipette and evenly spread with a sterile plastic inoculation loop ($\approx 0.32 \mu\text{l}$ CHX or water/mm²). In short-term exposure groups, after 1 minute of contact either with CHX or water, the drop was removed with a pipette and the sealers were placed in a dry incubator at 37°C for 20 minutes to liquid excess dry out, before allowed to set for 24 hours in a humidified atmosphere. For surface roughness test, in addition to no contact and CHX groups, measurements of the samples were also taken for 24 hours contact with Hanks' Balanced Salt Solution (HBSS; Sigma Aldrich, Gillingham UK) (Figure S4).

2.2.1 Assessment of setting time

The setting times of the sealers were analyzed in compliance to ISO 6876 (2012) [27]. A stopwatch was started after the preparation of the sealers and the placement of CHX or water upon them in the short-term and long-term exposure groups. The specimens were placed in an incubator at 37°C and 100% humidity until the end of setting. Setting of the sealers was assessed using an indentation technique with a Gilmore-type metric indenter, having a mass of 100.0 ± 0.5 g and a flat end of diameter 2.0 ± 0.1 mm. The sealers were

considered as set when the indenter was lowered gently onto the material surface and did not leave any visible round indentation on it.

2.2.2 Contact Angle Measurements

Contact angle measurement was used to investigate the wettability of the material surfaces. A 20- μ L drop of distilled water was placed with a syringe on the surface of the samples, and images were captured using a color video camera (JVC KY-F55B, JVCKENWOOD Corporation, Yokohama, Japan). The contact angle was measured using an image processing and analysis software (L.P. Optimas 6.5, Media Cybernetics Inc., Rockville, MD, USA).

2.2.3 Microhardness Test

Microhardness testing was carried out by applying an indentation technique, using a hardness-testing instrument (Struers A/S, Rødovre, Denmark). A pyramidal square-based diamond indenter was lowered onto the sealer surfaces and a load ranging up to 100 gf was applied for a dwell time of 5 s. At least two independent indentations at a distance of 5 mm selecting non-overlapping microscopical regions were performed on each sample and the Vickers hardness number (VHN) was recorded.

2.2.4 Assessment of Surface Roughness

Surface analysis of the samples was carried out using mechanical profilometry (Form Talysurf Series 2, Taylor Hobson, Leicester, UK) and scanning electron microscope (SEM) imaging (EVO MA10, Carl Zeiss Ltd, Cambridge, UK). The profiler used a precision motion system and a gauge head to measure the displacement at the sealer surface over a specified area. The region measured was 3 mm in a straight line at the center of each sample with a measurement taken every 2.5 μ m. The vertical axis range was set at 1 mm and a resolution

of 16 nm was used. The average arithmetic roughness (Ra) was recorded. Representative secondary electron scanning micrographs of the tested materials were made to picture the sealers' surface microstructure (magnification 100×).

2.3 Sealer characterization in *ex vivo* tooth model and endo training blocks

A split tooth model and endo training blocks (Endo-Training-Bloc, Dentsply Maillefer, Ballaigues, Switzerland) were used to simulate a clinical setting with irrigation of CHX as the last irrigant.

Twenty four, single-rooted anterior and posterior human teeth with one root canal (Ethical approval REC Ref 14/EM/1128), free of caries, were decoronated and the root length standardized at 15 mm, using a diamond saw (Buehler 11-1280-160 Isomet Low Speed Saw, Buehler, Lake Bluff, IL, USA) and consequently stored at 4°C in water until use. The root canals were instrumented with ProTaper rotary files (Dentsply Maillefer, Ballaigues, Switzerland) up to size F4, 1 mm shorter than the standardized root length. An irrigation protocol with 2 mL of 2.5% NaOCl between the changes of the rotary files with a 27 gauge Monoject 3cc Endodontic Syringe (CardinalHealth, Dublin, Ireland) was followed. The roots were split longitudinally along their long axis using a diamond saw and the two segments were repositioned and held tightly together by wrapping them up with Parafilm M. The root canals were irrigated with either 2 ml of saline or 2 ml of saline and then 2 ml of 2% CHX for 1 minute as the final irrigant. The root canals were dried with paper points before placement of sealers.

The tested sealers were placed inside the root canals using a lentulo spiral after which they were allowed to set for 24 hours in 37°C, 100% humidity. After the setting period, each root was unwrapped from the Parafilm M, and the root fragments were gently

detached with a use of a scalpel that was applied on the thin space formed between them. The sealers were exposed and gently retrieved intact from the dentin walls. After the separation process, the whole bulk of the sealer was adhered to one segment whilst the other segment was macroscopically free of sealer remnants. Hereafter, the exposed sealer on its half-tooth segment will be referred as sealer-tooth sample. The aforementioned preparation was also applied on endo training resin blocks (Figure S5). The exposed sealer on its resin block segment will be referred as sealer-block sample. The exposed sealers upon their substrates (either tooth or endo training blocks) were characterized by scanning electron microscopy (SEM) (tooth model) and energy dispersive spectroscopy (EDS) (tooth model), X-ray diffraction analysis (XRD)(tooth model and endo training blocks), and Fourier-transform infrared spectroscopy (FTIR) (tooth model and endo training blocks) (Figure S6).

2.3.1 Scanning Electron Microscopic examination-Elemental analysis

SEM examination was performed on sealer-tooth samples. These were mounted on aluminum stubs, carbon coated (Agar Scientific, Stansted, UK), and viewed with the scanning electron microscope (EVO MA10, Carl Zeiss Ltd, Cambridge, UK). Accelerating voltage ranged between 5–15 kV and the probe current between 125–300 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 rectangular areas of the intact sealers surface.

2.3.2 X-ray Diffraction analysis

Phase analysis of both the sealer-tooth and sealer-block samples was carried out. Cylindrical samples (10 mm diameter, 2 mm height) of sealers in no contact with CHX were also prepared and analyzed as controls. The surface analysis was performed using glancing angle X-ray diffraction analysis at a fixed angle of incidence of 5. The X-ray diffractometer (Bruker D8 Discover, Bruker, Billerica, Massachusetts, MA, USA) was operated in grazing-incidence asymmetric Bragg mode using Cu Ka radiation, an operating current of 40 mA and voltage of 45 kV for 15–458 2 θ with a sampling width 0.058, scan speed 18/min. The other settings included divergent slits at 1 mm, divergent height slit 10 mm, scintillator slit 8 mm and receiver slit 13 mm. Phase identification was accomplished using a search-match software utilizing Crystallography Open Database (COD) (Diffrac.Eva, Bruker, Billerica, Massachusetts, MA, USA).

2.3.3 Fourier transform infrared spectroscopy (FT-IR)

The measurements were performed in FT-IR spectrometer (Nicolet 6700, Thermo Scientific, Waltham, Massachusetts, USA) on sealer-tooth and sealer-block samples. The spectra were taken by the Smart MIRacle Accessory, Diamond setup. The data were analyzed by stacking the spectra retrieved from the FT-IR, using the accompanied software (Omnic).

2.4 Statistical analysis

The statistical analysis was performed with GraphPadPrism version 6.00 for windows (GraphPad software, La Jolla, CA, USA). The antibacterial assays were analyzed using the

nonparametric Kruskal–Wallis test and Dunn’s post-hoc method due to absence of normal distribution ($p<0.05$). Statistical analysis of the physical properties was performed using one-way analysis of variance, followed by Tukey multiple comparisons ($p<0.05$).

3. Results

3.1 Antibacterial Properties

No surviving planktonic bacteria of any species tested were recovered when CHX was applied in short- (1 minute) and long-term (along with setting process) for all sealers investigated and negative controls. For AH Plus without contact with CHX, there was no difference in the number of bacteria recovered from samples in saline or without saline for all tested species. BioRoot RCS without contact with CHX did not significantly reduce the numbers of surviving bacteria compared to controls except for *S. mutans* ($p<0.05$). Saline application on BioRoot RCS rendered the sealer to lose its antibacterial properties compared to control ($p>0.05$). PCS without CHX and saline did not exhibit any antibacterial properties against *E. faecalis*, *S. epidermidis* and *S. aureus* except for *S. mutans*. PCS with saline application eliminated all the bacteria of any species ($p<0.05$). The data for the antibacterial properties of sealers on planktonic bacteria are shown in Table 1.

The antibacterial activity was further investigated against bacteria in biofilms. For AH Plus, long-term exposure to CHX, increased the antibacterial activity against monospecies biofilms for all bacteria investigated ($p<0.05$). BioRoot RCS was effective against *S. epidermidis* biofilms in all conditions investigated ($p<0.05$). After long-term exposure to CHX, it was antibacterial against biofilms formed by any of the bacterial species investigated

($p < 0.05$). In addition, short-term contact with CHX improved the antibacterial efficacy of BioRoot RCS against *S. aureus* biofilms. PCS reduced bacterial survival in *E. faecalis* and *S. mutans* biofilms in all conditions ($p < 0.05$). Short- and long-term exposure to CHX had an antibacterial effect on *S. epidermidis* and *S. aureus* biofilms compared to controls ($p < 0.05$). In negative controls, only long-term exposure to CHX significantly reduced surviving bacteria in *S. mutans* biofilms ($p < 0.05$). No carryover effect was detected in the biofilm model (data not shown). The results for the antibacterial properties of sealers on biofilms are shown in Table 2.

3.2 Physical Properties

Contact with CHX did not affect the setting time of AH Plus compared to contact with water, while sealer without contact had a shorter setting time ($p < 0.05$). Long-term exposure to CHX increased the setting time of BioRoot RCS when compared with short-term exposure to CHX and both with short- and long-term exposure to water ($p < 0.05$). BioRoot RCS without contact with CHX or water set faster than the other conditions investigated ($p < 0.05$). No differences were observed in setting of PCS when in contact with either CHX or water. For PCS alone, the setting time was longer compared to CHX and water ($p < 0.05$).

Contact angles 81.1° and 80.9° were observed for AH Plus and PCS respectively, which were decreased after contact with CHX and water ($p < 0.05$), while BioRoot RCS presented increased contact angles only in contact with CHX ($p < 0.05$). Complete wetting (contact angle at 0°) of the BioRoot RCS surfaces was observed in short- and long-term water exposure groups. Long-term contact with CHX and water further decreased contact angles in AH Plus and PCS compared to short-term ($p < 0.05$).

The microhardness of all sealers was compromised by CHX and water both in short- and long-term exposure ($p<0.05$) compared to no contact groups, except for PCS that remained unaffected by contact with water compared to no contact groups. Long-term exposure to either CHX or water further reduced microhardness values of AH Plus in comparison with short-term exposure ($p<0.05$), while no significant differences were observed for BioRoot RCS and PCS. The physical properties (setting time, wettability and microhardness) of sealers are shown in Table 3.

Surface roughness was increased in AH Plus after long-term exposure to CHX ($p<0.05$) and in BioRoot RCS after both short- and long-term contact with CHX; long-term exposure to CHX further increased the surface roughness of BioRoot RCS compared to short-term ($p<0.05$). Crystalline lathe-like deposits were observed over the BioRoot RCS surface. For PCS, both short- and long-term exposure to CHX decreased surface roughness ($p<0.05$). Compared to CHX groups, setting in the presence of HBSS increased the surface roughness of AH Plus and BioRoot RCS ($p<0.05$), without affecting PCS ($p>0.05$). The surface roughness Ra values and sealers' microstructure after SEM are shown in Figure 2.

3.3 Materials characterization

To investigate how CHX may affect the surface characteristics and elemental composition of the different materials, scanning electron microscopy and EDS analyses were performed on exposed sealer surfaces retrieved from the sealer-tooth samples after splitting process. Based on the SEM micrographs (Figure 3), the CHX irrigation effected mostly the surface microstructure of AH Plus and BioRoot RCS. From the surface changes observed, changes in elemental composition were investigated after CHX and saline irrigation. AH Plus, in CHX irrigation group, exhibited extra peaks for magnesium, phosphorus and chlorine in

addition to silicon, calcium, zirconium and tungsten in saline group (Figure 3A). BioRoot RCS had two additional peaks, for magnesium and aluminium, to the ones identified in saline group, namely silicon, calcium, chlorine and zirconium (Figure 3B). PCS demonstrated phosphorus, calcium and chlorine peaks, when CHX was applied, together with aluminium, zinc and silver peaks, which were evident in saline groups (Figure 3C).

The FT-IR and XRD analyses were performed on sealer surfaces in tooth model and endo training blocks. FT-IR analysis showed the potential effects of CHX irrigation on sealers chemistry. AH Plus and BioRoot RCS were not affected by CHX irrigation in both resin block and tooth structure. The chlorhexidine irrigation in both tooth and resin model effected the chemistry of the PCS as indicated by changes in the 2000-2500 cm^{-1} region (Figure 4A). Furthermore, in the resin model the peak at 2900 cm^{-1} was obliterated and some changes were shown in the region of 1500 cm^{-1} (Figure 4A).

XRD analysis showed differences in the amounts of the phases and the crystallinity of the sealers (Figure 4B). The main crystalline phases in AH Plus were calcium tungstate (CT) (COD: 9009626) and zirconium oxide (ZO) (COD: 9016714) which were found in all conditions investigated. In tooth model under CHX irrigation, AH Plus exhibited an extra peak at $29^{\circ}20'$ of calcium carbonate (CC) (COD: 9015073), which was not observed in the resin block indicating that the changes were related to the effect of the CHX also on the tooth structure.

BioRoot RCS was observed to contain calcium hydroxide (CH) (COD: 9000113), dicalcium silicate (DCS) (COD: 9012789), tricalcium silicate (TCS) (COD: 1538413), and zirconium oxide (ZO) (COD: 9016714) as the main phases in the control. The sealer in contact with the resin block showed no changes while that in contact with the dentin in the root model presented calcium phosphate (CP) (COD: 1517238) peaks at 28° , 31° , $34^{\circ}20'$ in both

irrigation regimes. The peaks of calcium hydroxide were lower in the tooth model than in the control indicating possible reaction to form calcium phosphate, whilst dicalcium and tricalcium silicate retained.

The PCS had 3 peaks of zinc oxide (ZnO) (COD: 9008877) at 32°, 34°, 36°2θ and 2 of molecular silver (Ag) (COD: 9011607) at 38° and 44°2θ in the control. In contact with dentin two additional phases namely silver aluminum (SA) (COD: 1509038) and sodium silver chloride (SSC) (COD: 1509071) at 38°, 44° and 32°2θ respectively were formed for both saline and CHX exposures. The same 4 phases were also present in the resin blocks only when CHX was used.

4. Discussion

Hydraulic calcium silicate-based sealers interact with the dentin [28]. Thus, residuals of the final irrigating solution applied may affect the sealer integrity and antimicrobial properties. The use of CHX has been suggested to the irrigant of choice due to its substantivity and slow release over time, thus enhancing the antimicrobial properties of the obturation material [9, 10, 26].

In the present study, antibacterial properties of sealers after contact with CHX were investigated against planktonic bacteria and bacteria in biofilms. Different biofilm models have been used to assess the effectiveness of endodontic irrigants [29, 30]. However, the antibacterial properties of sealers against established biofilms is less investigated. Root canal sealers are placed in direct contact with dentin walls of the root canal and should ideally penetrate into dentinal tubuli. Both planktonic bacteria and bacteria in biofilms are hosted

into the root canal system, on dentin walls and dentinal tubuli where contact with sealer may occur [31, 32]. Post-treatment apical periodontitis has been associated with *E. faecalis*, *S. epidermidis* and *S. aureus* among others [33-35]. *S. mutans* is a caries-associated bacterium, which has been isolated from necrotic root canals [36, 37]. *S. mutans* was included in the present study in order to assess if bacteria associated with post treatment endodontic infection were more susceptible than species not commonly retrieved from such infections. Additionally, all the four bacterial species investigated are gram-positive; the comparisons between bacteria of the same Gram stain may be more accurate as among others they share similar characteristics regarding their cell envelope and thus susceptibility to antimicrobial agents [38].

To test the antibacterial properties against planktonic bacteria, the sealer surfaces were tested either with or without saline pre-treatment as a first approach to investigate whether the presence of moisture may affect the antibacterial activity of the materials, as it has been previously reported [39]. The DCT has been preferred to the classic agar diffusion test (ADT) to overcome the limitations of the latter: semiquantitative nature, limitation to distinguish between bacteriostatic and bactericidal activity, inability to detect the activity of insoluble components [40-42]. The DCT assay assesses the effect on planktonic bacteria, therefore a 48 hours-grown biofilm model was further developed using as substrate mixed cellulose esters (MCE) membrane filters. Bovine dentin or human dentin have been used to grow *E. faecalis* biofilms in previous studies [5, 41]. However, possible carryover effect or partial retrieval of bacteria can occur as the tested sealer may firmly adhere on dentin [39]. In the present study, high hydrophilicity of MCE material may have positively contributed to the biofilm-sealer separation process minimizing the disruption of biofilms. For measuring the antibacterial activity of the sealers against biofilms, a contact time shorter than

materials' setting may not be adequate and representative, while sealers maintain their antibacterial efficacy throughout the setting process [39]. Thus, the antibacterial properties of sealer surfaces against established biofilms were also tested for 24 hours contact time in the present study.

No inhibitory effect against both planktonic bacteria and biofilms were observed for AH Plus in the present study. Our findings corroborate with earlier literature, which indicates that AH Plus loses its antibacterial efficacy after setting of the material [39, 43]. CHX applied on the surface of the sealer improved the antibacterial properties of the AH Plus against planktonic bacteria, confirming the findings of a previous study investigating the antibacterial properties of AH Plus modified with CHX [44]. An increase in antibacterial activity was not observed when saline was used, as previously shown in another study using water upon sealers [39], confirming that the antibacterial activity was a result of CHX and not the effect of liquid interacting with the sealer. In our study, only long-term exposure to CHX improved the effectiveness of AH Plus against biofilms indicating a time- or dose-dependent action; it is well established that biofilms are more resistant compared to their planktonic counterparts [45].

Compared to AH Plus, increased antibacterial activity of BioRoot RCS was observed. The antibacterial activity was further improved after exposure to CHX. This finding may partially be explained by the proposed antibacterial mechanism of calcium-silicate cements that is predominantly related to high alkalinity (free hydroxyl ions). Calcium hydroxide, principally formed after hydration of calcium-silicate cements, subsequently releases calcium ions (Ca^{+2}) and hydroxyl ions (OH^-) in water which contribute to the antibacterial activity. A few studies have assessed the antibacterial effectiveness of BioRoot RCS against *E. faecalis*

with contradictory results. Conflicting results may partially be explained by differences in methodology [17, 46, 47]. High antibacterial efficacy for BioRoot RCS was shown using an *in vivo* simulated tooth model particularly after a final irrigation with EDTA [17]. The antibacterial efficacy of BioRoot RCS against *E. faecalis* biofilms in dentinal tubules presented fluctuations over time [46] and another study used a modified DCT in conjunction with ADT, concluding in moderate antibacterial properties for BioRoot RCS [47]. In terms of other hydraulic calcium silicate-based materials, earlier literature has shown increased antibacterial properties for mineral trioxide aggregate (MTA) mixed with CHX compared to unmodified MTA [48-51]. Studies on Biodentine, a calcium-silicate cement with similar chemistry to BioRoot RCS, showed improved antibacterial efficacy when combined with CHX compared to unmodified cement [49, 52]. Due to differences in methodology and bacteria tested in previous studies and the current, no direct comparisons can be performed and the extrapolation of conclusions cannot be carried out.

In the present study, PCS alone and in contact with CHX exerted the highest antibacterial efficacy among the sealers investigated. Our result for PCS alone are in agreement with the literature, as zinc oxide and eugenol sealers have been reported to be efficient in eliminating microorganisms during setting [5, 20, 53, 54]. Release of eugenol is the first contributing factor to the pronounced antibacterial efficacy of PCS, which is also consistent with the high antibacterial properties of PCS against both planktonic bacteria and bacteria in biofilms [55, 56]. In our study, characterization of PCS showed the presence of silver and zinc oxide, which may also contribute to the antibacterial properties of ZOE based cements [57, 58]. Increased antibacterial activity of ZOE based cements have been observed after incorporation of CHX in previous studies [59, 60]. Moreover, the silver chloride phase identified after last irrigation with saline or CHX in this study, is well documented for its

antibacterial properties and may have further contributed to the increased antibacterial efficacy of PCS [61].

Among the different bacterial species, *S. mutans control* was the one with the weakest growth both in planktonic and biofilm assays which is in accordance with the results of a study investigating antibacterial properties of sealers against the same four bacterial species [39]. This might explain *S. mutans'* higher susceptibility to BioRoot RCS and PCS with and without CHX compared to the other three bacterial species.

In the present study, the physical properties of the sealers were investigated in contact with CHX and water (setting time, wettability, microhardness) or HBSS (surface roughness). Exposure to water or HBSS were investigated in an attempt to assess whether it is CHX as the substance or the aqueous phase that yields the effects upon sealers' surfaces. HBSS also results in surface changes to the hydraulic materials caused by the deposition of calcium phosphate on the material surface which is one of the main features of these material types [62].

The slow setting time of a sealer may be connected with high washout and disintegration that may jeopardize the sealing ability and integrity of a root canal filling [63, 64]. Furthermore, longer setting times might permit more extensive interactions between sealers and irrigants [65]. As for antibacterial properties, the majority of sealers used in root canal treatments exhibit short-term effectiveness that is compromised after setting [66]. In our study, the setting time of AH Plus was found to be consistent with manufacturers' guidelines and previous publications [67, 68]. The setting of AH Plus in contact with CHX and water was prolonged in the present study while contrasting literature on this shows similar data [65] or opposing findings [69]. BioRoot RCS, which contains calcium chloride as setting

accelerator, exhibited the shortest setting time among the sealers tested in the present study. Corresponding short setting time has also been documented for the sealer in previous studies [70, 71]. Short- and long-term exposure to CHX almost doubled and tripled the setting time of BioRoot RCS in the present study. Contact with water, either short- or long-term, approximately doubled the setting time of the BioRoot RCS sealer explaining partially the prolongation may have occurred because of the aqueous phase of CHX aliquots applied on the sealer surface. Two publications have shown that addition of 2% CHX to MTA, also a calcium silicate- based cement, prevented setting of the material [72, 73]. Clearly, the chloride accelerator affected the regulation of setting favourably. In our study, PCS alone exhibited a mean setting time of 221 minutes. It is previously reported that this sealer has fluctuations in setting times [74, 75]. Our findings showed an accelerating setting process for PCS in contact with CHX and water compared to the sealer alone. This is in agreement with the fact that humidity shortens the setting time of ZOE cements given that CHX in aqueous presentation was applied upon the sealers [76].

Wettability, expressed in terms of contact angle (θ) between the drop of a liquid and the plane surface of the solid, is inversely associated with the surface free energy; surface free energy is the result of intermolecular attraction. A cutoff on the 90 degrees has been accepted to define hydrophobicity ($\theta > 90^\circ$) and hydrophilicity ($\theta < 90^\circ$) of materials' surfaces [77]. It has been shown that wettability of sealers on dentin can influence their ability to adhere, penetrate the dentinal tubules and thus exert their antimicrobial properties in contact with the entombed bacteria [21, 23, 43], which may affect indirectly their antibacterial efficacy. AH Plus is sensitive to moisture from residual substances derived from intracanal medications and irrigation solutions [65]. This was depicted in our wettability test, where we found that CHX and water rendered AH Plus more hydrophilic in an exposure

time-dependent manner. PCS, a highly hydrophobic material [71], was affected by CHX and water in similar pattern as AH Plus in wettability test. Presumably, the abundant moisture from the aqueous CHX increased sealers's hydrophilicity in a time-dependent manner. Regarding BioRoot RCS, exposure to CHX significantly increased hydrophobicity over time. This might be explained by the fact that CHX constitutes a positively charged hydrophobic molecule. Interestingly, this tendency was not shown in water groups as no drops could be formed and the surfaces were fully wetted.

Microhardness of a material is a measure of multiple properties. It can be used as an indicator of the setting process as well as to show how different setting conditions can affect the overall surface strength of a material [78]. AH Plus exhibited lower microhardness when in contact with CHX and water in the present study. Hydraulic calcium-silicate based cements, such as BioRoot RCS, present increased adsorption of water due to high hydrophilicity of their surfaces. A study comparing the physical properties of AH Plus, PCS and two calcium silicate-based sealers, BioRoot RCS and MTA Fillapex reported higher water sorption and porosity for BioRoot RCS [71]. Moreover, a study on setting of a premixed calcium phosphate silicate-based sealer (EndoSequence BC Sealer) documented a reduction in microhardness when additional water was included in the sealer [79]. In this respect, the differences reported in our study in microhardness assays are in accordance with the setting behaviour of BioRoot RCS under CHX and water exposure. The present study showed that PCS sealer exhibited low microhardness values, which was further compromised by CHX. This is in concordance with the low compressive strength previously reported for PCS sealer [80].

Surface roughness of substrates has been related to initial bacterial adhesion in the course of biofilm formation [81]. In addition to profilometry, qualitative SEM examination

was performed to show the changes on sealers' surfaces. In this study, higher values in surface roughness and more pores for BioRoot RCS compared to AH Plus were observed. This finding corroborates with two *ex vivo* studies where they found higher porosity for BioRoot RCS compared to AH Plus assessed by micro-computed tomography [82, 83]. Higher porosity was also reported for BioRoot RCS compared to AH Plus and PCS in another study [71]. BioRoot RCS as a hydraulic endodontic sealer is hydrophilic and exhibits high water sorption, which in turn increases porosity. This may be of clinical relevance as open pores in the bulk of endodontic sealers and at the sealer-dentin interface may serve as hubs and potentiate growth of residual bacteria [83]. Microleakage models using glucose as tracer have shown that nutrients could potentially enter the root canal from the oral cavity and travel through the bulk of filling materials via pores favouring the growth of entombed bacteria [84, 85]. PCS has displayed pronounced shrinkage when stored at 100% humidity [74], and a zinc oxide-eugenol impression material presented a maximum reduction in dimensions after disinfection with aqueous CHX solutions [86]. As a hydrophobic material, PCS does not favor water adsorption and consequently exhibits low porosity [71].

In the tooth model and resin blocks, sealers' surfaces were characterized with SEM analysis, X-ray diffraction and FT-IR spectroscopy. The purpose of using a tooth model and endo training blocks was to investigate how dentin as a substrate may affect the interactions between CHX and sealers. Dentin has been reported to effect material properties, both irrigation solutions and endodontic sealers and vice versa [5, 17]. Following irrigation with CHX or saline in the tooth model, SEM analysis provided detailed information on the elemental constitution. When CHX was used as the last irrigant in the present study, sealers further exhibited peaks of elements that can be found on dentin and on CHX. CHX may bind to dentin debris or dentin walls and may cross-link to sealers. This could be explained by the

fact that EDTA was not used to remove smear layer. This was favored in order to assess the pure effects of CHX irrigation on sealers avoiding any potential interactions with other irrigants; it has been reported that CHX interacts with EDTA and leads to the formation of a precipitate [87]. EDTA has also been shown to interact with the tricalcium silicate and affect hydration process of calcium silicate cements as BioRoot RCS [88]. XRD is useful to extract information on the crystallographic structure and was therefore used to identify the phase composition of sealers while the Fourier transform infrared spectroscopy assessed the chemical changes in the sealers after contact with CHX in tooth model and endo training blocks. FT-IR and XRD analysis showed no changes in AH Plus chemistry, but an extra calcium carbonate phase under CHX irrigation. AH Plus releases calcium from its calcium tungstate phase and the interaction with air may contribute to formation of calcium carbonate. In the same *ex vivo* tooth model, BioRoot RCS presented the calcium phosphate phase, both under CHX and saline irrigation. The peaks of calcium hydroxide were lower in the tooth model than in control, indicating reaction to form calcium phosphate. BioRoot RCS has been shown to penetrate the dentinal tubules with mineral tags forming a zone termed as mineral infiltration zone [82]. The formation of either calcium carbonate or calcium phosphate depends on the environmental conditions and the ease of combination of the carbonate or phosphate to the calcium. The precipitation of calcium phosphate is also pH-dependent [89]. Previous studies on BioRoot RCS have verified that irrigation with EDTA and smear layer removal do not promote the formation of calcium phosphate [17, 90]. Conversely, calcium phosphate has been proven to form when BioRoot RCS or other hydraulic calcium silicate cements were immersed in HBSS [90]. However, the use of simulated tissue fluid for testing material bioactivity in vitro has been questioned, as it does not completely replicate the clinical scenario [91]. These aforementioned findings hint toward the role of smear layer in

the calcium phosphate formation as a reservoir of minerals [92] and that CHX may not interfere in this process. In all, XRD and FT-IR analyses demonstrated unchanged chemistry for the sealer under CHX and saline irrigation.

PCS under CHX irrigation demonstrated two additional phases, namely sodium silver chloride and silver aluminum, which may partially explain the extra effects of CHX reported in mechanical properties of the sealer. FT-IR analysis indicated that the resin in the resin block altered the PCS chemistry. The interference of eugenol with setting of methacrylate resins is well known [93, 94]. The use of a resin block is thus not indicated for testing PCS.

Conclusions

The main hypotheses were rejected as contact with CHX increased the antibacterial activity of all sealers investigated and affected their physicomechanical performance. PCS alone and in contact with CHX exerted the highest antibacterial activity against both planktonic bacteria and biofilms. As for the physicomechanical properties, BioRoot RCS was the sealer to be mostly affected among the sealers investigated. Surface characterization showed that both AH Plus and BioRoot RCS remained unchanged under CHX irrigation, whilst two additional phases were observed for PCS. Further studies assessing the sealers' performance in more complex environments should be performed by using tooth models and multispecies biofilms. The potential interaction between CHX and endodontic sealers with various chemistries underlines the need for customization of a clinical protocol regarding irrigation techniques and materials used. The individualization of root canal

treatments will ensure that root canal fillings as a whole maintain their antimicrobial properties over time without compromising their physicochemical performance.

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Table 1

Planktonic bacteria	AH Plus		BioRoot RCS		PCS		Controls
	no saline	saline	no saline	saline	no saline	saline	
<i>E. faecalis</i>							
no CHX	7.591 (0.062)	5.072 (0.671)	4.041 (0.440)	5.227 (0.981)	4.204 (0.269)	0 (0)*	7.687 (0.334) /0 (0)* /0 (0)*
short CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	
long CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	
<i>S. mutans</i>							
no CHX	6.158 (0.962)	5.336 (0.628)	0 (0)*	5.47 (0.234)	0 (0)*	0 (0)*	6.878 (0.410) /0 (0)* /0 (0)*
short CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	
long CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	
<i>S. epidermidis</i>							
no CHX	7.635 (0.143)	5.403 (0.511)	5.447 (0.141)	5.563 (0.652)	5.681 (0.951)	0 (0)*	7.38 (0.512) /0 (0)* /0 (0)*
short CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	
long CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	
<i>S. aureus</i>							
no CHX	6.547 (0.341)	5.558 (0.978)	5.964 (0.688)	4.924 (0.095)	6.477 (0.747)	0 (0)*	7.968 (0.751) /0 (0)* /0 (0)*
short CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	
long CHX	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	

Table 1. Median Log (CFU+1)/mL and 25-75 interpercentile range of *E. faecalis*, *S. mutans*, *S. epidermidis*, and *S. aureus* in planktonic forms after direct contact for 1 hour with each sealer's surface. Controls are presented in the following order: **bacteria**/ short-term CHX/ long-term CHX. Blue asterisks indicate statistically significant differences between groups and the control of each bacterium (values in bold letters), p<0.05. In saline groups, 300 µl saline were transferred upon sealer surfaces for 24 hours at 37°C in relative humidity. Brackets and black asterisks indicate statistical significance between no saline and saline groups, p<0.05. (short: 1 minute contact time; long: continuous contact along with setting process)

Table 2

Biofilms	AH Plus	BioRoot RCS	PCS	Controls
<i>E. faecalis</i>				
no CHX	10.510 (0.279)	8.916 (1.306)	8.54 (0.819)*	10.540 (0.278) /9.944 (0.128)/8.505 (0.494)
short CHX	10.620 (0.178)	8.642 (0.805)	7.591 (0.886)*	
long CHX	7.447 (0.513)*	6.362 (0.541)*	7.759 (0.379)*	
<i>S. mutans</i>				
no CHX	9.960 (0.234)	8.472 (1.077)	4.778 (0.699)*	9.903 (0.257) /6.613 (1.123)/0 (0)*0 (0)*
short CHX	6.623 (0.284)	6.759 (1.263)	0 (0)*	
long CHX	0 (0)*	0 (0)*	0 (0)*	
<i>S. epidermidis</i>				
no CHX	10.599 (0.329)	6.477 (0.323)*	8.305 (0.485)	10.701 (0.299) /10.10 (0.248)/8.676 (0.311)
short CHX	10.49 (0.518)	6.778 (0.426)*	7.846 (1.099)*	
long CHX	7.929 (0.114)*	5.903 (0.517)*	6.699 (0.401)*	
<i>S. aureus</i>				
no CHX	10.748 (0.202)	8.346 (0.534)	8.833 (0.236)	10.731 (0.211) /10.38 (0.273)/8.675 (0.246)
short CHX	9.995 (0.191)	8.065 (0.992)*	8.022 (0.577)*	
long CHX	7.914 (0.222)*	7.477 (0.271)*	7.643 (0.584)*	

Table 2. Median Log (CFU+1)/mL and 25-75 interpercentile range of *E. faecalis*, *S. mutans*, *S.*

epidermidis, and *S. aureus* in monospecies biofilm after direct contact for 24 hours with sealers.

Controls are presented in the following order: **bacteria**/ short-term CHX/ long-term CHX. Blue asterisks indicate statistically significant differences between groups and the control of each bacterium (values in bold letters), $p < 0.05$. (short: 1 minute contact time; long: continuous contact along with setting process)

Table 3

Group	Setting time (min)	Contact angle (°)	Microhardness (VHN)
AH Plus			
no CHX/water	462 (9) ^a	81.1° (1.1) ^a	7.04 (0.4) ^a
short CHX	517 (9) ^b	68.9° (1.2) ^b	5.46 (0.29) ^b
long CHX	511 (11) ^b	46.8° (2.9) ^c	4.46 (0.78) ^c
short water	519 (8) ^b	66.2° (1.8) ^b	5.83 (0.62) ^b
long water	514 (10) ^b	43.6° (2.1) ^c	4.29 (0.56) ^c
BioRoot RCS			
no CHX/water	87 (6) ^a	13.9° (0.9) ^a	6.36 (0.43) ^a
short CHX	162 (5) ^b	29.2° (1.2) ^b	2.09 (0.28) ^b
long CHX	289 (13) ^c	37.5° (1) ^c	1.67 (0.17) ^b
short water	154 (8) ^b	0° (0) ^d	4.82 (0.31) ^c
long water	165 (9) ^b	0° (0) ^d	4.59 (0.62) ^c
PCS			
no CHX/water	221 (10) ^a	80.9° (0.7) ^a	1.51 (0.16) ^a
short CHX	175 (6) ^b	41.5° (1.2) ^b	0.70 (0.19) ^b
long CHX	180 (3) ^b	34.4° (2) ^c	0.40 (0.05) ^b
short water	161 (5) ^b	42.3° (0.5) ^b	1.49 (0.22) ^a
long water	163 (7) ^b	35.2° (1.4) ^c	1.40 (0.31) ^a

Table 3. Mean and standard deviation of physical properties (setting time, wettability, microhardness). Read vertically, the same superscript letter shows no statistically significant differences within the same materials ($p>0.05$). (short: 1 minute contact time; long: continuous contact along with setting process)

Figure legends

Figure 1. Indicative confocal laser scanning microscopic images of *E. faecalis*, *S. mutans*, *S. epidermidis*, and *S. aureus* 48-hours monospecies biofilms grown on membrane filters. The scanning was performed from the top of the biofilm to the membrane surface using a 60× water lens, 0.5 µm step size, and a format of 512 x 512 pixels corresponding to an area of 88 x 88 µm.

Figure 2. Representative secondary electron scanning micrographs of the tested sealers after contact with CHX and HBSS (magnification 100×). Mean and standard deviation of surface roughness Ra. Read horizontally, the same superscript letter shows no statistically significant differences within the same materials ($p>0.05$).

Figure 3. High magnification scanning electron micrographs (2000×) of tested sealers retrieved from the split tooth model after saline or CHX last irrigation. Elemental analysis of sealer surfaces and their spectra. CHX irrigation affected the surface microstructure of AH Plus (A), BioRoot RCS (B) and PCS (C).

Figure 4. (A) Fourier transform infrared spectroscopic scans of sealers placed in a tooth model and resin blocks after final irrigation with saline (blue) and chlorhexidine (red). The black arrows denote the regions (cm^{-1}) where changes in scan patterns are observed (B) X-ray diffraction analysis: Green letters are used for the compounds that can be found in all conditions and controls, black only in controls, red in tooth model and blue in resin blocks. CT, calcium tungstate; ZO, zirconium oxide; CC, calcium carbonate; CH, calcium hydroxide; TCS, tricalcium silicate; DCS, dicalcium silicate; CP, calcium phosphate; ZnO, zinc oxide; Ag, cubic silver; SA, silver aluminium; SSC, sodium silver chloride

Biofilm formation

E.faecalis

S.mutans

S.epidermidis

S.aureus

Figure 2

AH Plus

BioRoot RCS

PCS

AH Plus

A

BioRoot

B

PCS

C

Figure 4

Supplementary material

1. **Figure S1: Planktonic bacteria assay**
2. **Figure S2: Biofilm formation**
3. **Figure S3: Biofilms assay**
4. **Figure S4: Physical properties**
5. **Figure S5: Tooth model and endo-training blocks**
6. **Figure S6: Chemical properties**

Figure S1

Figure S1. Schematic representation of planktonic bacteria assay. The bottoms of a 96-well microtiter plate were coated with each sealer. A fixed amount of 15 μ L CHX was applied on the sealer surfaces (a). In short-term exposure groups, after 1 minute of contact with CHX, the drop was removed with a pipette and the sealers were placed in a dry incubator at 37°C for 20 minutes to let any liquid excess dry out, before allowed to set (b). The sealers were tested against planktonic bacteria for three different groups of exposure to CHX: group 1, no CHX (no contact with CHX); group 2, short CHX (1-minute contact with CHX); group 3, long CHX (continuous contact with CHX along with setting process) (c). The bacteria were suspended in Phosphate Buffered Saline (PBS) to an optical density at 600 nanometers (OD₆₀₀) of 1.0 (d). An amount of 10 μ L from each bacterial suspension was carefully placed on the surface of the materials for 1 hour contact (e). Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates (f).

Figure S2

Figure S2. Schematic representation of biofilm formation. The bacteria were suspended in PBS to an optical density at 600 nanometers (OD600) of 1.0 (**a**). Membrane filters were cut in circular 3-mm diameter pieces and placed upon TSB agar plates. A droplet of 2 µl of each bacterial inoculum OD600 1.0 was applied upon the outer surface of membranes (**b**). The agar plates were incubated at 37°C in a 5% CO₂ supplemented atmosphere for 48 hours (**c**). The monospecies biofilms were established and verified with the use of confocal laser scanning microscopy (**d**).

Figure S3

Figure S3. Schematic representation of biofilms assay. Caps of 0.2-mL polypropylene thin wall PCR tubes were cut and filled with the mixed sealers (**a**). A fixed amount of 10 µL CHX was applied on the sealer surfaces. In short-term exposure groups, after 1 minute of contact with CHX, the drop was removed with a pipette and the sealers were placed in a dry incubator at 37°C for 20 minutes to let any liquid excess dry out, before allowed to set (**b**). The sealers were tested against bacteria in biofilms for three different groups of exposure to CHX: group 1, no CHX (no contact with CHX); group 2, short CHX (1-minute contact with CHX); group 3, long CHX (continuous contact with CHX along with setting process) (**c**). After biofilm formation, the filter membranes were positioned upon the sealers with the established biofilms facing their surfaces (**d**). Parafilm M was applied around the caps to ensure tight contact between membranes and sealer surfaces (**e**). After 24 hours of contact, each membrane with its corresponding cap was transferred to vials containing 5 ml PBS and vigorously vortexed with glass beads (**f**). After serial dilutions in PBS, CFUs were counted after overnight incubation (**g**).

Figure S4

Figure S4. Cylindrical specimens, measuring 10 mm in diameter and 2 mm in height, were prepared into molds for each sealer. For both CHX and water exposure groups, a drop of 25 μ l was applied upon the sealers with a pipette. In short-term exposure groups, after 1 minute of contact with either CHX or water, the drop was removed with a pipette and the sealers were placed in a dry incubator at 37°C for 20 minutes to liquid excess dry out, before allowed to set for 24 hours in a humidified atmosphere (**a**). The physical properties were assessed by testing the setting time (**b**), wettability (contact angle measurements) (**c**), microhardness (**d**), and surface roughness of sealers (**e**).

Figure S5

Figure S5. Schematic representation of tooth model and endo-training blocks. Twenty four presterilized, single-rooted human teeth, free of caries, were decoronated and the root length standardized at 15 mm, using a diamond saw (a). The root canals were instrumented with ProTaper rotary files up to size F4, 1 mm shorter than the standardized root length. An irrigation protocol with 2 mL of 2,5% NaOCl between the changes of the rotary files was followed (b). The roots were split longitudinally along their long axis using a diamond saw (c) and the two segments were repositioned and held tightly together by wrapping them up with Parafilm M (d). The root canals were irrigated with either 2 ml of saline or 2 ml of saline and then 2 ml of 2% CHX for 1 minute as the final irrigant. The root canals were dried with paper points before placement of sealers. The tested sealers were placed inside the root canals (e) and they were allowed to set for 24 hours (f). The aforementioned preparation was also applied on endo-training resin blocks.

Figure S6

Figure S6. Assessment of chemical properties. After the setting period, each root was unwrapped from the Parafilm M, and the root fragments were gently detached with a use of a scalpel that was applied on the thin space formed between them **(a)**. The sealers were exposed and gently retrieved intact from the dentine walls. After the separation process, the whole bulk of the sealer was adhered to one segment whilst the other segment was macroscopically free of sealer remnants. The aforementioned preparation was also applied on endo-training resin blocks. The exposed sealers upon their substrates (either tooth or endo training blocks) were characterized by scanning electron microscopy (SEM) and energy dispersive spectroscopy, X-ray diffraction analysis (XRD), and Fourier-transform infrared spectroscopy (FT-IR) **(b)**.