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Effect of chlorhexidine digluconate on antimicrobial activity, cell viability and physicochemical properties of three endodontic sealers

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ABSTRACT

Objective: Assess the biological and physicochemical properties of AH Plus, BioRoot RCS and Pulp Canal Sealer (PCS) leachates with and without chlorhexidine (CHX).

Methods: The sealers were studied in no contact and 1-minute contact with CHX. For biological properties (antibacterial activity and cytotoxicity), leachates were formed in saline of freshly mixed, 1-, 7- and 28 days set sealers. The antibacterial properties of sealer leachates were investigated for planktonic and biofilm growth of *E. faecalis*, *S. mutans*, *S.epidermidis* and *S.aureus*. The 3-(4,5 dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate murine fibroblast cell viability after exposure to the leachates. The physical properties (water uptake, sorption, solubility, porosity, surface characteristics) of sealers and the pH of the immersion liquid (saline or distilled water) were also assessed over a 28-days period.

Results: CHX improved the antibacterial properties of the sealer leachates and reduced cell viability for all sealer leachates, except for freshly mixed PCS. BioRoot RCS leachates presented the highest antibacterial properties and cell viability with and without CHX contact. PCS was the material most affected by CHX in terms of physical properties, whereas for AH Plus, solubility was increased. CHX did not affect the physical properties of BioRoot RCS, except for solubility that was decreased. CHX contact did not change sealers' alkalinity in distilled water whereas it increased it for AH Plus and BioRoot RCS in saline.

Significance: CHX improved the antibacterial efficacy of sealer leachates and either compromised or did not affect cell viability. CHX affected to various extent sealers' physicochemical properties.

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1. Introduction

Irrigation solutions and root canal obturation materials are important for long-lasting clinical success of endodontic treatment [1]. Following irrigation, an endodontic sealer is applied in direct contact with dentinal walls to provide a bacteria-tight seal in the root canal space [2]. Endodontic sealers based on different chemical compositions, such as zinc oxide eugenol, resin, silicone or calcium silicate are available [3]. These materials should ideally offer many biological and physicochemical properties such as antimicrobial activity, remain unaffected by the irrigating solutions, keep a long-term dimensional and physicochemical stability inside the root canal space [1,4–6], remain insoluble, and not induce cytotoxic effects to surrounding periapical tissues [7].

Irrigation liquids may be left in the root canal system (dentinal walls and tubules) after drying, notably in the apical part or anatomical irregularities [8,9]. In addition, compounds from irrigation liquids are observed on dentin after irrigation [10]. Irrigants and constituents from irrigation liquids may potentially interact with sealers and affect their physicochemical and biological properties. Studies on interactions between sealers and irrigants have mainly focused on sealer properties such as sealing ability, microleakage and wettability [11–15]. Until present, few studies have investigated the effect of irrigation liquids on the antimicrobial properties [16–19], and cytotoxicity.

Different irrigation solutions such as sodium hypochlorite (NaOCl), chlorhexidine digluconate (CHX), 17% ethylene diamine tetraacetic acid (EDTA), citric acid and MTAD are used in endodontic treatments [20,21]. Depending on the irrigation protocol followed, these solutions may be used as last irrigants during chemical preparation. In particular, chlorhexidine digluconate (CHX) possesses potent-broad antimicrobial properties and is often used in endodontics as the last irrigation solution [22,23]. It acts by binding to dentine (a property known as substantivity), it releases gradually [24], and thus may interact with the sealer and modify its properties [19].

Contact between tissue fluids or irrigation liquids and sealer may cause leaching of constituents from the sealer. Leachates could potentially migrate to patent dentinal tubules, lateral canals or to periapical tissues through the bulk of filling materials or the dentine-sealer interface [2,25–27].

Leachates of endodontic materials have attracted the attention in regard to antibacterial properties and cytotoxicity [28]. The antibacterial properties of leachates may aid in eradication of residual planktonic bacteria or bacteria in biofilms in untouched areas after chemo-mechanical preparation such as apical ramifications, lateral canals, and isthmuses [29–36]. At the same time, the leachable compounds should ideally not induce cytotoxic effects to the periapical tissues as this may retard the healing process and thus jeopardise the clinical success of root canal therapies [7,37].

A recent literature review on standardisation of antimicrobial testing of dental materials suggests characterisation of elution/degraded materials along with cytocompatibility testing [38]. There is lack of literature on both sealer leachates and the effects of CHX to endodontic sealers with respect to antimicrobial efficacy, cytotoxicity and physicochemical properties.

The aim of this study was to assess the antibacterial activity and cytotoxicity (cell viability) of the leachates of three sealers with and without chlorhexidine contact and investigate the effect of CHX on sealers' water uptake, sorption, solubility, porosity, surface characteristics and pH of the immersion liquid. The null hypothesis tested was that exposure to CHX will not yield any changes in sealers' properties.

2. Materials and methods

An epoxy resin-based sealer, AH Plus (Dentsply International Inc, York, PA, USA), a tricalcium-silicate based sealer, BioRoot™ RCS (Septodont, Saint-Maur-des-Fossés, France), and a zinc oxide eugenol sealer, Pulp Canal Sealer (PCS) (Kerr Corporation, Romulus, MI) 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 (SDW) and standardised to 2%.

2.1. *Biological properties-leachate preparation*

The bottoms of a 96-well microtiter plate (Costar, Flat bottom, Ultra low attachment, Corning Incorporated, Corning, NY, USA) were coated with each sealer by using a small size round ended dental instrument (Fig. S1a). Two groups were formed according to exposure to CHX: group 1, no CHX (no contact); group 2, CHX (short-term exposure: 1 min contact time). For CHX group, after sealer preparation a drop of 15 μl CHX was applied upon the fresh materials with a pipette and evenly spread with a sterile plastic inoculation loop. After 1 min of contact with CHX, the drop was sucked up with a pipette and the sealers were placed in a dry incubator at 37 °C for 20 min to let any excess dry out (Fig. S1c). The same amount of CHX within the same application times was also transferred into uncoated wells. Sealer leachates were initiated to form for freshly mixed, 24 h (1 day), 7 days and 28 days set sealers (Fig. S1b): 300 μl sterile 0.9% saline solution (saline) were applied upon the sealers' surfaces into the wells for 24 h to form leachates at 37 °C in a 100% humidified atmosphere (Fig. S1d).

2.1.1. *Antibacterial assays*

The sealer leachates were tested against both planktonic bacteria and bacteria in biofilms. 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 h in Tryptone Soya Broth (TSB) at 37° C, 5% CO_2 supplemented atmosphere.

The antimicrobial activity of sealer leachates were investigated against planktonic bacteria. Briefly, the bacteria were suspended in phosphate buffered saline (PBS) to an

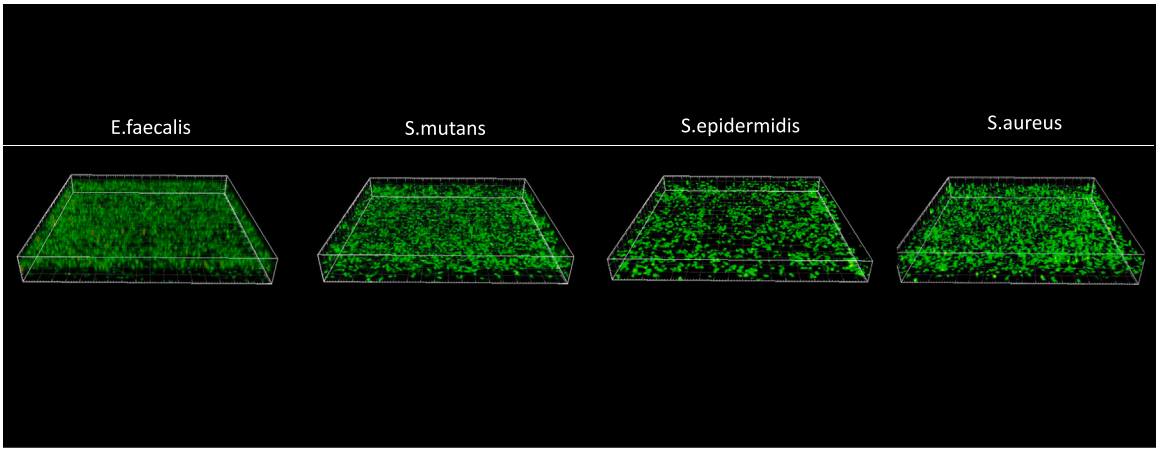

Fig. 1 – Representative confocal laser scanning microscopic images of *E. faecalis*, *S. mutans*, *S. epidermidis*, and *S. aureus* 48-h monospecies biofilms grown on polyester coverslips. The scanning was performed from the top of the biofilm to the membrane surface using a 60 × water lens, 0.5 µm step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

optical density at 600 nanometres (OD_{600}) of 1.0, corresponding to approximately 2×10^8 Colony Forming Units (CFUs)/mL. After leaching process, 90 µl of each leachate was transferred into new 96 wells and mixed with 10 µl of each bacterial suspension (OD 1.0) (Fig. S2b). The same amount of 10 µl from each bacterial suspension was mixed with 90 µl of saline and served as positive controls. The specimens were incubated at 37 °C for 1 h. Colonies of surviving bacteria were calculated after serial dilution in PBS and plating on TSB agar plates incubated overnight at 37 °C, 5% CO_2 supplemented atmosphere (Fig. S2c).

For biofilm assays, polyester coverslip discs (13 mm, Nunc™ Thermanox™ Coverslips, Thermo Fisher Scientific, Waltham, MA, USA) were placed on the bottoms of 24-well plates (Costar, Flat bottom, Ultra low attachment, Corning Incorporated, Corning, NY, USA). Bacteria grown overnight for 18 h in TSB were mixed with fresh medium in a fixed rate 1/10. Two mL of each bacterial suspension were transferred into the 24-well plates and covered sufficiently the coverslip discs (Fig. S3a). The plates were incubated at 37 °C in a 5% CO_2 supplemented atmosphere for 48 h and monospecies biofilms were established (Fig. S3b). After incubation period, the discs were washed gently with PBS to remove loosely attached bacteria. Sealer leachates were extracted as it was aforementioned (Fig. S1) and 100 µl of each leachate were applied on the discs for one hour at 37 °C in contact with the biofilms (Fig. S3d). One hundred µl saline were also transferred upon discs and served as positive controls. After contact time, each disc was transferred to vials containing 5 mL PBS and vigorously vortexed with glass beads (Fig. S3e). After serial dilutions in PBS, CFUs were counted after overnight incubation at 37 °C in a 5% CO_2 supplemented atmosphere (Fig. S3f). Carry over effect of the method was also assessed. Polyester coverslip discs with established biofilms served as positive controls and were placed in vials containing 5 mL PBS. The sealers' leachates were also transferred in the same vials with positive controls. These samples were vigorously vibrated with glass beads. Possible carryover effect was measured after serial dilutions and CFUs were calculated as described previously. The formation of biofilms was verified using a confocal laser-scanning microscope (Olympus FluoView FV1200, Olympus Corporation, Tokyo, Japan). The coverslip discs were covered with Syto-9/Propidium iodide (PI) (FilmTracer™ LIVE/DEAD Biofilm Viability kit, Thermo Fisher Scientific Inc., Waltham, MA, USA) staining to colour any present biofilms. A diode laser emitting at 473 nanometres (nm) was used and 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 × 512 pixels corresponding to an area of 88 × 88 µm (Fig. 1).

2.1.2. *Cell viability*

The cell viability was tested by assessing the cell metabolic activity in contact with sealers' leachates. Leachates from freshly mixed, 24 h, 7 days and 28 days set sealers with and without 1 min contact with CHX were filtrated under sterile conditions, as was aforementioned (Fig. S1). L929 murine fibroblast cell line was cultured in 75 cm^2 flasks (Falcon® Rectangular Canted Neck Cell Culture Flask, Corning, NY, US) in cell culture medium (Dulbecco modified Eagle medium) supplemented with 5% foetal bovine serum, 100 units/mL penicillin G, and 100 µg/mL streptomycin at 37 °C in air with 5% CO_2 in a humidified incubator under ambient atmospheric pressure. At 70–80% confluence, cells were detached under trypsinization at 37 °C for 2–3 min and subcultured or seeded for the experimental procedures. The L929 cell number was standardised to 75.000 cells/mL and 200 µl were transferred to 96 wells (Fig. S4a). After 1 day of incubation, the supernatant medium was aspirated and 100 µl mixture of each leachate with cell culture medium in a 1:1 ratio was applied upon the seeded cells for another 24 h (Fig. S4b). For negative controls, 100 µl mixture of saline with cell culture medium in a 1:1 ratio was transferred upon the seeded cells. The 3-(4,5 dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma M2128) was employed to evaluate cell metabolic function [39]. The mixtures were decanted and

100 mL MTT was transferred into each well and incubated for 1 h (Fig. S4c). After aspiration, 100 mL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals that formed and absorption was read at 570 nm (Synergy H1, BioTek, Winooski, VT, USA) (Figs. S4d and 4e).

2.2. Physical properties of sealers

Water uptake, sorption, solubility, porosity of sealers with and without CHX contact were evaluated following a modification of ISO 4049; 2019 [40] regarding the manufacturing of sealer specimens. Normally in ISO 4049, specimens measuring 15 mm in diameter, 1 mm in height are immersed in 10 mL defining a "≈ 40.06 mm^2/ mL" immersion ratio per specimen. In our study, inert teflon cylindrical moulds (10 mm diameter, 1 mm height) with bottom and side walls (Fig. S5a) were manufactured in such way to cover the bottom face and side surfaces of the sealer samples and leave free the top face of the materials. Each mould was weighted before sealer placement to an accuracy of ± 0.1 μg. The sealers were placed into the moulds (Fig. S5a) and a glass microscope slide was applied upon them to achieve flat, uniform surfaces. The sealers into the moulds were either allowed to set independently (no CHX) or in contact with CHX. In CHX exposure group, a drop of 25 μl CHX was applied upon half of the sealer samples with a pipette and evenly spread with a sterile plastic inoculation loop (Fig. S5b). After 1 min of contact with CHX, the drop was removed with a pipette (Fig. S5b) and the sealers were placed in a dry incubator at 37 °C for 20 min to let any liquid excess dry out, before allowed to set (Fig. S5c). After sample preparation (n = 9 for each experimental group) (Fig. S5c), the sealers were allowed to set into the moulds for a time period 50% longer than the setting time stated by the manufacturers (t_0) and each specimen was weighted to an accuracy of ± 0.1 μg (denoted as "m"). The volume 'V' of each specimen was calculated by measuring the mean diameter and the thickness of each specimen to an accuracy of 0.01 mm using a digital caliper (Mitutoyo 500-197-30, Mitutoyo, IL, US). The specimens were immersed at time point t_0 into snap vials (ND18, VWR International, PA, USA) containing 1.960 mL water (milli-Q water; Elix Essential 5 UV Water Purification System, Merck KGaA, Darmstadt, Germany) to comply with the immersion ratio per specimen (≈ 40.06 mm^2/mL) applied by ISO 4049 (Fig. S6a). The specimens were then removed after 1 day, dried using filter paper, waved in the air for 15 s and then weighed 1 min after removal from the immersion solution to an accuracy of 0.1 μg (Fig. S6b). Their mass was recorded as 'm_1'. The water uptake of each specimen could be recorded using Eq. (1).

$$W_{uptake} = \frac{m_1 - m}{V} \quad (1)$$

Subsequently the specimens were re-immersed and the aforementioned process was repeated to measure the water uptake of the specimens after 7, 14, 21 and 28 days. After 28 days, the mass of the specimens (fully saturated with water) was recorded as 'm_2'. The specimens were stored in a desiccator maintained at 23 ± 1 °C for at least 24 h using silica gel as desiccant until a constant mass could be recorded (Fig. S6c). This constant mass was recorded as 'm_3'. Water sorption (W_{sp}) for each sample was calculated using Eq. (2).

$$W_{sp} = \frac{m_2 - m}{V} \quad (2)$$

Water solubility (Wsl) for each sample was calculated using Eq. (3).

$$W_{sl} = \frac{m - m_3}{V} \quad (3)$$

The porosity of each specimen was calculated using Eq. (4):

$$porosity(\%) = \left[\left(\frac{m_2}{m}\right) - 1\right] \times 100 \quad (4)$$

The mass of the water absorbed by the pores of each specimen could be quantified on the basis of the Archimedes principle. The difference in mass (g) between each sample when dry and when submerged in solution, can be expressed as ¨volume¨ of the pores present in each sample.

2.3. Microscopy of sealer surfaces

Optical microscopy (NexiousZoom, Euromex, Arnhem, The Netherlands) was performed to investigate the microstructure of the 28 days specimens that were evaluated for ISO 4049. In addition, specimens with the same dimensions were prepared as aforementioned (Fig. S5), incubated at 37 °C, 100% humidity and also evaluated under optical microscopy. The micrographs were captured using a digital camera (Leica DFC 290, Leica Microsystems, Danaher Corporation, Washington DC, USA).

2.4. Chemical properties–assessment of pH

The sealers' alkalinity in contact or not with CHX was assessed measuring the pH of sealers' leachates derived from the assays both for biological (Fig. S1e) and physical properties (Fig. S6b). The pH values were assessed with a pH metre (Sension+ PH31; Hach, Loveland, CO, USA), previously calibrated using buffer solutions of pH 4, 7, and 14.

2.5. Statistical analysis

The statistical analysis was performed with IBM SPSS Statistics software version 27 (IBM, Armonk, USA). Before each statistical analysis, the data were assessed for normality with the Shapiro-Wilk test and homogeneity of variance with Levene's test. Statistical analysis of the physical (water uptake, sorption, solubility, porosity), chemical (pH assessment) properties and cytotoxicity was performed using Tukey's (for equal variances across groups) and Dunnett's C (for unequal variances across groups) multiple comparison test ($p < 0.05$). In case of pairwise comparisons of two groups, parametric t-tests were performed ($p < 0.05$). The antibacterial assays were analysed using the nonparametric Kruskal–Wallis and Dunn's test due to absence of normal distribution of data ($p < 0.05$).

3. Results

3.1. Biological properties

3.1.1. Antibacterial assays of sealer leachates

Leachates from BioRoot RCS eliminated the planktonic bacteria for all species and conditions investigated ($p < 0.05$). Exposure to CHX enhanced the antibacterial activity of leachates from AH Plus ($p < 0.05$). Leachates from PCS reduced the number of CFUs for planktonic *S. mutans* and *S. epidermidis* for all experimental conditions investigated compared to control ($p < 0.05$). Against planktonic *E. faecalis* and *S. aureus*, leachates from PCS eliminated the numbers of bacteria up to 24 h setting with and without exposure to CHX ($p < 0.05$), whilst only leachates from PCS in contact with CHX exhibited antibacterial properties up to 28 days ($p < 0.05$). The data for the direct contact test with planktonic bacteria is shown in Table 1.

Leachates from PCS with and without exposure to CHX showed antibacterial activity against all biofilms up to 7 days ($p < 0.05$), while exposure to CHX improved the antibacterial properties against *E. faecalis* and *S. mutans* biofilms up to 28 days ($p < 0.05$). Exposure to CHX enhanced the antibacterial activity of leachates from AH Plus against biofilms ($p < 0.05$), while no difference in antibacterial activity was observed for AH Plus without CHX contact compared to control. BioRoot RCS leachates reduced the number of bacteria in *E. faecalis* and *S. mutans* biofilms for all conditions up to 7 days ($p < 0.05$). The results for the antibacterial properties of sealers on biofilms are shown in Table 2.

3.1.2. Cell viability

Only 28-days set AH Plus and BioRoot RCS presented cell viability higher than 70% in accordance with the threshold set by ISO 10993-5;2009 [41]. For each condition (sealer and sealer + CHX) and setting time (freshly mixed, 24 h, 7 days, 28 days) investigated, reduced cell viability was observed for leachates from AH Plus and PCS compared to BioRoot RCS ($p < 0.05$) except for 7- and 28-days set AH Plus without CHX ($p > 0.05$). Exposure to CHX significantly decreased all sealer leachates' viability for each setting time compared to leachates from sealers without CHX ($p < 0.05$), however, this was not observed for freshly mixed PCS. The results of the MTT assay are presented in Fig. 2.

3.2. Physical properties

Constant mass m_3 was achieved after 1 extra day in desiccator for AH Plus and PCS while 2 extra days were needed for BioRoot RCS after the initial 24 h-desiccating.

BioRoot RCS with and without CHX exposure had the highest water uptake compared to other sealers for all immersion periods investigated ($p < 0.05$). No statistically significant differences were observed for AH Plus and BioRoot RCS with and without exposure to CHX for all immersion periods tested ($p > 0.05$). PCS with CHX exposure presented significantly lower elution compared to PCS for each immersion period ($p < 0.05$). For all sealers both with and without CHX contact, most of water uptake occurs in the first 24 h of immersion. The data for water uptake are shown in Fig. 3 and Table S1.

Water sorption, solubility and porosity were highest for BioRoot RCS both with and without CHX contact compared to the other sealers investigated ($p < 0.05$). CHX did not affect the water sorption and porosity compared to no contact for BioRoot RCS ($p > 0.05$), however solubility was significantly decreased ($p < 0.05$). For AH Plus, contact with CHX increased the solubility of the sealer ($p < 0.05$), whereas sorption and porosity remained unaffected ($p > 0.05$). PCS with CHX contact exhibited increased sorption and porosity, while solubility was decreased compared to no CHX contact ($p < 0.05$). The data for sorption, solubility and porosity are shown in Table 3.

3.3. Microscopy of sealer surfaces – qualitative analysis of surface properties

The representative images of the sealer surfaces viewed under the optical microscope are shown in Fig. 4. Non-immersed AH Plus with and without CHX contact did not present any characteristic features upon their surfaces; only few voids were present for AH Plus with CHX (Fig. 4b). Immersed AH Plus surfaces exhibited mainly air entrapped voids (Fig. 4c) whilst AH Plus with CHX contact had both air entrapped and capillary voids (Fig. 4d); the surfaces of AH Plus with CHX contact were rough presenting whitish depositions. Non-immersed BioRoot RCS surfaces with and without CHX contact were partially covered by crystal-like depositions (Figs. 4e and 4f). Immersed BioRoot RCS with and without CHX contact demonstrated many capillary voids of various sizes (Figs. 4g and 4h). Non-immersed PCS presented flat, even surfaces with a grey background whereas contact with CHX changed the topography and the colour of the surfaces (Figs. 4i and 4j). Following immersion, PCS surfaces with and without CHX contact appeared with a brighter more yellowish hue. The immersed PCS without CHX presented dry surface texture with a significant amount cracks in the bulk of the material, a declare of extensive shrinkage (Fig. 4k). Contact with CHX reduced the amount of cracks on the surfaces, while more capillary voids were (became) evident (Fig. 4l).

3.4. Chemical properties–assessment of pH

As for the pH assessment of the sealer leachates for biological properties (extraction vehicle: saline), BioRoot RCS had the highest pH for all the setting times (freshly mixed, 1 day, 7 days, 28 days) of the sealers with and without CHX contact ($p < 0.05$). Regarding AH Plus, the freshly mixed sealer with and without CHX contact presented the highest pH with a decreasing trend over setting time ($p < 0.05$), whilst CHX did not affect the pH values for each setting time tested compared to AH Plus alone. Freshly mixed and 28 days PCS with and without CHX exhibited the lowest (acidic) pH values compared to 1- and 7 days of setting when the pH was slightly alkaline ($p < 0.05$). No significant differences were found between PCS alone and with CHX contact for all setting times tested ($p > 0.05$). The results for measurement of pH of the

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 h with each sealer's leachate. Controls are presented in the following order: *bacteria*/short-term CHX. Asterisks indicate statistically significant differences between groups and the control of each bacterium (values in bold letters), $p < 0.05$ (nonparametric Kruskal–Wallis and Dunn's test).

Planktonic bacteria	AH Plus				BioRoot RCS				PCS				Controls
	Freshly mixed	*24 h*	*7 days*	*28 days*	*Freshly mixed*	*24 h*	*7 days*	*28 days*	*Freshly mixed*	*24 h*	*7 days*	*28 days*	
E. faecalis													
No CHX	7.239 (0.44)	7.834 (0.212)	7.758 (0.239)	7.663 (0.342)	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	7.365 (0.107)	7.193 (0.255)	**7.465 (0.355)**/
CHX	0 (0)*	4.193 (0.133)*	6.508 (0.481)*	6.292 (0.26)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	4.696 (2.16)*	5.556 (0.548)*	0 (0)*
S. mutans													
No CHX	6.949 (0.278)	7.297 (0.09)	7.694 (0.365)	7.589 (0.334)	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	3.857 (1.456)*	5.262 (0.312)*	**7.497 (0.04)**/
CHX	0 (0)*	0 (0)*	6.146 (0.287)*	5.579 (0.365)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*
S. epidermidis													
No CHX	7.106 (0.129)	7.666 (0.143)	7.471 (0.235)	7.317 (0.372)	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	6.387 (0.409)*	6.466 (0.49)*	**7.230 (0.337)**/
CHX	0 (0)*	4.857 (0.345)*	6.423 (0.21)*	6.230 (0.343)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	5.643 (0.429)*	0 (0)*
S. aureus													
No CHX	6.843 (0.378)	7.376 (0.23)	7.221 (0.423)	7.312 (0.443)	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	7.024 (0.264)	7.051 (0.796)	**7.106 (0.49)**/
CHX	0 (0)*	4.806 (0.514)*	6.483 (0.103)*	6.554 (0.101)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	0 (0)*	5.505 (0.564)*	0 (0)*

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 1 h with sealer leachates. Controls are presented in the following order: *bacteria*/short-term CHX. Asterisks indicate statistically significant differences between groups and the control of each bacterium (values in bold letters), $p < 0.05$ (nonparametric Kruskal–Wallis and Dunn's test).

Bacteria in biofilms	AH Plus				BioRoot RCS				PCS				Controls
	Freshly mixed	*24 h*	*7 days*	*28 days*	*Freshly mixed*	*24 h*	*7 days*	*28 days*	*Freshly mixed*	*24 h*	*7 days*	*28 days*	
E. faecalis													
No CHX	8.82 (0.205)	8.932 (0.732)	8.946 (0.721)	8.820 (0.205)	7.477 (0.982)*	7.752 (0.599)*	7.806 (0.833)*	8.889 (0.381)	7.58 (0.578)*	7.716 (0.525)*	7.408 (1.02)*	8.734 (0.444)	**9.48 (0.46)**/ 7.326 (0.39)*
CHX	7.633 (0.121)*	7.424 (0.455)*	8.101 (0.635)*	7.959 (0.421)*	7.308 (0.405)*	7.212 (0.459)*	7.628 (0.689)*	8.129 (0.448)	5.079 (0.311)*	6.859 (0.349)*	6.494 (0.744)*	7.900 (0.313)*	
S. mutans													
No CHX	8.681 (0.139)	8.602 (0.322)	8.612 (0.655)	8.681 (0.139)	7.079 (0.35)*	7.204 (1.077)*	7.244 (0.386)*	8.489 (0.184)	6.301 (0.168)*	6.164 (0.385)*	6.355 (0.527)*	7.724 (0.337)	**8.505 (0.525)**/ 7.101 (0.212)*
CHX	7.322 (0.844)*	7.360 (0.438)*	7.123 (1.138)*	7.618 (0.742)*	7.100 (0.096)*	7.253 (0.665)*	7.016 (0.266)*	8.301 (0.36)	5.805 (0.824)*	6.188 (0.248)*	5.380 (0.633)*	6.190 (0.407)*	
S. epidermidis													
No CHX	7.778 (0.402)	7.987 (0.633)	8.032 (0.393)	7.878 (0.402)	8.681 (2.067)	8.854 (1.047)	8.704 (0.451)	8.716 (0.534)	6.903 (0.558)*	6.128 (1.101)*	6.318 (0.456)*	7.763 (0.554)	**8.146 (1.417)**/ 7.255 (1.092)*
CHX	6.61 (0.252)*	7.001 (0.344)*	7.122 (0.629)*	7.289 (0.779)*	7.054 (0.512)*	7.351 (1.176)*	6.988 (1.259)*	8.037 (0.363)	6.546 (0.113)*	6.101 (1.080)*	6.291 (0.574)*	7.849 (0.709)	
S. aureus													
No CHX	8.591 (0.385)	8.834 (0.588)	8.947 (0.369)	8.591 (0.385)	7.849 (0.65)	7.942 (0.989)	7.834 (0.55)	8.824 (0.251)	5.778 (0.778)*	5.987 (0.956)*	7.204 (0.412)*	8.103 (0.325)	**9.00 (0.297)**/ 7.447 (0.954)*
CHX	6.724 (0.074)*	6.511 (0.441)*	6.422 (0.265)*	7.531 (0.337)*	7.278 (1.141)*	6.799 (0.425)*	6.771 (0.235)*	8.415 (0.556)	6.204 (0.121)*	6.531 (0.179)*	6.107 (0.737) *	7.904 (0.445)	

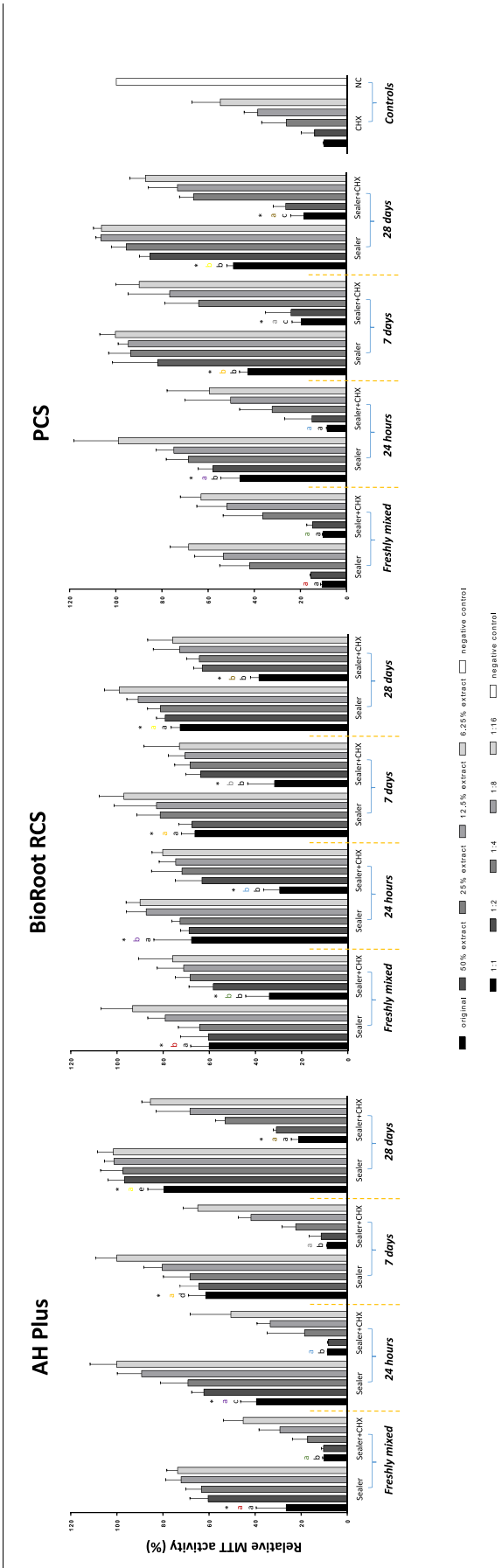

Fig. 2 – Mean relative MTT activity and standard deviation of L929 murine fibroblasts after 24-h exposure to undiluted sealer leachates and their dilutions. Statistical analysis was performed in undiluted leachates. The statistical differences between the leachates of each group and CHX control are denoted with black asterisks ($p < 0.05$). Same black letters signify no statistical differences between different groups in each sealer tested. Same coloured letters indicate no statistical differences between the same groups within all the sealers tested ($p > 0.05$). Red: Freshly mixed sealers, CHX; Purple: 24-h set sealers; Blue: 24-h set sealers, CHX; Orange: 7-d set sealers; Grey: 7-d set sealers, CHX; Yellow: 28-d set sealers; Brown: 28-d set sealers, CHX. The results are presented as percentage ratios compared to the negative control.

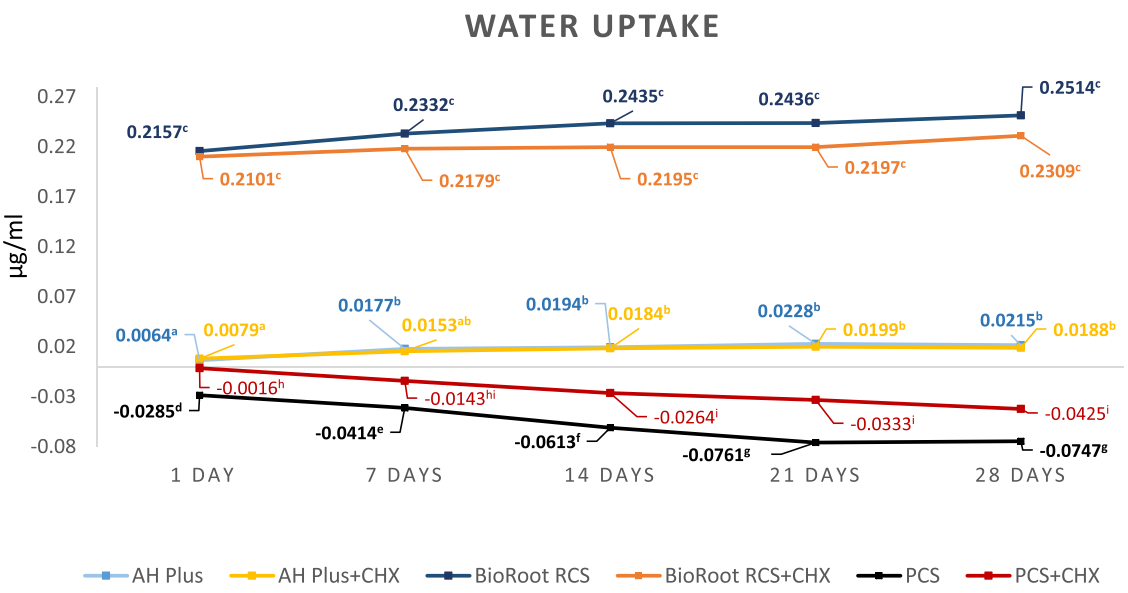

Fig. 3 – Mean water uptake values for test sealers with and without CHX contact. Read horizontally (within the same sealer and experimental condition, between different immersion periods, Tukey's multiple comparison test) and vertically (within the same immersion period, between different sealers and experimental conditions, parametric t-tests and Dunnett's C multiple comparison test), the same superscript letter shows no statistically significant differences, $p > 0.05$.

sealer leachates for the different setting times are shown in Fig. 5a and Table S2.

As for the pH assessment of the sealer leachates for physical properties (ISO 4049) (extraction vehicle: water), BioRoot RCS both with and without CHX contact exhibited the highest values for all immersion periods (1, 7, 14, 21 and 28 days) followed by AH Plus and PCS that had the most acidic pH ($p < 0.05$). CHX did not affect the pH of any of the sealers tested for any of the immersion periods ($p > 0.05$). The results for measurement of pH of the sealer leachates for the different setting times are shown in Fig. 5b and Table S3.

4. Discussion

Contact and interactions between endodontic sealers and remnants of irrigation solutions and tissue fluids may occur during and after root filling procedures. This may promote leaching of constituents from endodontic sealers. The characterisation of sealer leachates may thus be of clinical relevance. Moreover, the assessment of leachates of endodontic materials have attracted attention and the characterisation of elution/degraded materials along with cytocompatibility should also be tested in vitro [38].

The antimicrobial properties of leachates have been mainly tested for pulp capping materials or root-end filling materials [28,42]. The antimicrobial effects of endodontic sealers' leachates (liquid constituents) are investigated herein for the first time. In addition, there is little or no study investigating the effects of irrigation on the cytotoxicity of sealers. A few studies have assessed the leaching of sealers and characterised their leachates [25,43–45].

Endodontic sealers with different chemistry were evaluated in the present study to assess the biological properties of sealer leachates (antimicrobial properties and cell viability) and leaching of the materials (physical properties). AH Plus is a well-documented resin based endodontic sealer that is often selected in studies as a benchmark for comparisons [2,46]. BioRoot RCS, a calcium silicate based sealer, possesses biological properties, both high antibacterial efficacy [16] and

Table 3 – Mean sorption, solubility and porosity values with standard deviation for test sealers with and without CHX contact after 28 days of immersion. Read vertically (between different experimental conditions, parametric t-tests and Dunnett's C multiple comparison test), the same superscript letter shows no statistically significant differences, $p > 0.05$.

	Condition	28 days		
		Sorption (µg/mL)	Solubility (µg/mL)	Porosity (%)
AH Plus	No CHX	0.1353 (0.0351)^{a}	-0.0050 (0.0036)^{a}	1.82 (0.74)^{a}
	CHX	0.1614 (0.038)^{a}	0.0002 (0.0039)^{b}	2.25 (0.54)^{a}
BioRoot RCS	No CHX	0.3869 (0.0557)^{b}	0.2162 (0.042)^{c}	6.36 (0.82)^{b}
	CHX	0.3965 (0.0634)^{b}	0.1661 (0.027)^{de}	6.31 (0.85)^{b}
PCS	No CHX	0.1050 (0.0389)^{ca}	0.1429 (0.0051)^{e}	1.54 (0.57)^{ca}
	CHX	0.2188 (0.0346)^{d}	0.1029 (0.0089)^{f}	3.70 (1.45)^{d}

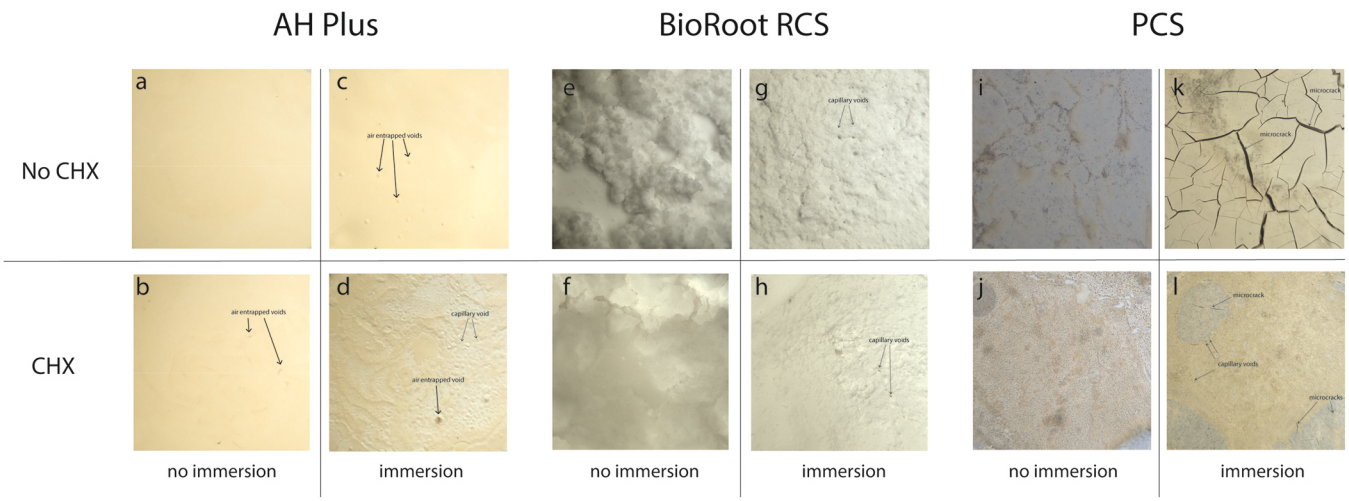

Fig. 4 – Representative microscopical images of the immersed or non-immersed sealer surfaces showing different features including air entrapped voids, capillary voids, crystal-like formations: (a) AH Plus, no immersion, no CHX; (b) AH Plus, no immersion, CHX; (c) AH Plus, immersion, no CHX; (d) AH Plus, immersion, CHX; e) BioRoot RCS, no immersion, no CHX; (f) BioRoot RCS, no immersion, CHX; (g) BioRoot RCS, immersion, no CHX; (h) BioRoot RCS, immersion, CHX; i) PCS, no immersion, no CHX; (j) PCS, no immersion, CHX; (k) PCS, immersion, no CHX; (l) PCS, immersion, CHX.

low cytotoxicity [47], however the sealer is affected by the environment due to its hydraulic properties [44]. PCS is a conventional zinc-oxide eugenol sealer, which has been in clinical use for a long time and has antibacterial properties [19] but high cytotoxicity due to eugenol release [47–49]. Regarding the choice of CHX, it has been suggested as a last irrigant before the root filling [22,23] and thus is likely to interact with endodontic sealers.

In endodontic infections, the root canals can be hosted by planktonic bacteria and bacteria in biofilms, on dentin walls and into dentinal tubuli [32,35,36,50]. After chemomechanical preparation, residual planktonic bacteria or biofilms can remain in remote areas such as apical ramifications, lateral canals, and isthmuses [29–34]. In this study, the antibacterial properties of sealer leachates were assessed against both planktonic bacteria and bacteria in monospecies biofilms. *E. faecalis*, *S. epidermidis* and *S. aureus* have been associated with post-treatment apical periodontitis [51–53]. *S. mutans*, a pathogen associated with caries, has been also reported in necrotic root canals [54,55] and it was included in the present study as a reference to evaluate the susceptibility of species not commonly retrieved from such infections [19,56]. The selection of gram-positive bacterial species serves the fact that comparisons between bacteria of the same Gram stain may be more accurate due to similarities in characteristics such as their cell membrane and thus susceptibility to antimicrobial agents [57].

The antibacterial properties of sealer leachates were assessed with the means of direct contact tests between the leachates and the bacteria in planktonic forms and biofilms and statistical analysis was performed on the CFUs calculation, which constitutes a well-documented method to quantify the bactericidal effect of antimicrobials [19,56,58].

The cytotoxicity of sealer leachates was evaluated with the use of MTT assay, which is widely used to assess cell viability of such materials [59–61]. It is a standardised method and reliable indicator of the cellular metabolic activity [62].

It is also important that irrigation solutions favour the biological properties of sealers without altering their physicomechanical behaviour and chemical constitution [19]. In the present study, the ISO 4049 was selected to be performed as it allows the assessment of various parameters (water uptake, sorption, solubility) with the same study design. It further enables the evaluation of porosity based on a previously described gravimetric method [63] and the measurement of pH of the soaking (immersion) liquids. Thus, in our study ISO 4049 was selected to assess the physical properties of the sealers, albeit ISO 4049 is not intended for root canal sealers. The ISO 4049 (water uptake, sorption, solubility) suggest the use of cylindrical specimens where the whole surface area of cylinders participates in dissolution and elution or liquid uptake. In our study, the aim was to examine the physical properties of the sealers focusing on the leaching of the sealer surfaces in contact with CHX. CHX is a water-based solution, thus contact with the water solvent may have affected the materials investigated. Based on the results for water uptake, 2% aqueous CHX solution did not have a statistically significant effect on AH Plus and BioRoot RCS, but affected PCS. Taking this into account and in order to investigate whether water alone exerts an effect on PCS, a follow-up experiment was conducted where only distilled water was applied during setting similarly to the procedure followed for the CHX solution (see materials and methods). The water uptake (μg/mL) after 1 day for PCS in contact with water was calculated and compared to PCS with CHX solution contact and PCS without any liquid contact. Water uptake was assessed only after 1 day, given that all the materials tested did not present any fluctuations over time in all conditions tested. No significant effect of water alone was

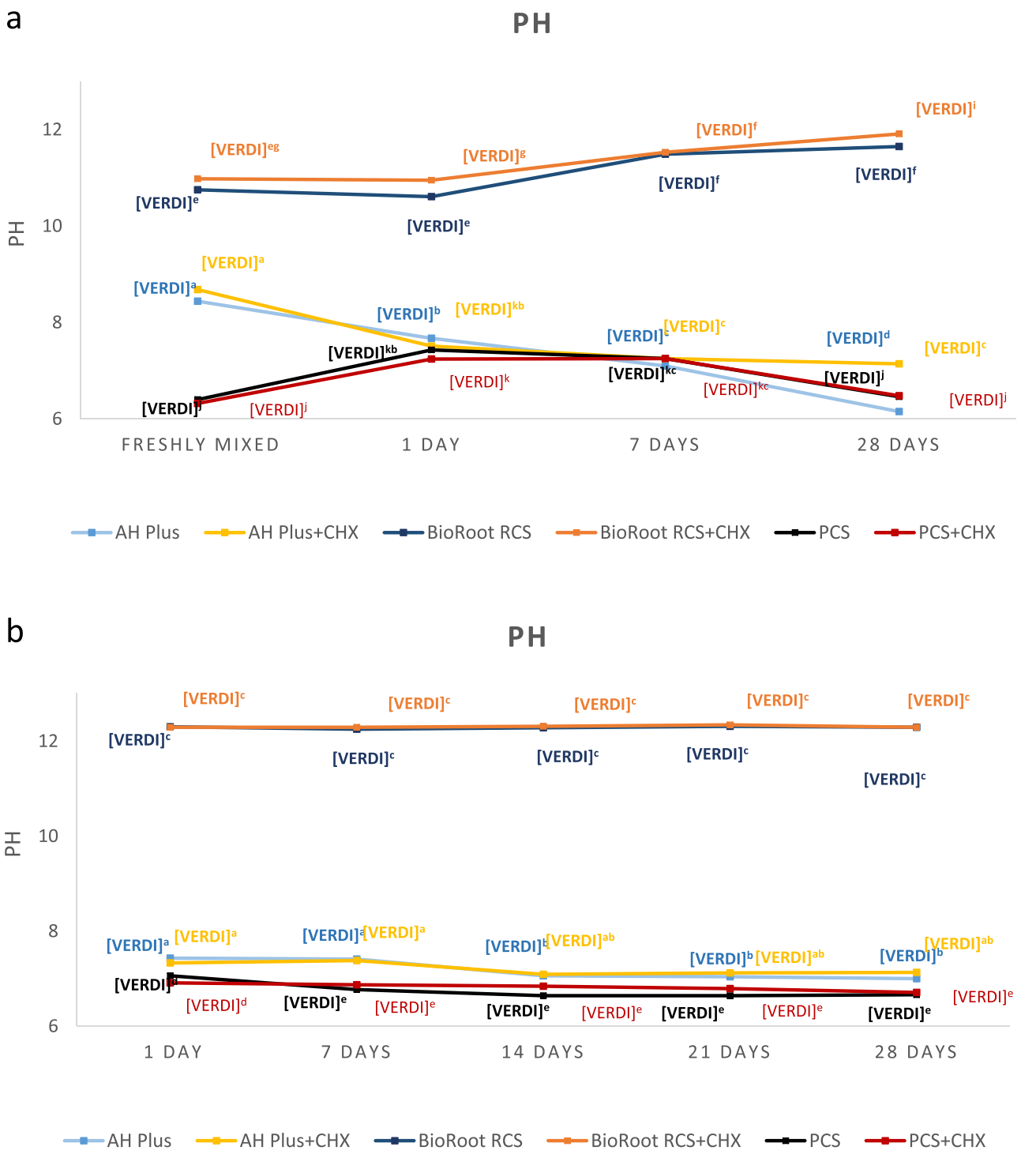

Fig. 5 – Mean pH values of freshly mixed, 24 h, 7 days and 28 days set sealers' leachates (for biological properties) in contact or not with CHX (pH = 5.98 ± 0.11). Saline 0.9% (pH = 5.6 ± 0.09) used as the extraction vehicle (a). Mean pH values of sealers' leachates in contact or not with CHX (pH = 5.98 ± 0.11) with distilled water (6.89 ± 0.15) used as the extraction vehicle (b). Read horizontally (within the same sealer and experimental condition, between different immersion periods, Tukey's multiple comparison test) and vertically (within the same immersion period, between different sealers and experimental conditions, parametric t-tests and Dunnett's C multiple comparison test), the same superscript letter shows no statistically significant differences, p > 0.05.

observed compared to no contact, but lower than after contact with the CHX solution (data not shown).

Immersion to water (suggested by ISO 4049) further degraded the materials in time, especially BioRoot RCS as a hydraulic cement, but also served to simulate contact with tissue fluids. Thus, the use of an immersion liquid (water) was a necessity to assess these properties. Inert teflon cylindrical moulds (with bottom and side walls) (Fig. S5a) were manufactured in such way to cover the bottom face and side surfaces of the sealer samples and leave free the top face of the materials. Thus, this mould design enabled us to expose only the sealer surface of interest in the immersion liquid.

In the present study, CHX improved the antibacterial properties of the sealer leachates and mostly compromised or at least did not affect the cell viability. It has been shown that CHX is an efficient antimicrobial agent [60,64–66] while studies show various findings for cytotoxicity [60,67]. A recent publication evaluating the cytotoxicity of AH Plus, MTA Fillapex (hydraulic calcium silicate based cement) and PCS with incorporated CHX nanoparticles also demonstrated compromised cell viability for the modified sealers [60]. Leachates from sealers without CHX contact presented an increasing cell viability over setting time (freshly mixed, 24 h, 1 day, 7 days, 28 days) which confirms previous scientific data that set materials are less cytotoxic than freshly mixed [61]. Overall, the sealer leachates were less effective against biofilms compared to their planktonic counterparts [68].

BioRoot RCS eliminated all the planktonic bacteria for all setting times, while it showed antibacterial activity up to 7 days against *E.faecalis* and *S.mutans* biofilms. The high antibacterial properties of BioRoot RCS leachates can be associated with the proposed antibacterial mechanism of hydraulic calcium-silicate cements: when in contact with water, the calcium hydroxide, formed during hydration process, releases calcium ions (Ca^{2+}) and hydroxyl ions (OH^-), which in turn increases alkalinity and contributes to potent antimicrobial properties [28,69]. The high alkalinisation effect of BioRoot RCS was also reported in this study, a finding that is consistent with previous scientific data [44]. Earlier literature has reported moderate antibacterial properties for BioRoot RCS [70,71], whilst three recent studies showed antimicrobial activity [19,72,73]. Nevertheless, direct comparisons between previous literature and the current study cannot be performed, due to differences in methodology. CHX contact did not compromise the antibacterial properties of BioRoot RCS against planktonic bacteria and improved its efficacy against biofilms (*S. epidermidis, S. aureus*). This is also in accordance with the results of BioRoot RCS for pH, as CHX increased the alkalinity of the leachates. Additionally, a study assessing the effect of CHX on the antibacterial properties of three sealers reported improved efficacy for BioRoot RCS after CHX contact [19]. In the same direction, studies have found enhanced antimicrobial properties for calcium silicate based cements with incorporation of CHX compared to the unmodified [64,65,74–76]. The enhanced antimicrobial behaviour of hydraulic cements after modification or contact with CHX may be further explained by the synergistic release of calcium/hydroxyl ions and CHX, given their high solubility [60]. Furthermore, BioRoot RCS leachate exerted the lowest cytotoxicity among the sealers tested, which can also be associated with pronounced calcium ion release and the high alkalinisation potential of hydraulic cements [42]. Previous studies on sealer cytotoxicity have also showed less cytotoxicity for BioRoot RCS compared to AH Plus and PCS [47,77]. Interestingly, BioRoot RCS with CHX contact was the only sealer that presented lower cytotoxicity compared to CHX positive control for all setting times.

AH Plus leachates did not exhibit any antibacterial properties even derived from freshly mixed material. Earlier literature on the antimicrobial efficacy of AH Plus bulk material or surfaces indicates that the sealer maintains its efficacy only as unset [56,78]. An explanation to this is AH Plus' physical properties and that is chemically stable [79,80]. Any compounds that potentially have antimicrobial effect may be entrapped in the resinous matrix [81]. The consistent physicochemical behaviour of AH Plus was shown also in our study with low solubility and pH values which were setting time-dependent. Contact with CHX rendered AH Plus leachate antibacterial against both planktonic bacteria and bacteria in biofilms for all setting times. This enhancement in antibacterial efficacy of AH Plus leachates after CHX contact up to 28 days setting time may indicate a possible mechanism of crosslinking between the antimicrobial agent (substantivity of CHX) and the sealer surface, which confers long-lasting efficacy. Earlier literature has also demonstrated improved antibacterial properties of AH Plus surfaces after CHX contact [19] or incorporation of CHX [82]. As for cytotoxicity, AH Plus exposure resulted in low cell viability especially as freshly mixed with a gradual improvement along with the setting time. Our findings are in concordance with many studies that have also found pronounced cytotoxicity for AH Plus especially when unset [46,47,60,77,83–85]. AH Plus contains epoxy resin that is cytotoxic [86], and this may explain the pronounced cytotoxic effect of the sealer particularly as freshly mixed [83].

PCS leachate alone exerted antibacterial efficacy among the sealers investigated and contact with CHX improved sealer's properties especially against biofilms. These findings for PCS alone are in agreement with the literature evaluating ZOE based sealers [30,87–91]. In addition, previous publications have shown enhanced antibacterial properties for ZOE sealers either modified with CHX [92,93] or after CHX contact [19]. Regarding its antimicrobial mechanism, release of eugenol is the first contributing factor [90,91], which was also indicated in our study, given the negative water uptake values and the yellowish colour of PCS leachates. Furthermore, the silver and zinc oxide may also contribute to the antibacterial properties of PCS [94,95]. A recent study has identified silver chloride phase in PCS after contact with CHX, which may have further contributed to the improved antibacterial properties of PCS [96]. PCS leachate exhibited higher cytotoxicity as freshly mixed and in contact with CHX, which corroborates with previous scientific data [47,60]. The release of eugenol has been also associated with cytotoxicity, biocompatibility/cell viability [97].

The physical properties of the sealers with and without CHX contact was evaluated according to ISO 4049. Another study has also employed ISO 4049 to assess the physical properties of AH Plus, MTA Fillapex, BioRoot RCS, Endoseal following immersion in various liquids [44]. The findings for

AH Plus and BioRoot RCS are in accordance with our study: BioRoot RCS had the highest water uptake, sorption, solubility and porosity while AH Plus was the material least affected. Hydraulic calcium-silicate based cements, such as BioRoot RCS, presented high hydrophilicity of their surfaces [19] which in turn leads to increased adsorption of water and porosity. Moreover, its hydraulic nature and the formation of calcium hydroxide renders the sealer susceptible to the environmental conditions [98]. The microscopic images further confirmed these differences in physical behaviour as BioRoot RCS appeared porous with capillary voids and AH Plus was slightly affected by immersion. Besides poor physical properties, open pores in the bulk of endodontic sealers may serve as hubs and favour bacterial growth [99]. Moreover, nutrients entering the root canal may find pathways through the bulk of filling materials via pores and facilitate the growth of entombed bacteria [100,101]. PCS was the material to be mostly affected by CHX in terms of physical properties, whereas AH Plus and BioRoot RCS remained unaffected, except for their solubility which was increased for AH Plus and decreased for BioRoot RCS. This was also verified under the optical microscope where PCS without CHX presented dry surface texture with a significant amount of cracks in the bulk of the material, a declare of extensive shrinkage. Contact with CHX reduced the amount of cracks on the surfaces, while more capillary voids were evident. Release of eugenol, speculated to occur by the yellowish colour change of the PCS leachates in conjunction with the negative water uptake values, may be associated with the presence of microcracks and shrinkage. Pronounced shrinkage for PCS has been observed when stored at 100% humidity [5], as well as the dimensions of a zinc oxide-eugenol impression material were reduced after disinfection with aqueous CHX solutions [102]. Additionally, PCS is a hydrophobic material [19] and thus does not promote water adsorption and consequently exhibits low porosity [103], findings that corroborate with the present study.

Regarding chemical properties and pH assessment, differences in pH values between the leachates for biological properties and ISO 4049 may be attributed to the different soaking liquids (saline for biological assays: pH: 5.6 ± 0.09; distilled water for ISO 4049: pH: 6.89 ± 0.15), different immersion times and specimen surface to immersion liquid ratio. Alkalinity of sealer leachates did not change after CHX contact in distilled water whilst in saline significant differences were shown after 28 days for AH Plus and after 1- and 28 days for BioRoot RCS. AH Plus and PCS presented pH values closer to neutral while BioRoot RCS maintained high alkalinity over time. These results are in accordance with earlier literature [44,60].

The key point of this study was to evaluate the performance of sealer leachates following interaction with CHX in terms of biological properties and the sealers' physical properties. There is scant scientific data about the potential interactions between endodontic sealers and irrigation solutions. Future efforts should include the evaluation of other irrigation solutions that are suggested for use as last irrigants before sealer placement in the root canal system such as EDTA and sodium hypochlorite. Sealer leachates should be investigated further, including thorough chemical characterisation of the eluates. As for antimicrobial properties, multispecies biofilms of various maturation stages should also be evaluated, as young biofilms are more susceptible to antimicrobial agents than mature ones [104,105]. Further studies involving more complex environments such as tooth models and the use of human cells or clinical bacterial isolates may give insight of the role of sealer leachates in therapeutics of endodontic pathosis.

5. Conclusions

The main hypothesis of the study was rejected as exposure to CHX affected sealers' properties. CHX in contact with sealer surfaces improved the antibacterial properties of the sealer leachates and reduced cell viability for all sealer leachates, except for freshly mixed PCS. Among the tested sealers, BioRoot RCS leachates presented the highest antibacterial properties and cell viability with and without CHX contact. Regarding chemical properties and pH assessment, alkalinity of sealer leachates did not change after CHX contact in distilled water whilst in saline CHX increased alkalinity after 28 days for AH Plus and after 1- and 28 days for BioRoot RCS. PCS was the material most affected by CHX in terms of physical properties, whereas AH Plus remained unaffected except for solubility which was increased. Although BioRoot RCS presented the highest values for water uptake, water sorption, solubility and porosity, CHX did not affect the sealer, except for solubility that was decreased.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dental.2022.04.013.

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